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SUBARCHNOID HEMORRHAGE
AND INFLAMMATION

Habilitation thesis

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Preface

The objective of this work is to compile selected scientific publications to which I have contributed as the first or corresponding author. The compilation covers review articles as well as clinical and experimental research articles related to the topic of subarachnoid hemorrhage (SAH).

Presented articles are organized thematically to put them into the context of SAH pathophysiology. The first chapter is an introduction covering the definition, epidemiology, clinical and research aspects of SAH. The second is dedicated to major complications such as cerebral vasospasms or delayed cerebral ischemia (DCI). Chapter three provides deeper insight into pathophysiology of SAH through the description of early brain injury and inflammation as its crucial contributor. Chapter four covers some problematic aspects of inflammation related to diagnostics and treatment, such as variability and deregulation of the inflammatory response. Finally, vagus nerve stimulation, which is a promising way to activate cholinergic anti-inflammatory pathway (CHAIP) and control inflammation, is discussed.

SAH is a hemorrhagic subtype of stroke, which is predominantly caused by rupture of a cerebral aneurysm. Aneurysmal SAH represents a severe medical condition associated with high morbidity and mortality. Symptoms of SAH are very variable, from headache to coma. Treatment of an aneurysm is a medical priority, and the most frequent treatment options are clipping or coiling. Elimination of an aneurysm from blood flow prevents re-rupture, which is the most severe complication associated with SAH. Other life-threatening complications include acute hydrocephalus and DCI.

DCI is a severe late complication, which usually occurs from day 4 to day 14 after SAH. DCI affects approximately 30% of patients with SAH and significantly contributes to worsening outcomes. Cerebral vasospasm was considered the only cause of DCI; however, DCI may occur independently of vasospasm development. The concept of early brain injury (EBI) was elaborated to explain the pathophysiology of DCI.

EBI represents pathologies that may cause direct damage to brain tissue or lead to DCI/vasospasm development. EBI includes pathologies like transient global cerebral ischemia, oxidative stress, blood-brain barrier disruption, brain edema, endothelial/neuronal apoptosis, and inflammation. Inflammation plays a key role in the pathophysiology of EBI and SAH. Inflammation interconnects EBI-related pathologies, and inflammation is also associated with both DCI and cerebral vasospasm. One of the most extensively studied proinflammatory markers associated with SAH is IL-6.

The methods used in our studies were diverse and combined both experimental and clinical approaches.

In experimental conditions, we used the perforation model of SAH in Sprague-Dawley rats. Subarachnoid hemorrhage was induced by perforation of the internal carotid artery in the bifurcation by a sharpened nylon suture introduced through the vessel in the neck. The

neurological impact of SAH was evaluated using the “Modified Garcia Score” assessment, which is designed to quantify both neurological deficit and impairment of consciousness. After euthanasia, the brain was removed, and a picture of the underside of the brain was taken to evaluate SAH grade using the “Sugawara Grading System”. In order to predict the severity of SAH, we have developed “Anesthesia Recovery Time System”, which is based on time to recover of vital reflexes/functions (spontaneous ventilation, pain reaction, consciousness recovery).

Clinical research was conducted in the Department of Neurosurgery, The University Hospital Brno and Faculty of Medicine, Masaryk University. The patients with a diagnosis of SAH who underwent surgical, endovascular, and conservative treatments were typically enrolled in studies presented in this compilation. The neurological impact of SAH was usually characterized by the Hunt-Hess scale, and the severity of bleeding was typically expressed using the Fischer scale. Cerebral vasospasm was assessed by transcranial dopplerometry, and brain oxygen partial pressure was monitored in some patients using the Licox system. Outcomes of the patients were evaluated using the Glasgow Outcome Scale.

Laboratory samples included brain tissue and blood in experimental research, while cerebrospinal fluid and blood were assessed in clinical conditions.

Various laboratory methods were used to process laboratory samples; protein expressions were assessed using Western blot or ELISA, and mRNA expressions were evaluated by RT-PCR. Additionally, in some experimental studies, we evaluated brain water content to estimate cerebral edema and immunohistochemistry coupled with TUNEL staining to estimate the level of neuronal apoptosis.

The results of the presented studies are mainly related to two major topics: partial pressure of oxygen in brain tissue (P_{btO_2}), which was an important subject of our previous research and inflammation, which represents a main topic of our recent research.

Regarding P_{btO_2} , we found that the dynamics of P_{btO_2} is significantly higher in patients without cerebral vasospasm when increasing the fraction of inspired oxygen to 100%, demonstrating the effect of cerebral vasospasm on blood supply to the brain. We also described that P_{btO_2} correlates with those parameters of transcranial dopplerometry, which reflect conditions related to vessel resistance and microvasculature, suggesting the role of small vessels in the development of delayed cerebral ischemia. Finally, we found a moderate negative correlation between P_{btO_2} and IL-6 levels in cerebrospinal fluid of SAH patients, implying the role of inflammation in the pathophysiology of SAH.

Inflammation is an important contributor to early brain injury. Our studies are focused on the early inflammatory response after SAH. We found that the best potential marker of SAH-related inflammation is IL-6, and the best compartment is cerebrospinal fluid (CSF). These findings are in accordance with other studies. We also described that IL-6 is massively increased in CSF early after SAH, and the levels correlate with poor patient outcomes. However, the variability of IL-6 levels is too high to establish a threshold for diagnostics. Subsequently, we studied the SAH-associated inflammatory response in experimental conditions and found that the inflammatory response is triggered early after SAH and is significantly pronounced in

severe cases. Additionally, we described that severe SAH is associated with an inflammatory response in the whole brain, not only in areas affected by the hematoma. This is an important finding suggesting that SAH may have a global impact on the brain or that severe SAH may lead to deregulation of inflammatory response.

In the field of experimental research, we also described that the simultaneous collection of CSF and blood may lead to cerebral edema formation. Other methodical research was focused on the effect of SAH severity on anesthesia recovery time. This may be useful for prediction of SAH severity. Finally, the most complex experimental research was focused on the role of $\alpha 7$ nicotinic acetylcholine receptor in the pathophysiology of SAH. We described in this study that pharmacological stimulation of $\alpha 7$ nicotinic acetylcholine receptor reduces SAH-associated neuronal apoptosis. This was one of the first reports suggesting the role of cholinergic anti-inflammatory pathway (CHAIP) in the pathophysiology of SAH.

In conclusion, subarachnoid hemorrhage is a severe condition associated with high morbidity and mortality. Inflammation plays a crucial role in the pathophysiology of SAH. Inflammatory response is triggered early after SAH, and severe subarachnoid hemorrhage is associated with inflammation affecting the whole brain. IL-6 represents the best marker of SAH-associated inflammation; however, there is a high variability in IL-6 levels after SAH. This is a problem for the effective treatment of inflammation after SAH. CHAIP is a promising target for the treatment of the inflammatory response after SAH.

List of abbreviations

ACM	– Arteria cerebri media
BBB	– Blood-brain barrier
CHAIP	– Cholinergic anti-inflammatory pathway
CVS	– Cerebral vasospasm
CSF	– Cerebrospinal fluid
CT	– Computed tomography
CTA	– Computed tomography angiography
CBF	– Cerebral blood flow
DCI	– Delayed cerebral ischemia
DSA	– Digital subtraction angiography
EBI	– Early brain injury
EDV	– End-diastolic velocity
FiO ₂	– Fraction of inspired oxygen
GCS	– Glasgow coma scale
GOS	– Glasgow Outcome Score
HH	– Hunt-Hess scale
ICH	– Intracerebral hemorrhage
ICP	– Intracranial pressure
ISCH	– Ischemic stroke
MRA	– Magnetic resonance angiography
mRS	– Modified Rankin Scale
PbtO ₂	– Partial pressure of oxygen in brain tissue
PI	– Pulsatility index
PNS	– Parasympathetic nervous system
PSV	– Peak systolic velocity
RI	– Resistance index
RNS	– Reactive nitrogen species
SAH	– Subarachnoid hemorrhage
SNS	– Sympathetic nervous system
TCD	– Transcranial dopplerometry
V _{mean}	– Mean Velocity
VNS	– Vagus nerve stimulation

1. Introduction – Stroke and subarachnoid hemorrhage

1.1 Stroke

Stroke is a severe medical condition associated with high morbidity and mortality. Each year, around 12,2 million new cases of stroke occur worldwide (incidence around 158/100 000) and there are over 101 million surviving patients who have suffered from stroke (prevalence about 1002/100 000)¹. Stroke accounts for 6,5 million deaths each year (mortality rate around 85/100 000) representing the second leading cause of death worldwide¹.

The majority of strokes are of ischemic etiology, which develops due to cerebral vessel occlusion, ischemic strokes account for over 62% of strokes¹. The rest 38% of strokes are of hemorrhagic etiology, which develops due to non-traumatic cerebral vessel rupture either in cerebral parenchyma or outside the parenchyma in subarachnoid space.

The rupture of the parenchymal vessel leads to intracerebral hemorrhage, which accounts for around 75% of hemorrhagic strokes¹. The rupture of the extraparenchymal vessel, which is localized in subarachnoid space, results in subarachnoid hemorrhage (SAH), which represents around 25% of hemorrhagic strokes. Figure 1 shows the number of new cases and deaths in 2019. Although the ischemic subtype represents a majority of strokes (A), hemorrhagic stroke is associated with higher mortality, and the total numbers of deaths due to ischemic stroke and hemorrhagic stroke are similar (B).

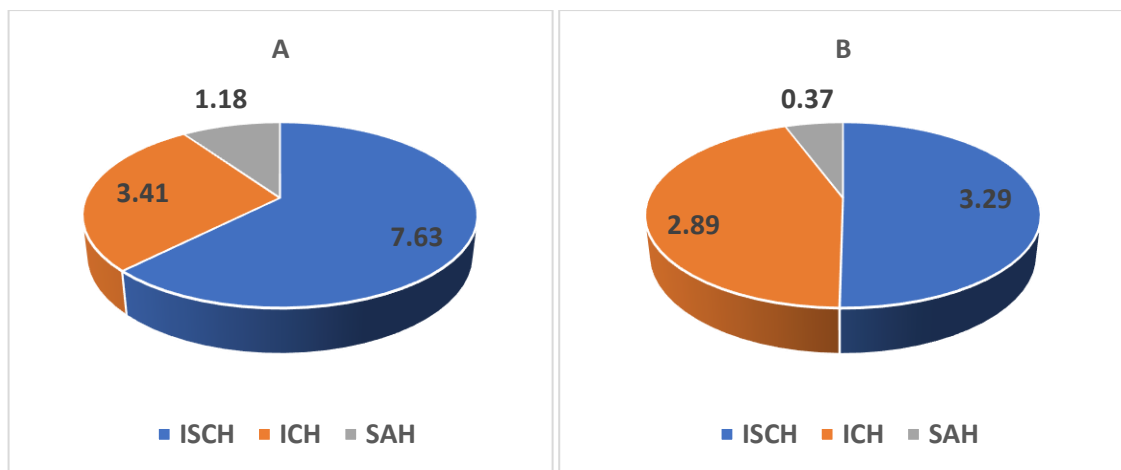


Figure 1: A - A total number of patients (in millions) suffering from ischemic stroke (ISCH), intracerebral hemorrhage (ICH), and subarachnoid hemorrhage (SAH) in 2019 worldwide. **B** – The total number of deaths (in millions) due to ischemic stroke (ISCH), intracerebral hemorrhage (ICH), and subarachnoid hemorrhage (SAH) in 2019 worldwide. The graphs are based on data in: Feigin VL, Brainin M, Norrving B, Martins S, Sacco RL, Hacke W, Fisher M, Pandian J, Lindsay P. World Stroke Organization (WSO): Global Stroke Fact Sheet 2022. *Int J Stroke*. 2022;17:18–29.

1.2 Subarachnoid hemorrhage

Subarachnoid hemorrhage represents just a minority of strokes, global incidence is around 15/100 000, and prevalence is 109/100 000¹. However, SAH is very important from the clinical point of view, because it affects relatively young patients and is associated with high morbidity and mortality. Approximately 2% of deaths due to ischemic strokes occur in patients younger than 50 years, 9% of deaths from intracerebral hemorrhage are in patients younger than 50 years, while 16% of deaths from SAH occur in patients younger than 50 years¹. The peak incidence of SAH is between 50 and 60 years and women are affected approximately 1,6 times more often than men². Modifiable risk factors for SAH development represent hypertension, smoking, excessive alcohol intake, while non-modifiable risk factors are age, sex, polycystic kidney disease, family history and genetic factors (4q31.23 (EDNRA), 8q11.23-q12.1 (SOX17), 9p21.3 (CDKN2A/CDKN2B), 10q24.32 (CNNM2), 12q22 (NDUFA12/NR2C1/FGD6/VEZT), 13q13.1 (KL/STARD13) and 18q11.2 (RBBP8))²⁻⁹. There are also geographic factors associated with SAH, the highest incidence is in Japan (22,7/100 000) and Finland (19,7/100 000)⁶.

Around 85% of SAH cases are of aneurysmal etiology, 10% of cases are non-aneurysmal (perimesencephalic) and the rest 5% represent bleeding caused by a wide range of other pathologies, such as arteriovenous malformations, other vascular pathologies, tumors, infections and other causes². Perimesencephalic SAH represents pathology that may be considered relatively benign, while aneurysmal SAH may potentially have a very bad prognosis^{2,10}.

A cerebral aneurysm represents a bulge on the vessel wall which has a tendency to grow due to pressure inside the vessel and this mechanism represents a typical vicious circle possibly resulting in aneurysm rupture. The most common shape of an aneurysm is saccular (90%) and predominant localization is in the anterior part of the Willis circle (85%), especially at the branching sites¹¹. As the aneurysm grows, the wall becomes weaker and weaker, while the tension in the wall increases according to Laplace law and once it reaches a critical level, the aneurysm ruptures resulting in subarachnoid hemorrhage. The annual risk of rupture is 0,05%/0,5% for aneurysms smaller than 10mm in patients without/with a history of a ruptured aneurysm, and it is 6% for aneurysms larger than 25mm¹². However, the majority of ruptured aneurysms are smaller than 1cm, van Gijn explains it as follows: "90% of all aneurysms are small and the small fraction of this majority that ruptures outnumber the greater fraction of the minority of large aneurysms that ruptures"¹³.

Aneurysmal SAH represents a severe medical condition. Detailed pathophysiological mechanisms will be described in Chapter 3, but it is necessary to mention two important aspects right now. First, aneurysmal SAH is of arterial origin (high blood pressure), the highest blood pressure in cerebral circulation is in Willis circle and the most often localization of cerebral aneurysms is on vessels of Willis circle or nearby branches¹³.

Second, the subarachnoid space is a relatively free space filled with cerebrospinal fluid, which does not represent significant resistance to bleeding. Thus, SAH associated with rupture of cerebral aneurysm usually represents massive bleeding.

1.2.1 Diagnostics and treatment of SAH

Symptoms of SAH are very variable, from headache to coma. Sudden onset of headache which is referred to be “the most severe headache in the life” is a typical feature of SAH which is in approximately 50% of patients the only symptom of SAH, other symptoms are nausea, vomiting, loss of consciousness, and focal neurological deficit².

Several classifications reflecting clinical symptoms were established. Based on the character of headache, neurological deficit, and level of consciousness, the classification of SAH severity was elaborated in 1968 by William Hunt and Robert Hess¹⁴ (Table 1).

Category	Description
Grade I	Asymptomatic, or minimal headache and slight nuchal rigidity.
Grade II	Moderate to severe headache, nuchal rigidity, no neurological deficit other than cranial nerve palsy.
Grade III	Drowsiness, confusion, or mild focal deficit.
Grade IV	Stupor, moderate to severe hemiparesis, possibly early decerebrate rigidity and vegetative disturbances.
Grade V	Deep coma, decerebrate rigidity, moribund appearance.

Table 1: Classification published by Hunt Hess in 1968, originally entitled: “Classification of patients with intracranial aneurysms according to surgical risk”¹⁴. The classification is nowadays titled the Hunt-Hess scale.

World Federation of Neurological Surgeons elaborated the SAH grading scale based on the Glasgow coma scale (GCS). Glasgow coma scale (Table 2) was elaborated in 1974, WFNS classification (Table 3) was originally developed in 1988 and modified in 2015¹⁵⁻¹⁷.

Response	Scale	Score
Eye-opening	Spontaneously	4
	To verbal stimulus	3
	To pain	2
	No eye opening	1
Verbal response	Oriented	5
	Confused	4
	Inappropriate, words discernible	3
	Incomprehensible	2
	No verbal response	1
Motor response	Obeys commands	6
	Purposeful movement to pain	5
	Withdraws from pain	4
	Flexion	3
	Extension	2
	No motor response	1
Minor Brain Injury: 13-15 points; Moderate Brain injury: 9-12 points; Severe Brain Injury: 3-8 points		

Table 2: Glasgow coma scale

Grade	GCS
I	15
II	14
III	13
IV	7 - 12
V	3 - 6

Table 3: Modified WFNS scale

The second step in the diagnostic algorithm is a non-contrast computed tomography (CT) scan which represents the main diagnostic tool to confirm SAH. In case of negative CT findings, lumbar puncture may be used to confirm the SAH if clinical symptoms are obvious. The sensitivity of CT to detect SAH is 100% within 6 hours and more than 97% within 72 hours after onset of bleeding². Based on CT findings Fisher, Kistler, and Davies elaborated the SAH scoring system in 1980, which is nowadays known as the Fisher scale (table 4)¹⁸.

Grade	The character of bleeding on CT
1	No blood in subarachnoid space
2	Diffuse or vertical layers <1mm thick
3	Localized clot or/and vertical layers ≥1mm
4	Intracerebral or intraventricular clot with diffuse or no SAH

Table 4: Fisher scale

When the diagnosis of SAH is confirmed, the next step of medical management is to search for a source of bleeding. The gold standard is digital subtraction angiography (DSA) with 3D reconstruction, other options are CT angiography (CTA) and magnetic resonance angiography (MRA). Cerebral aneurysms account for approximately 85% of SAH².

Treatment of an aneurysm is a crucial part of medical management because a damaged vessel is prone to re-rupture if there is blood flow in the aneurysm. Thus, the main goal the of treatment is to obliterate the aneurysm to prevent blood flow to it. The most often treatment options are clipping and coiling. Clipping represents surgical treatment; typically, the neck of the aneurysm is closed by the clip eliminating the aneurysm from circulation. Coiling represents an endovascular solution; filling the aneurysm with coils leads to aneurysm thrombotisation and elimination from circulation. The selection of treatment modality depends on several factors including shape, size, localization of aneurysm, clinical condition, comorbidities, or age². If possible, the aneurysm treatment should be performed as soon as possible to prevent rebleeding.

Following aneurysm treatment, neurointensive care is required to prevent possible complications and eventually treat them if they occur. Neurological complications include rebleeding, hydrocephalus, and cerebral vasospasm/delayed cerebral ischemia. These major complications will be discussed in the next chapters. Non-neurological complications include stress cardiomyopathy, neurogenic pulmonary edema, and hyponatremia. Stress cardiomyopathy and pulmonary edema are associated with excessive activation of the sympathetic nervous system and related adrenergic surge, while hyponatremia is linked to cerebral salt-wasting syndrome and syndrome of inappropriate antidiuretic hormone secretion¹⁹. Other goals of neurointensive care are to prevent infection and to control fever. Non-infectious SAH-associated fever is a frequent symptom, which is believed to be caused by irritation of the hypothalamus by blood¹⁹.

The outcome of the patients is usually expressed as a Glasgow Outcome Score (GOS) and modified Rankin Scale (mRS). Glasgow Outcome Score (Table 5) was elaborated as a classification of brain injury by Jennett and Bond in 1975²⁰.

Grade	Description
1	Death
2	Vegetative state
3	Severe disability
4	Moderate disability
5	Low or no disability

Table 5: Glasgow Outcome Score

Rankin Scale was introduced in 1957 for the classification of cerebrovascular events and a modified version (table 6), which added grades 0 and 6 to the original scale, is used from 2005^{21,22}. The modified Rankin Scale is nowadays considered to be a gold standard for outcome assessment in clinical trials²³.

Grade	Description
0	No symptoms
1	No significant disability. Able to carry out all usual activities, despite some symptoms.
2	Slight disability. Able to look after own affairs without assistance, but unable to carry out all previous activities.
3	Moderate disability. Requires some help, but able to walk unassisted.
4	Moderately severe disability. Unable to attend to own bodily needs without assistance, and unable to walk unassisted.
5	Severe disability. Requires constant nursing care and attention, bed-ridden, incontinent.
6	Death

Table 6: Modified Rankin Scale

1.2.2 Subarachnoid hemorrhage in clinical and experimental research

Subarachnoid hemorrhage is an important topic of both clinical and experimental research. The most widely studied pathologies are cerebral vasospasm and early brain injury. Besides basic clinical characteristics and radiologic findings, blood and cerebrospinal fluid are the only two compartments accessible for laboratory analyses in clinical settings.

In experimental conditions, more consistent SAH may be produced, and all the organ compartments are accessible for laboratory analyses. The first experimental use of animals for experimentally induced SAH was published by Bagley in 1928²⁴.

Recently, the most widely used models of SAH are the blood injection model and perforation model in rats²⁵. In the blood injection model, the autologous blood is injected into the cisterna magna. This model is relatively easy and is associated with a constant blood volume in the subarachnoid space as well as with a low mortality rate²⁵. The blood injection model, however, does not mimic the clinical situation, which is better reproduced by the perforation model. In the perforation model, a nylon suture is pushed through the internal carotid artery and SAH is induced by perforation of the artery (Figure 2). Real bleeding caused by vessel puncture leads to important pathological changes, such as an acute increase of intracranial pressure with subsequent transient ischemia, which plays a major role in the pathophysiology of SAH²⁶. The perforation model is for this reason considered to be more appropriate than the blood injection model for SAH studies, even though the perforation model has several disadvantages including surgical difficulty, no possibility to control blood volume in subarachnoid space, and high mortality rate²⁵.

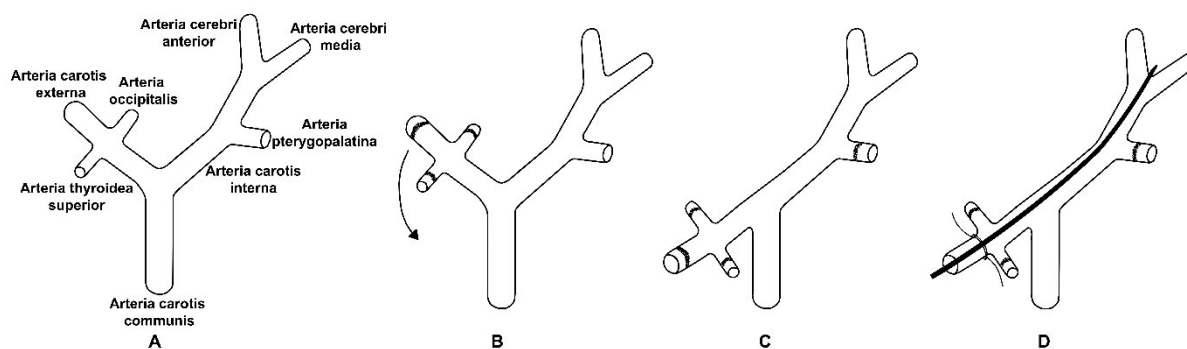


Figure 2: Perforation model of SAH. A – Arteria carotis communis, externa, interna, and their branches. B – Preparation of arteria carotis externa, preparation, coagulation, and cutting of arteria occipitalis and arteria thyroidea superior, ligation, coagulation, and cutting of arteria carotis externa. C – Mobilization of the stem of arteria carotis externa, preparation of arteria carotis interna, coagulation and cutting of arteria pterygopalatine, D – Temporary ligation of the stem of arteria carotis externa, introduction of nylon suture into the vessel, perforation of arteria carotis interna in bifurcation.

The impact of SAH on experimental animals may be evaluated using various behavioral tests. The most widely used neurological scoring system for experimental stroke research in rats was elaborated by Garcia²⁷. This system was later modified by Sugawara to better reflect neurological deficit produced by the SAH model²⁸. This scoring system known as the modified Garcia score, primarily designed to assess neurological deficit 24 hours after SAH, is depicted in Table 7.

	0	1	2	3
Spontaneous activity (in cage for 5 min)	No movement	Barely moves position	Moves but does not approach at least 3 sides	Moves and approaches at least 3 sides
Spontaneous movements of all limbs	No movement	Slight movement	Moves all limbs but slowly	Move all limbs same as pre-SAH
Movements of forelimbs (out-stretching)	No out-stretching	Slight out-stretching	Outstretching limited and less than pre-SAH	Outstretch same as pre-SAH
Climbing wall of wire cage	Fall	Fails to climb	Climbs weakly	Normal climbing
Touch of trunk	-----	No response	Weak response	Normal response
Vibrissae touch	-----	No response	Weak response	Normal response

Table 7: Modified Garcia score

The severity of SAH is the most important characteristic of the SAH perforation model. Evaluation of SAH grade in living animals is not possible without sophisticated tools. A simple tool to assess SAH grade after animal sacrifice was elaborated by Sugawara²⁸. It is based on the snapshot of the basal part of the brain and subsequent visual semiquantitative analysis of the amount of blood in the subarachnoid space (Figure 3). The base of the brain is divided into six parts and each part is scored according to the amount of blood as follows: Grade 0 – no blood, Grade 1 – minimal amount of blood, Grade 2 – moderate blood clot with recognizable vessels, Grade 3 – massive blood clot, vessels are not visible. The total score is calculated as a sum of all six sub-scores. Scores 13 - 18 represent severe SAH, scores 6-12 mild/moderate SAH, and animals with a score lower than 6 are usually excluded for insufficient SAH²⁹.

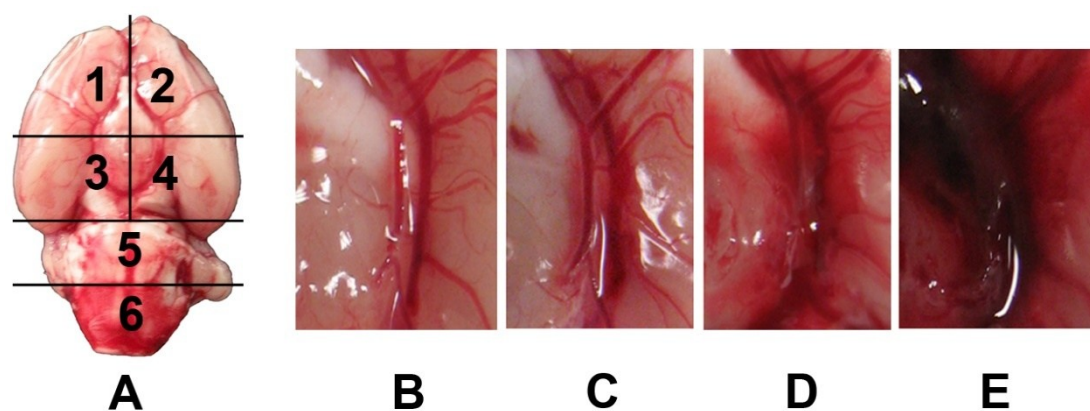


Figure 3: SAH grading system. A – Segments used for SAH evaluation. B – Grade 0: no blood in subarachnoid space. C – Grade 1: minimal amount of blood in subarachnoid space. D – Grade 2: moderate amount of blood in subarachnoid space with a recognizable vessel, E – Grade 3: large amount of blood in subarachnoid space, vessels are not recognizable.

Despite the differences between human beings and animal models, the basic mechanisms of pathophysiological reactions are similar between all the mammals. Experimental models of SAH allow us to study the pathophysiology of SAH in fine detail, in homogenous animal groups, and strictly defined conditions.

2. Clinical complications related to subarachnoid hemorrhage

2.1 Rebleeding and hydrocephalus

Rebleeding is a re-rupture of an aneurysm, and it is one of the most serious complications associated with SAH. Rebleeding rate is around 20% within 72h of SAH onset and around 70% of rebleeding cases occur within 6h after SAH³⁰. Rebleeding worse outcome in 40%-80% of patients and has a mortality rate of 20%-60%³⁰. Successful treatment of aneurysm is the best prevention of rebleeding. That is why the aneurysm should be treated as soon as possible.

Hydrocephalus is another severe complication of SAH which may develop early after the onset of bleeding or in a delayed manner. Acute hydrocephalus may develop within hours after onset of bleeding. Blood clots may obstruct the liquor system causing intracranial hypertension and subsequent cerebral ischemia leading to quick deterioration of neurologic functions and eventually death of the patient, if not treated. The risk of acute hydrocephalus development is more related to the presence of intraventricular blood rather than to the volume of cisternal hemorrhage³¹. Acute hydrocephalus affects approx. 20% of patients with SAH and acute intervention, implantation of external ventricular drainage, is required in patients with WFNS grade 2 or higher². Chronic hydrocephalus usually develops over weeks after SAH due to impaired resorption of cerebrospinal fluid (CSF), which causes slow deterioration of neurologic functions. Around 30% of patients after SAH need implantation of shunting system, most often ventriculoperitoneal shunt³².

2.2 Delayed cerebral ischemia and cerebral vasospasm

Delayed cerebral ischemia (DCI) is a severe late complication that affects around 30% of SAH patients³³. DCI most typically develops between 4th and 14th day after the onset of bleeding and significantly contributes to worsening outcomes^{33,34}. Cerebral vasospasm (CVS) develops in approximately 70% of patients with SAH, around 50% of these patients will be symptomatic due to cerebral ischemia, and finally, cerebral infarction develops in 50% of symptomatic patients³⁵. A larger amount of blood in the subarachnoid space is more associated with vasospasm development, while poor clinical status at admission is more associated with DCI development^{33,36,37}. Digital subtraction angiography is the gold standard for diagnostics of CVS, however, there are several other diagnostic tools to assess and monitor vasospasm development including transcranial doppler (TCD), CTA, and MRA. Complex neurointensive care, which is among others focused on the management of blood pressure, volume, and tonicity therapy, is crucial for the prevention and medical management of cerebral vasospasm³⁸. Thanks to modern neurointensive care, the risk of death or permanent disability due to CVS was reduced to less than 10%, however, the vasospasm is still a significant cause of poor outcome³⁵.

2.2.1 The author's publications related to the topic of vasospasms

2.2.1.1 Evaluation of changes in brain tissue oxygen levels (PbtO₂) for detection of vasospasm in patients after subarachnoid haemorrhage

Duris K, Smrčka M, Ševčík P, Gál R, Juráň V, Neuman E, Sova M, Vybíhal V, Kýr M. Evaluation of changes in brain tissue oxygen levels (PbtO₂) for detection of vasospasm in patients after subarachnoid haemorrhage. *Anesteziologie a Intenzivní Medicina*. 2009;20:102–106.

Type of article: Research article

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In this research, we aimed to evaluate the effect of SAH-associated CVS on oxygen levels in brain tissue (PbtO₂). PbtO₂ was monitored using the Licox system which represents a tool for direct local measurement of partial pressure of oxygen in tissue based on the principle of polarography. Licox probe is a Clark-type electrode isolated by Teflon membrane, which is permeable only to molecular oxygen. When oxygen reaches the cathode, it is electrolytically reduced generating a certain voltage. The area of monitoring is around 15mm² and the physiological value of PbtO₂ is above 20mmHg^{39,40}.

Twenty-six PbtO₂ records from seven patients with severe SAH (HH3-HH5) due to rupture of an aneurysm on arteria cerebri media (ACM) were analyzed. The PbtO₂ monitoring was performed for approximately ten days. The vasospasm screening was performed daily using TCD which represents a routine tool for vasospasm detection. Due to severe SAH, all the patients required mechanical ventilation, and a clinical experiment was performed following TCD assessment: Fraction of inspired oxygen (FiO₂) was increased to 100% for 10 minutes and the response in PbtO₂ was evaluated. Following the rise of FiO₂, there was a significantly higher increase in PbtO₂ in patients without CVS. The dynamics of PbtO₂ were also associated with clinical status at admission (HH) and outcome (GOS). Better HH as well as better GOS were associated with a higher increase in PbtO₂.

FiO₂ manipulation seemed to be an interesting option in CVS monitoring for patients with PbtO₂ monitoring, however, later research indicated that high concentration of oxygen may be an important contributor to oxidative stress⁴¹. Further research on the diagnostic use of FiO₂ manipulations was discontinued because of controversies around oxidative stress, though the use of 100% oxygen for a short period was still considered safe in intensive care at that time^{42,43}.

PŮVODNÍ PRÁCE

Možnosti využití mozkové tkáňové oxymetrie v detekci vazospasmů u pacientů po subarachnoidálním krvácení

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Souhrn

Cíl studie: Zhodnotit vliv klinického stavu pacienta při přijetí (Hunt Hesse, HH), léčebného výsledku (Glasgow Outcome Score, GOS) a přítomnosti vazospasmů na změny parciálního tlaku kyslíku v mozkové tkáni (PbtO₂) v závislosti na změnách frakce kyslíku ve vdechované směsi (FiO₂) u pacientů na umělé plicní ventilaci (UPV).

Typ studie: Prospektivní intervenční studie.

Typ pracoviště: Neurochirurgická klinika FN, Klinika anesteziologie, resuscitace a intenzivní medicíny.

Materiál a metoda: U pacientů se subarachnoidálním krvácením při ruptuře aneurysmatu bylo prováděno 10denní monitorování PbtO₂. Opakovaně byla prováděna diagnostika přítomnosti vazospasmů pomocí transkraniální dopplermetrie (TCD). U pacientů na UPV byl prováděn klinický pokus, při kterém bylo zvýšeno FiO₂ na 1,0 a sledována dynamika změn PbtO₂. Takto jsme pořídili 26 kontinuálních záznamů od 7 pacientů s diagnózou aneurysmatu *arteria cerebri media* (ACM). Hodnotili jsme vliv vazospasmů, klinického stavu pacienta při přijetí (HH) a outcome (GOS) na změny PbtO₂ při zvýšení FiO₂. Vyhodnocení bylo provedeno pomocí smíšených modelů.

Výsledky: Prokázali jsme statisticky významný rozdíl v dynamice změn PbtO₂ při zvýšení FiO₂ v závislosti na HH, GOS a přítomnosti vazospasmů. U pacientů v lepším klinickém stavu (HH3) a s lepším outcome (GOS5) dochází k výraznějšímu nárůstu PbtO₂ po zvýšení FiO₂ na 1,0 ($p < 0,001$ u obou parametrů). U pacientů bez vazospasmů dochází k výraznějšímu nárůstu PbtO₂ po zvýšení FiO₂ než u pacientů s vazospasmy ($p < 0,001$).

Závěr: Prokázali jsme vliv vazospasmů na dynamiku změn PbtO₂ při zvyšování FiO₂. Změny PbtO₂ při zvyšování FiO₂ mají souvislost i s dalšími faktory (HH, GOS).

Klíčová slova: tkáňová oximetrie – vazospasmy – subarachnoidální krvácení – aneurysma mozkových tepen

Abstract

Evaluation of changes in brain tissue oxygen levels (PbtO₂) for detection of vasospasm in patients after subarachnoid haemorrhage

Objective: Evaluation of the impact of the admission clinical status of subarachnoid haemorrhage (SAH) patients (Hunt Hesse Score, HH3), the outcome (Glasgow Outcome Score, GOS) and the presence of vasospasm on the changes in the brain tissue oxygen levels (PbtO₂), in relation to changes of FiO₂ in mechanically ventilated patients.

Design: Prospective interventional study.

Setting: Neurosurgical Department of University Hospital, Department of Anaesthesia and Resuscitation, University Hospital.

Materials and methods: We monitored PbtO₂ in patients with SAH due to aneurysm rupture for 10 days. Presence of vasospasms was repeatedly assessed with transcranial doppler (TCD). In patients on mechanical ventilation we performed the clinical trial. We increased the FiO₂ to 1.0 and measured the dynamics of changes of PbtO₂ simultaneously. Thus we obtained 26 continual measurements in the study group of 7 patients. The influence of vasospasm, the admission HH3 and GOS on changes of PbtO₂ after an increase of FiO₂ were evaluated. Mixed models analyses were used to evaluate the measured data.

Results: In the study group we observed a significant difference in the dynamics of PbtO₂ levels after an increase of FiO₂ in relation to the patients' admission clinical status (HH3), outcome (GOS) and the presence of vasospasm. The levels of PbtO₂ after an increase in FiO₂ increased more in the patients with the better clinical status (HH3) and better outcome (GOS) ($p < 0,001$ in both parameters). In patients without vasospasm we found a significantly higher increase of PbtO₂ levels after increasing FiO₂ than in patients with vasospasm ($p < 0,001$).

Conclusion: The dynamics of PbtO₂ levels were influenced by vasospasm. The changes of PbtO₂ levels after increasing FiO₂ are also associated with other factors (HH, GOS).

Keywords: brain tissue oximetry – vasospasm – subarachnoid hemorrhage – aneurysm of brain vessels

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Úvod

Závažnou komplikací u pacientů po subarachnoidálním krvácení (SAK) je výskyt vazospasmů, které vedou k ischemizaci mozkové tkáně. Vyskytují se až u 30 % pacientů [1] a mají až 30% mortalitu [2]. Příčina vazospasmů není doposud známa. Podle současné hypotézy oxyhemoglobin, uvolňovaný z rozpadajících se erytrocytů v subarachnoidálním prostoru, aktivuje gen pro endotelin-1, což je jeden z nejsilnějších známých vazokonstriktorů. Zároveň se oxyhemoglobin váže na molekuly oxidu dusnatého (NO), snižuje množství NO v cévní stěně a tím dále umožňuje nástup vazospasmů [3]. Vazospasmy vedou k ischemizaci mozkové tkáně. Ischemická tkáň propadá nekróze, a to postupně od oblasti s nejmenším kolaterálním oběhem (jádro infarktu) k oblasti s lepším kolaterálním oběhem (penumbra). Dokud penumbra existuje, je potenciálně zachránitelná [4]. Diagnostika vazospasmů je obtížná. Běžně používané monitorování nitrolebního tlaku (ICP) umožní diagnostikovat následky vazospasmů, kterými je zvýšení ICP v důsledku proběhlé ischemie; ICP tedy o vazospasmech informuje nepřímo a pozdě [5]. Přímou diagnostiku vazospasmů umožňuje transkraniální dopplerometrie (TCD), která však zatím neumožňuje kontinuální monitorování. Některé pacienty lze totiž TCD vyšetřit obtížně, navíc TCD umožňuje měřit průtok krve jen v proximálních kmenech mozkových cév. K časné detekci vazospasmů se v poslední době experimentálně užívá monitorování oxygenace mozkové tkáně [6]. Informaci o oxygenaci mozkové tkáně můžeme získat pomocí tkáňové oxymetrie. Tkáňová oxymetrie umožňuje lokální měření parciálního tlaku kyslíku (PbO_2) v libovolné části mozku. Byly vyvinuty dva systémy měření tkáňové oxymetrie. Systém Neurotrend (Codman) pracuje na bázi luminiscence, zatímco systém Licox (GSM Integra) funguje na principu polarografie. Mezi hodnotami naměřenými pomocí těchto dvou systémů existuje určitá diskrepance. Podle dosud publikovaných studií přináší přesnější a stabilnější výsledky systém Licox [7, 8, 9], který je v současnosti také rozšířenější. Systém Neurotrend již v současnosti není distribuován. Monitorování PbO_2 nachází uplatnění zejména v neurotraumatologii, kde slouží k detekci ischemických změn vedoucích k sekundárnímu poranění mozku [5, 7, 10, 11, 12, 13]. U pacientů se SAK (subarachnoidální krvácení) z ruptury aneurysmatu se tkáňová oxymetrie experimentálně užívá k časné detekci ischemických změn následkem vazospasmů [6, 13, 14], své uplatnění ovšem nachází i v peroperačním monitorování [15]. Čidlo se zavádí z malého trepanačního návrtníku, asi 2 cm pod *dura mater* do neokrevní oblasti mozku (oblast, ve které nejsou soustředěny důležité mozkové funkce). Rozsah měřené oblasti činí 5–17 mm². Fyziologické hodnoty jsou 25–35 mm Hg [16, 17, 18], hodnoty signalizující ischemii jsou nižší než 15 mm Hg [18]. U pacientů po kraniocerebrálním poranění bylo prokázáno, že

s rostoucí dobou poklesu PbO_2 pod 15 mm Hg nebo s poklesem PbO_2 pod 6 mm Hg, roste pravděpodobnost úmrtí [19]. U pacientů po SAK, kteří zemřeli, byl popsán vyšší výskyt poklesu středních hodnot PbO_2 pod 10 mmHg než u pacientů, kteří přežili [6]. Cílem práce je zhodnotit vliv: klinického stavu pacienta při přijetí (Hunt Hesse, HH) [20], výsledného stavu pacienta (Glasgow Outcome Score, GOS) [20] a přítomnosti vazospasmů na dynamiku změn PbO_2 v závislosti na změnách frakce kyslíku ve vdechované směsi (FiO_2) u pacientů na umělé plicní ventilaci (UPV).

Materiál a metoda

Do souboru byli zařazeni pacienti po SAK z ruptury aneurysmatu mozkové tepny. Pacienti s prokázaným SAK, jejichž stav nevyžadoval umělou plicní ventilaci, byli přijati na neurochirurgickou kliniku. Pacienti v těžkém klinickém stavu, kteří byli na UPV, byli přijati na kliniku anestezie, resuscitace a intenzivní medicíny. Po přijetí byla provedena angiografie, v případě nálezu aneurysmatu bylo u pacientů v klinickém stavu HH1–HH3 léčebnou metodou první volby operační řešení. U pacientů v klinickém stavu HH4 a HH5 byl léčebnou metodou první volby *coiling*. Pro zařazení pacienta do studie byl získán informovaný souhlas pacienta, v případě pacientů v bezvědomí byl souhlas se studií vyjádřen podpisy dvou lékařů, kteří se nepodíleli na tomto grantu. Studie byla schválena etickou komisí. U operovaných pacientů bylo čidlo k měření tkáňové oxymetrie zavedeno večer před operací, aby byla zajištěna dostatečně dlouhá doba pro stabilizaci čidla. V případě pacientů, u kterých byl proveden *coiling*, bylo čidlo implantováno 4. den po SAK, kdy začíná období největšího rizika vzniku vazospasmů trvající do 14. dne po SAK. Čidlo bylo vždy zavedeno 2 cm pod *dura mater*, do oblasti povodí mozkové tepny, na které bylo prokázáno aneurysma. Při monitorování povodí *arteria cerebri anterior* (ACA) bylo místo implantace mediálně od Kocherova bodu, při sledování povodí *arteria cerebri media* (ACM) to bylo laterálně od Kocherova bodu. Poloha čidla byla zpětně ověřena na kontrolním CT mozku. Po zavedení čidla následovalo 6hodinové období stabilizace čidla, údaje naměřené v tomto časovém úseku nebyly do hodnocení zahrnuty.

Monitorování PbO_2 probíhalo po dobu 10 dnů, u operovaných pacientů navíc probíhalo peroperační monitorování. Kontrolní CT mozku bylo prováděno první den po ošetření aneurysmatu a dále v intervalu 3–4 dnů, vyšetření TCD bylo prováděno v 2–3denním intervalu. Operovaní pacienti a pacienti s prokázanými vazospasmy mozkových tepen byli udržováni v mírné řízené hypotermii (34 °C), která byla u pacientů s vazospasmy aplikována nejlépe po dobu trvání vazospasmů, avšak maximálně do rozvoje nežádoucích komplikací hypotermie. V rámci grantu bylo do souboru celkem zařazeno 39 pacientů, 4 pacienti byly po sléze vyřazeni pro chybné zavedení čidla.

Do zkoumaného souboru pro účely této práce bylo zařazeno 7 pacientů na UPV, u kterých byl v návaznosti na vyšetření TCD (v intervalu 2–3 dnů) prováděn klinický pokus, při kterém bylo po 3minutovém klidovém záznamu na 10 minut zvýšeno FiO_2 na 1,0 a sledována reakce PbtO_2 . Takto jsme pořídili 26 kontinuálních záznamů. Jako hlavní parametr jsme hodnotili vliv vazospasmů na dynamiku změn PbtO_2 při zvýšení FiO_2 . Jako doplňující parametry byly vyhodnoceny HH (jako ukazatel klinického stavu) a GOS (jako výsledný stav pacienta). U všech pacientů zkoumaného souboru bylo zavedeno monitorování intrakraniálního tlaku (ICP) parenchymovým čidlem nebo pomocí zevní komorové drenáže. Během periody klidového záznamu i během experimentálního zvyšování FiO_2 se pohybovaly hodnoty středního arteriálního tlaku a intrakraniálního tlaku v rozmezí fyziologických hodnot.

Vzhledem k charakteru dat byla hodnocená proměnná PbtO_2 logaritmicky transformována, což umožnilo následné parametrické vyhodnocení. Na základě grafického zhodnocení dat jsme k longitudinální analýze použili data z třímínutového klidového záznamu před zvýšením FiO_2 a data získaná v období 6 minut po zvýšení FiO_2 na 1,0. Po tomto časovém intervalu již dochází k linearizaci křivek PbtO_2 a další nárůst není patrný. Analýza longitudinálních dat (opakovaná měření PbtO_2 v čase u jednotlivých kontinuálních záznamů) byla provedena pomocí smíšených modelů – s interceptem a lineárním, popř. kvadratickým efektem času – modelovaných jako náhodné pro každý dvouúrovňový model. Použili jsme dvouúrovňový model. Na první úrovni byla závislá proměnná $y = \log(\text{PbtO}_2)$ uvažována jako kvadratická funkce času s náhodnými parametry pro každý kontinuální záznam. Na druhé úrovni modelu byly náhodné parametry z první úrovně vysvětlovány zkoumanými faktory modelovanými jako fixní efekty; hodnotili jsme HH, GOS, výskyt vazospasmů. Pro detailnější popis a použití smíšených modelů odkazujeme čtenáře na citovanou literaturu [21, 22]. Pro snazší pochopení výsledků uvádíme grafickou reprezentaci modelů s modelovaným průměrem trendů. Za hladinu významnosti jsme standardně považovali hodnotu $\alpha = 0,05$.

Výsledky

Ve zkoumaném souboru jsme pořídili 26 kontinuálních záznamů sedmi pacientů s diagnózou aneurysma ACM, u šesti žen a jednoho muže. Průměrný věk činil 44,6 let (min. 29 let, max. 62 let). Operování byli tři pacienti, coiling byl proveden u čtyř pacientů. Charakteristika souboru podle HH je v tabulce 1, charakteristiku

Tabulka 1. Charakteristika souboru podle HH

HH	Počet pacientů	Počet záznamů
3	2	8
4	3	12
5	2	6

Tabulka 2. Charakteristika souboru podle přítomnosti GOS

GOS	Počet pacientů	Počet záznamů
1 (smrt)	4	14
2,3	1	4
4,5	2	8

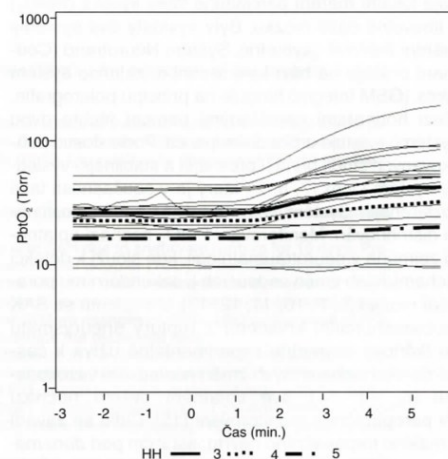
Tabulka 3. Charakteristika souboru podle přítomnosti vazospasmů

Vazospasmy	Počet pacientů	Počet záznamů
Ano	1	3
Ne	2	8
Obojí	4	15

Kategorie: ANO – vazospasmy přítomny při všech TCD vyšetřeních u daného pacienta; NE – vazospasmy nebyly přítomny při žádném TCD vyšetření u daného pacienta; Obojí – u daného pacienta část TCD vyšetření s průkazem vazospasmů a část vyšetření bez průkazů vazospasmů

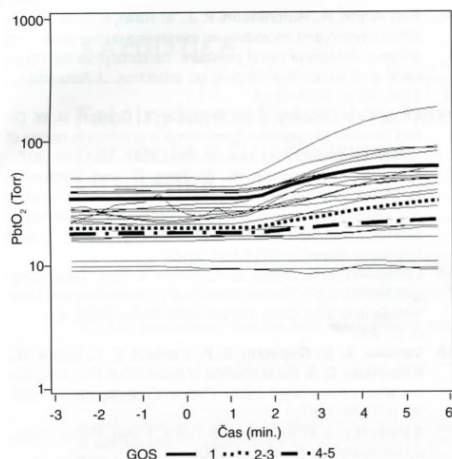
souboru podle GOS uvádí tabulka 2 a charakteristiku souboru podle výskytu vazospasmů tabulka 3.

Ve zkoumaném souboru jsme prokázali signifikantní rozdíl v dynamice změn PbtO_2 u skupin dělených podle HH (graf 1), GOS (graf 2) a přítomnosti vazospasmů (graf 3). Po zvýšení FiO_2 roste hladina PbtO_2 u skupiny HH3 rychleji než u ostatních pacientů ($p < 0,001$) a také u skupiny HH4 dochází ve srovnání se skupinou HH5 k rychlejšímu nárůstu PbtO_2 ($p < 0,001$). Rozdíl ve vstupní hladině PbtO_2 mezi výše uvedenými skupinami nebyl statisticky významný ($p = 0,86$). U pacientů ve skupině GOS 5, 4 (dobrý outcome) dochází po zvýšení FiO_2 k rychlejšímu nárůstu než u ostatních pacientů ($p < 0,001$) a také u pacientů s GOS 2, 3 (špatný outcome) dochází k rychlejšímu nárůstu PbtO_2 než u skupiny GOS 1 (smrt) – $p < 0,001$. Rozdíl ve vstupní hladině PbtO_2 mezi skupi-



Graf 1. Modely dynamiky změn PbtO_2 po zvýšení FiO_2 (čas 0) u skupin podle klinického stavu při přijetí (klasifikováno podle Hunt-Hesse)

Dynamika změn hladin PbtO_2 je mezi skupinami statisticky významně rozdílná ($p < 0,001$).



Graf 2. Modely dynamiky PbtO₂ po zvýšení FiO₂ (čas 0) u skupin podle GOS
Dynamika změn hladin PbtO₂ je mezi skupinami statisticky významně rozdílná ($p < 0,001$).

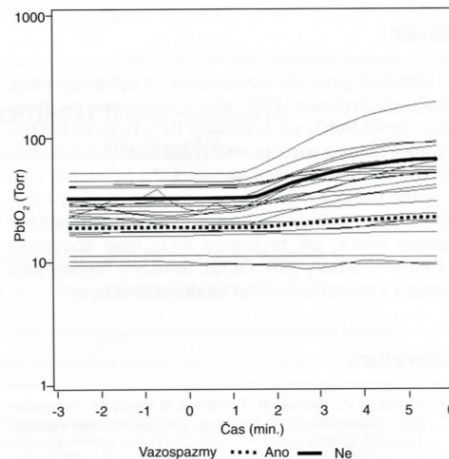
nami podle GOS byl statisticky významný ($p = 0,003$). U pacientů s vazospasmy, které byly prokázány pomocí TCD (rychlost toků nad 120 cm · s⁻¹), dochází po zvýšení FiO₂ k minimálnímu nárůstu hladiny PbtO₂, zatímco u pacientů bez vazospasmů PbtO₂ výrazně narůstá. Rozdíl v dynamice změn PbtO₂ mezi oběma skupinami je statisticky významný ($p < 0,001$), stejně jako rozdíl ve vstupní hladině PbtO₂ ($p < 0,001$). Ten je u skupiny s vazospasmy nižší než u skupiny bez vazospasmů.

Diskuse

Vazospasmy jsou závažnou komplikací SAK z ruptury aneurysmatu, které jsou spojeny s vysokou mortalitou. Diagnostika vazospasmů je obtížná.

Monitorování PbtO₂ může vést k časné detekci ischemických událostí [5, 23] a je považováno za bezpečné a spolehlivé [24]. Pokus o diagnostiku vazospasmů pomocí monitorování PbtO₂ u umělé ventilovaných pacientů zatím proveden nebyl, avšak změny PbtO₂ při zvyšování FiO₂ již byly popsány [23, 25, 26]. Předpokládá se, že hlavní podíl na tomto jevu má kyslík rozpouštěný v krvi. Ten sice tvoří pouze 2 % kyslíku transportovaného krví, avšak zvyšuje uvolňování kyslíku z hemoglobinu, a tím se dostane víc kyslíku do tkáně [27].

U pacientů s vazospasmy nedochází k patrnému nárůstu hodnot PbtO₂ po zvýšení FiO₂, jak je patrné u pacientů bez vazospasmů, kde dochází k výraznému nárůstu (viz graf 3). Toto zjištění je dále podporováno pozorováním, že u pacientů v horším klinickém stavu (HH5) nebo s horším outcome (GOS1) také nedochází k výraznému nárůstu PbtO₂ po zvýšení FiO₂ (viz grafy 2 a 3). Předpokládáme, že výskyt vazospas-



Graf 3. Modely dynamiky PbtO₂ po zvýšení FiO₂ (čas 0) u skupin podle výskytu vazospasmů
Dynamika změn hladin PbtO₂ je mezi skupinami statisticky významně rozdílná ($p < 0,001$).

mů koreluje s horším stavem (HH5) a outcome (GOS1).

Reakce PbtO₂ po zvýšení FiO₂ se projeví s latencí 1–2 minut, jak je patrné z grafů, a po 5–6 minutách dochází k linearizaci křivek PbtO₂; další nárůst již není patrný.

Jsme si vědomi možného zkreslení výsledků, které je dáno hodnocením 26 záznamů od sedmi pacientů. Charakteristika každého pacienta může mít významnější vliv na dynamiku hodnoceného parametru, než je vliv každého konkrétního záznamu. Toto zkreslení předpokládáme spíše u vlivu faktorů HH a GOS. Důležitějším závěrem je však prokázaný význam vazospasmů na vliv změn dynamiky PbtO₂. Vzhledem k tomu, že každý pacient může mít kontinuální záznam pořízený v situaci s vazospasmy nebo bez nich (což je klinicky důležitý předpoklad). Zároveň předpokládáme, jak naznačuje tabulka 3, že při hodnocení tohoto parametru je minimální vliv charakteristiky jednotlivého pacienta a naopak relevantní hodnocení jednotlivých kontinuálních záznamů v kontextu klinické aplikace. V této práci má hodnocení HH a GOS doplňující charakter.

Použití kvadratického modelu v periodě do 6 minut po zvýšení FiO₂ považujeme za jednoduchý a zároveň dostatečně reprezentativní model. Smíšené modely nacházejí v medicíně uplatnění zejména při hodnocení růstových křivek [28] nebo dynamiky zánětlivých parametrů [29]. Výhodu smíšených modelů k vyhodnocení našich dat vidíme zejména v elegantním řešení vzájemné korelace opakovaných měření, ve vyřazení se s rozdílnými vstupními hodnotami FiO₂ před započítáním experimentu (implicitní charakteristika smíšených modelů) a zároveň v možnosti klasického regresního modelu [21, 22].

Závěr

Prokázali jsme vliv vazospasmů na dynamiku změn PbtO_2 při zvyšování FiO_2 . Vliv vazospasmů na dynamiku změn PbtO_2 při zvyšování FiO_2 by mohl být přínosný k detekci vazospasmů u pacientů po SAK, kteří jsou na umělé plicní ventilaci. To by mohlo vést k využití tkáňové oxymetrie k rychlé a jednoduché diagnostice u pacientů po SAK z ruptury aneurysmatu. Změny PbtO_2 při zvyšování FiO_2 mají souvislost i s dalšími faktory (HH, GOS). Smíšené modely jsou vhodné k vyhodnocení dat tohoto charakteru.

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2.2.1.2 Correlation between Brain Tissue Oxygen Monitoring Parameters and Transcranial Dopplerometry in Patients with Severe Subarachnoid Hemorrhage

Duris K, Neuman E, Mrlian A, Vybihal V, Juran V, Kyr M, Smrcka M. Correlation between Brain Tissue Oxygen Monitoring Parameters and Transcranial Dopplerometry in Patients with Severe Subarachnoid Hemorrhage. *Cesk. Slov. Neurol. Neurochir.* 2014;77:196–201.

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The goal of this research was to evaluate the correlation between TCD parameters and PbtO₂ in patients suffering from SAH.

Several parameters of TCD reflect CVS of which the most important are velocities reflecting the blood flow in a particular vessel: peak systolic velocity (PSV), end-diastolic velocity (EDV), and mean velocity (V_{mean}). Mean velocities higher than 120cm/s in ACM, 90cm/s in ACI, and 70 cm/s in basilar artery are considered as thresholds for cerebral vasospasm⁴⁴. Other important parameters are the pulsatility index (PI) and resistance index (RI) which are based on the difference in velocity between systolic blood flow and diastolic blood flow reflecting flow resistance. PI is a complex parameter calculated as follows: $PI = (PSV - EDV) / V_{\text{mean}}$ and the physiological value is below 1.2⁴⁵. When simplified, we may say that PI reflects overall resistance to blood flow, more accurately it reflects a complex interplay among cerebrovascular resistance/compliance and cerebral perfusion pressure⁴⁶. RI is calculated as follows: $RI = (PSV - EDV) / PSV$ and the physiological value is below 0.75⁴⁵. RI reflects resistance to blood flow distally to probe, for example in microvessels⁴⁵.

Forty-two PbtO₂ records from twenty-two patients with severe aneurysmal SAH (HH4-5) were analyzed. Periods of +/-10 or +/-60 minutes of PbtO₂ monitoring, with a node point corresponding to TCD assessment, were analyzed. No association of PbtO₂ parameters with any of the velocities was found, however, there was a significant correlation of both PI and RI with PbtO₂ in both 10 and 60 min. periods.

Since PbtO₂ is a direct tissue measurement, it reflects conditions related to microvasculature/vessel resistance/cerebral perfusion pressure in a more complex manner than just blood flow velocities.

Vztah mezi parametry transkraniální dopplerometrie a tkáňovou oxymetrií u pacientů s těžkým subarachnoidálním krvácením

Correlation between Brain Tissue Oxygen Monitoring Parameters and Transcranial Dopplerometry in Patients with Severe Subarachnoid Hemorrhage

Souhrn

Cíl: Cílem práce bylo zjistit, zda existuje korelace mezi hodnotami PbtO₂ a parametry transkraniální dopplerometrie (TCD) u pacientů s těžkým subarachnoidálním krvácením (SAK). **Soubor a metodika:** Do souboru byli zařazeni pacienti s těžkým subarachnoidálním krvácením na podkladě ruptury aneurysmatu mozkové tepny, kteří byli v klinickém stavu Hunt Hess 4 (HH4, sopor) a Hunt Hess 5 (HHS, kóma). U pacientů byl kromě standardních medicínských postupů, které zahrnují i TCD, prováděn monitoring oxygenace mozkové tkáně (partial pressure of oxygen in brain tissue – PbtO₂, systém Licox). Hodnocena byla korelace mezi PbtO₂ a parametry TCD. **Výsledky:** Celkem jsme do souboru zařadili 27 pacientů, pět pacientů bylo následně vyřazeno pro špatnou pozici nebo předčasnou malfunkci PbtO₂ čidla. U zkoumaného souboru korelovaly hodnoty PbtO₂ s pulzatilním a rezistentním indexem v intervalech ± 10 min (PI: $r = -0,4077$, $p = 0,0074$; RI: $r = -0,4055$, $p = 0,0077$) a ± 60 min (PI: $r = -0,4145$, $p = 0,0064$; RI: $r = -0,4089$, $p = 0,007$), přičemž výchozí bod hodnocení odpovídal vyšetření TCD. U zkoumaného souboru nebyla signifikantní korelace mezi PbtO₂ a rychlostmi toků na zkoumaných cévách. **Závěr:** PbtO₂ monitoring poskytuje informace o stavu oxygenace mozkové tkáně a její mikrovaskulatury. Nicméně neexistuje jednoduchá souvislost mezi hodnotami PbtO₂ a rychlostmi toků na magistralních cévách, které charakterizují cévní vazospazmy. Protože neznáme vztah mezi rychlostmi toků na magistralních cévách a hodnotami PbtO₂, jeví se v klinické praxi u pacientů s těžkým SAK vhodné paralelní užití TCD i PbtO₂ monitoringu.

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subarachnoidální krvácení – transkraniální dopplerometrie – cerebrální vazospazmy

Key words

subarachnoid hemorrhage – transcranial Doppler sonography – cerebral vasospasm

Abstract

Objectives: The aim of this study was to evaluate correlation between brain tissue oxygen level and transcranial dopplerometry (TCD) parameters in patients with severe subarachnoid hemorrhage (SAH). **Patients and methods:** Patients with subarachnoid hemorrhage from a rupture of cerebral vessel aneurysm and clinical status within the Hunt-Hess scale grade 4 (sopor) or 5 (coma) were enrolled into the study. Brain tissue oxygen monitoring (PbtO₂, Licox system) was performed in addition to the standard ICU monitoring that includes TCD. The correlation between TCD and PbtO₂ parameters was evaluated. **Results:** We enrolled a total of 27 patients, five patients were subsequently excluded for malposition or malfunction of the PbtO₂ probe. There was a significant correlation between PbtO₂ and both pulsatility index and resistivity index in ± 10 minute (PI: $r = -0.4077$, $p = 0.0074$; RI: $r = -0.4055$, $p = 0.0077$) and ± 60 minute intervals (PI: $r = -0.4145$, $p = 0.0064$; RI: $r = -0.4089$, $p = 0.007$) with a node point corresponding to TCD measurement. There was no significant correlation between PbtO₂ and any of the velocity parameters. **Conclusion:** PbtO₂ monitoring provides information on brain tissue oxygenation and its microvasculature. However, there is no direct link between PbtO₂ values and TCD velocities, the main characteristics of cerebral vasospasm. While we do not know the dynamics between velocities of the main cerebral vessels and PbtO₂, the use of both PbtO₂ monitoring and TCD seems to be appropriate for follow up of patients with severe SAH in clinical setting.

Úvod

Subarachnoidální krvácení (SAK) je závažná patologie, která ročně postihuje asi 10/100 000 obyvatel a která je spojena až s 50% mortalitou [1]. Nejčastěji je příčinou SAK ruptura aneuryzmatu mozkové tepny, jež odpovídá za cca 85 % případů [2]. Zásadní léčebný postup je ošetření aneuryzmatu (operačně nebo endovaskulárně) mající za cíl předejít opakovanému prasknutí aneuryzmatu, které je spojeno s úmrtností až 80 % [2]. Vedle opakovaného krvácení jsou nejzávažnější komplikací SAK cévní vazospazmy vedoucí k ischemizaci mozkové tkáně. Vazospazmy typicky vznikají mezi 4. a 14. dnem po atace krvácení, postihují asi 30 % pacientů a jsou zatíženy až 30% mortalitou [3–5]. Přesná příčina vzniku vazospasmů není známa, ale byla popsána celá řada faktorů, které se na jejich vzniku podílejí. Mezi nejdůležitější patří porucha kontraktility cév, dysfunkce endotelu, toxicita rozpadových produktů hemoglobinu a zánět [6,7]. V léčbě vazospasmů se používá HHH terapie (hypertenze, hemodiluce, hypervolemie), nimodipin a magnezium, avšak léčba vazospasmů je i dnes problematická a není dostatečně účinná [8]. Proto jsou vazospazmy předmětem intenzivního studia v experimentální i klinické rovině. Vedle objasnění patofyziologie jsou důležitým cílem výzkumu možnosti časné detekce vzniku vazospasmů.

Zlatým standardem v diagnostice vazospasmů je digitální subtrakční angiografie (DSA), která však není vhodná k monitoringu pacientů z důvodu inva-

zivitu a radiační zátěže [9]. K diagnostice a sledování vazospasmů se u pacientů po SAK v klinické praxi nejčastěji užívá transkraniální dopplerometrie (TCD). Ta není v diagnostice vazospasmů tak přesná jako DSA, je to však neinvazivní metoda umožňující relativně rychle vyšetření u lůžka pacienta [9,10]. TCD zatím rutinně neumožňuje kontinuální monitorování, metoda je velmi závislá na vyšetřujícím lékaři, někteří pacienti jsou obtížně vyšetřitelní, a proto jsou zkoumány další metody sloužící k časné detekci vazospasmů [11]. Jednou z nadějných metod je tkáňová oxy-metrie, která nalézá své uplatnění nejen u pacientů po SAK [12–14].

Cílem naší práce bylo zjistit, zda existuje korelace mezi hodnotami PbtO₂ a parametry TCD u pacientů s těžkým subarachnoidálním krvácením.

Soubor a metodika

Projekt i všechny postupy byly schváleny etickou komisí FN Brno. Do souboru byli zařazeni pacienti s těžkým subarachnoidálním krvácením na podkladě ruptury aneuryzmatu mozkové tepny, kteří byli v klinickém stavu HH4 (sopor) a HH5 (kóma). Všechny léčebné postupy včetně ošetření aneuryzmatu, péče na jednotce intenzivní péče i vyšetření pomocí TCD probíhaly obvyklým způsobem. Obecným cílem léčby bylo ošetřit aneuryzma co nejdříve a dále udržovat systolický tlak minimálně na 140 torrů u pacientů bez vazospasmů a minimálně na 180 torrů u pacientů s vazospazmy. Léčba však byla v jednotlivých případech individualizována s ohledem na věk, celkový

stav pacienta s důrazem na kardiovaskulární onemocnění. U pacientů zařazených do studie byl navíc prováděn monitoring PbtO₂ (systém Licox). Čidlo bylo zavedeno čtvrtý den po SAK a kontinuální monitorování probíhalo tak, aby bylo pokryto období nejvyššího rizika vzniku vazospasmů. Monitoring byl ukončen v optimálním případě po 10 dnech od zavedení čidla nebo dříve v případě, že čidlo přestalo měřit (záporné nebo nulové hodnoty po dobu několika hodin). Monitorovanou oblastí bylo povodí arterie cerebri media ipsilaterálně k lokalizaci aneuryzmatu. V případě středočárových aneurysmat bylo nejprve zhodnoceno, zda lze identifikovat dominantní cévu zásobující AcoA. Bylo-li možné určit dominantní cévu, bylo čidlo zavedeno ipsilaterálně. Pakliže dominantní cévu nebylo možné určit, bylo čidlo zavedeno do nedominantní hemisféry.

Parametry PbtO₂ byly hodnoceny jednak z celého období monitoringu (v dalším textu označován jako PbtO₂ monitoring) a jednak z omezených časových úseků ± 10 min a ± 60 min v návaznosti na TCD vyšetření (v dalším textu označován jako korelace PbtO₂ k TCD).

Hodnoty PbtO₂ monitorování byly hodnoceny v souvislosti s následujícími klinickými parametry: klinický stav při přijetí (Hunt Hess skóre, dále jen HH), rozsah hematomu dle vstupního CT (Fischer skóre, dále jen Fischer) a výsledný stav pacienta (Glasgow Outcome Scale, dále GOS). Hodnoty PbtO₂ byly pro každého pacienta zprůměrovány a průměrné hodnoty byly použity k další analýze. Ke statistickému

Tab. 1. Parametry GOS v souvislosti s přítomností vazospazmů. Je patrná tendence k vyššímu výskytu vazospazmů u pacientů s horším výsledným stavem, avšak k validnímu statistickému zpracování dat není dostatečný počet pacientů.

GOS	Vazospazmy ano	Vazospazmy ne
1 (smrt)	6	2
2 + 3 (stav neuspokojivý)	6	5
4 + 5 (stav uspokojivý)	1	2

zpracování dat byl použit Studentův t-test a one-way ANOVA.

Hodnocení korelace PbtO₂ ve vztahu k TCD probíhalo ze záznamů odpovídajícím intervalům ± 10 min a ± 60 min, přičemž za uzlový bod bylo považováno vyšetření TCD. Průměrné hodnoty PbtO₂ za daný časový interval byly korelovány s následujícími parametry TCD: maximální systolická rychlost toku (Peak Systolic Velocity, PSV), rychlost toku na konci diastoly (End Diastolic Velocity, EDV), střední průtoková rychlost (mean Velocity, Vmean), index pulzatility (Pulsatility Index, PI), index rezistence (Resistivity Index, RI). Korelace výše uvedených parametrů byla popsána pomocí Pearsonova korelačního koeficientu. Za hladinu významnosti byla považována hodnota $\alpha = 0,05$.

Výsledky

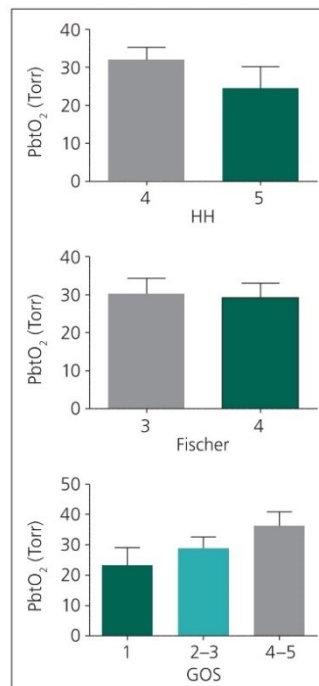
Charakteristika souboru

Celkem bylo do souboru zařazeno 27 pacientů. Pět pacientů bylo následně vyřazeno. Důvodem k vyřazení byla poloha čidla v subdurálním prostoru (dva pacienti) nebo předčasná malfunkce čidla (tři pacienti). Z hodnoceného souboru 22 pacientů bylo 17 žen a pět mužů, přičemž průměrný věk souboru činil 53,3 let (minimum 28, maximum 70). Charakteristika souboru dle klinického stavu při přijetí: 15 pacientů bylo v klinickém stavu HH4 (sopor), v klinickém stavu HH5 (kóma) bylo sedm pacientů.

Charakteristika souboru dle tíže krvácení: u šesti pacientů odpovídal CT nález stupni Fischer 3 (šířka hematomu nad 1 mm), u 16 pacientů byl nález odpovídající stupni Fischer 4 (hemocefalus). Charakteristika souboru dle lokalizace aneuryzmatu: aneuryzma bylo lokalizováno na ACM v 12 případech, u šesti pacientů bylo aneuryzma na AcoA a na AcoP bylo aneuryzma lokalizováno u čtyř pacientů. Operováno bylo 14 pacientů, zatímco coiling byl použit u osmi pacientů. Charakteristika souboru dle výsledného stavu: GOS 4 a 5 (dobrý výsledný stav) – tři pacienti, GOS 3 a 2 (neuspokojivý výsledný stav) – 11 pacientů, GOS 1 (smrt) – osm pacientů. Tab. 1 uvádí parametry GOS v souvislosti s přítomností vazospazmů. Za kritérium vazospazmů byla považována hodnota Vmean nad 120 cm/s na arteria cerebri media a hodnota nad 90 cm/s na arteria cerebri anterior [15]. Z tabulky je patrná tendence k vyššímu výskytu vazospazmů u pacientů s horším výsledným stavem, avšak k validnímu statistickému zpracování dat jsme neměli k dispozici dostatečný počet pacientů. Následující statistické analýzy byly provedeny za použití parametrických statistických metod vzhledem k přibližně normálnímu rozložení dat (zjištěno na základě grafické analýzy).

PbtO₂ monitoring

Průměrná délka monitoringu PbtO₂ činila u zkoumaného souboru 7,59 dní, přičemž



Obr. 1. Parametry PbtO₂ monitoringu v závislosti na HH, Fischer a GOS.

Je patrná tendence k vyšším hladinám PbtO₂ u pacientů v lepším klinickém stavu při přijetí (HH4), u pacientů s lepším výsledným stavem (GOS 4–5), avšak rozdíl není statisticky významný. Není patrná žádná tendence k rozdílu v hodnotách PbtO₂ u skupin dělených dle tíže krvácení (Fischer). Data jsou vyjádřena jako průměr (m) a standardní chyba průměru (SEM).

nejkratší záznam trval 4,60 dne a nejdelší 10 dní. K hodnocení bylo použito celkem 22 záznamů. Parametry PbtO₂ monitoringu v závislosti na HH, Fischer a GOS uvádí obr. 1. U zkoumaného souboru

Tab. 2. Charakteristiky hodnot PbtO₂ v intervalech ± 10 min a ± 60 min u souboru jako celku a dále u skupin pacientů s a bez vazospazmů. Rozdíly mezi hodnotami PbtO₂ u skupin s vazospazmy a bez vazospazmů nejsou statisticky významné ani v jednom z intervalů. Data jsou vyjádřena jako průměr (m) a standardní chyba průměru (SEM).

Interval	± 10 min				± 60 min	
Skupina	Celek	Vazospazmy ano	Vazospazmy ne	Celek	Vazospazmy ano	Vazospazmy ne
m	31,82	32,56	31,51	32,76	32,65	32,84
SEM	2,85	4,84	3,07	2,71	4,50	3,45

Tab. 3. Výsledky korelační analýzy mezi PbtO₂ a parametry TCD pro intervaly ± 10 min a ± 60 min. Signifikantní je korelace PbtO₂ s PI a RI v obou časových intervalech. U ostatních parametrů TCD (PSV, EDV, Vmean) korelace signifikantní není ani v jednom časovém intervalu.

		PSV	EDV	Vmean	PI	RI
± 10 min	Pearson r	0,1119	0,2355	0,2056	-0,4077	-0,4055
	p	0,4804	0,1333	0,1915	0,0074	0,0077
± 60 min	Pearson r	0,06897	0,2056	0,1756	-0,4145	-0,4089
	p	0,6643	0,1914	0,2661	0,0064	0,0072

je patrná tendence k vyšším hladinám PbtO₂ u pacientů v lepším klinickém stavu při přijetí (31,99 torru u skupiny HH4 vs 24,44 torru u skupiny HH5), u pacientů s lepším výsledným stavem (36,31 torru pro skupinu GOS 4 + 5; 28,85 torru pro GOS 3 + 2 a 23,27 pro skupinu GOS 1), avšak rozdíl není statisticky významný ($p = \text{NS}$ pro všechny výše uvedené skupiny). U skupin dělených dle tíže krvácení není patrná žádná tendence k rozdílu v hodnotách PbtO₂ ($p = \text{NS}$).

Korelace PbtO₂ a TCD

Hodnocení bylo provedeno z hodnot PbtO₂ v intervalu ± 10 a ± 60 min v návaznosti na vyšetření TCD. K hodnocení bylo použito celkem 42 záznamů od 22 pacientů. Tab. 2 ukazuje charakteristiky hodnot PbtO₂ intervalů ± 10 min a ± 60 min u souboru jako celku a dále u skupin pacientů s a bez vazospazmů. Za kritérium vazospazmů byla považována hodnota Vmean nad 120 cm/s na ipsilaterální arteria cerebri media. Rozdíly mezi hodnotami PbtO₂ u skupin s vazospazmy a bez vazospazmů nejsou statisticky významné ani v jednom z výše uvedených intervalů ($p = \text{NS}$). Tab. 3 a obr. 2 ukazují výsledky korelační analýzy mezi jednotlivými parametry TCD a průměrnými hodnotami PbtO₂ v daných časových intervalech. U zkoumaného souboru byla signifikantní korelace mezi PbtO₂ a PI v intervalu ± 10 min ($r = -0,4077$, $p = 0,0074$) i v intervalu ± 60 min ($r = -0,4145$, $p = 0,0064$). Dále byla u zkoumaného souboru signifikantní korelace mezi hodnotami PbtO₂ a RI v intervalu ± 10 min ($r = -0,4055$, $p = 0,0077$) i v intervalu ± 60 min ($r = -0,4089$, $p = 0,0072$). U ostatních parametrů TCD (PSV, EDV, Vmean) korelace u zkoumaného souboru signifikantní nebyla ani v jednom časovém intervalu ($p = \text{NS}$ pro všechny uvedené parametry).

Diskuze

Cílem této práce bylo zjistit, zda existuje korelace mezi hodnotami PbtO₂ a parametry TCD u pacientů s těžkým subarachnoidálním krvácením z ruptury aneurysmatu mozkové tepny.

Tkáňová oxymetrie slouží ke kontinuálnímu měření parciálního tlaku kyslíku v tkáni (PbtO₂). Jedná se o invazivní metodu umožňující lokální měření PbtO₂. Systém Licor, který používáme na naší klinice, funguje na principu polarografie a umožňuje měřit hodnoty PbtO₂ z plochy o rozměrech 7,5–15 mm² [16]. Klinicky významné vazospazmy jsou spojeny s ischemizací mozkové tkáně [17], a proto se tkáňová oxymetrie jeví jako vhodný nástroj k časné detekci následků vazospazmů. Problém však spočívá v tom, že dosud nebyly definitivně stanoveny meze fyziologických hodnot PbtO₂. Například studie, kterou uveřejnili Jödicke, Hübner a Böcker uvádí jako dolní mez normy 19,5 torru [18], Meixensbergerova studie uvádí jako fyziologické hodnoty 48 torrů [19]. Za mez signalizující ischemii jsou však všeobecně považovány hodnoty 15–20 torrů, přičemž důležitá je doba, po kterou je mozek kritickým hodnotám vystaven [20,21]. U pacientů po kraniocerebrálním poranění bylo prokázáno, že s rostoucí dobou poklesu PbtO₂ pod 15 torrů rostou pravděpodobnost úmrtí [20]. U pacientů, kteří zemřeli následkem SAK, byl ve srovnání s přeživšími pacienti popsán vyšší výskyt středních hodnot PbtO₂ pod 10 torrů [22]. Další studie prokázala, že dlouhodobý pokles PbtO₂ pod 20 torrů je asociovan s horším výsledným stavem [21]. Souvislost mezi parametry TCD a hodnotami PbtO₂ však zatím popsána nebyla.

Monitorovaná oblastí bylo ve všech případech povodí ACM, neboť zavést čidlo do tohoto povodí je možné s vyso-

kou mírou jistoty, což neplatí pro zavádění čidla do povodí ACA a monitoring v povodí ACP se na našem pracovišti neprovádí často. Znalost povodí monitoringu byla důležitá, protože hodnocení bylo provedeno na základě párování záznamů pořízených pomocí TCD a systému Licor. Z hlediska hodnocení praktického využití metody bylo tedy nejlepší provádět monitoring v povodí ACM, i když bylo aneurysma lokalizováno v jiném povodí.

U námi zkoumaného souboru se u PbtO₂ monitoringu průměrné hodnoty pohybovaly nad 20 torrů. To si vysvětlujeme tím, že monitoring byl zahájen poměrně časně po SAK (4. den), tedy v době, kdy ještě vazospazmy u většiny pacientů nejsou vyvinuty a dá se předpokládat, že hodnoty PbtO₂ budou v tomto období vyšší než v období, kdy jsou již vazospazmy plně vyvinuty. Monitoring trval nejdéle po dobu 10 dní, potom bylo čidlo zrušeno, aby bylo minimalizováno riziko infekčních komplikací. Takový postup je považován za bezpečný a je spojen s minimálním rizikem pro pacienta [23]. Období monitoringu mezi 4. a 14. dnem po SAK bylo zvoleno proto, aby byla pokryta perioda největšího rizika vzniku vazospazmů, která je důležitá z klinického hlediska.

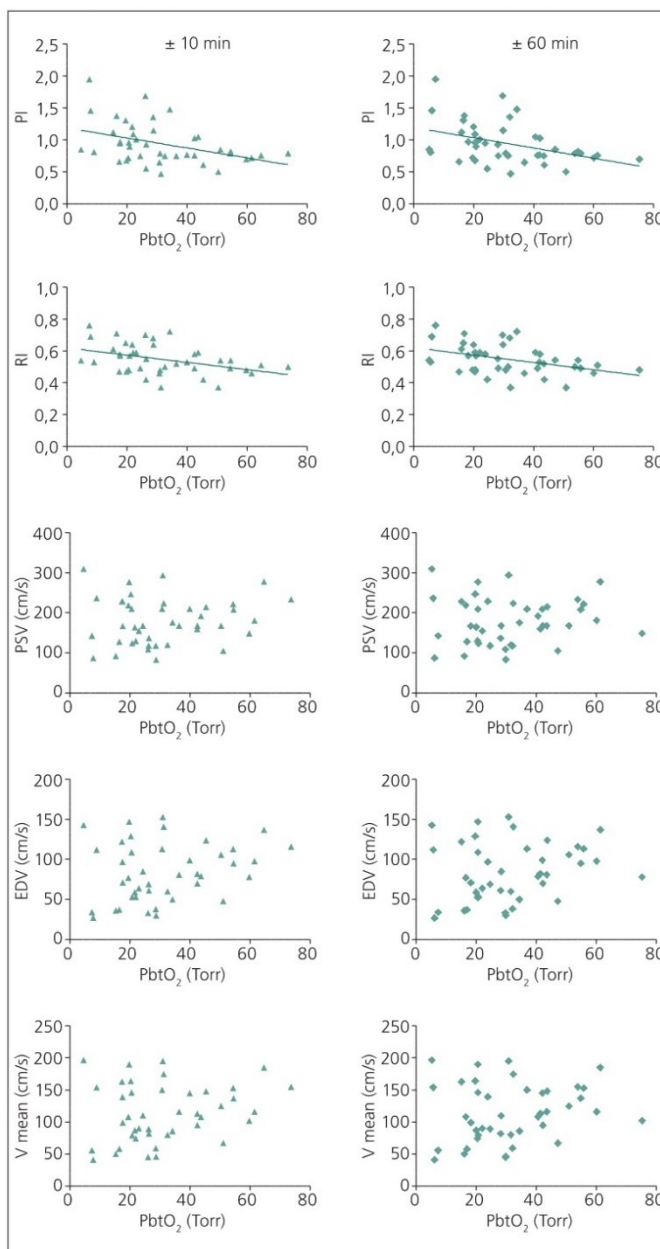
Průměrné hodnoty PbtO₂ monitoringu nebyly u zkoumaného souboru významně rozdílné v závislosti na HH, Fischer ani GOS, přestože byla zjevná tendence k nižším hodnotám PbtO₂ u pacientů v horším klinickém stavu při přijetí a s horším výsledným stavem. I tato skutečnost může být způsobena časnou aplikací monitoringu PbtO₂, neboť se dá předpokládat, že hodnoty PbtO₂ u pacientů v horším stavu a s horším výsledným stavem budou díky závažnému klinickému průběhu, který významně ovlivňuje také vazospazmy, nižší v pozdějším období.

VZTAH MEZI PARAMETRY TRANSKRANIÁLNÍ DOPPLEROMETRIE A TKÁŇOVOU OXYMETRIÍ

U zkoumaného souboru jsme hodnotili korelaci mezi parametry TCD a hodnotami $PbtO_2$ v časových intervalech ± 10 min a ± 60 min. Tyto intervaly jsme zvolili, aby byl pokryt kratší i delší časový interval v návaznosti na TCD vyšetření, neboť dynamika mezi tokem v magistralních cévách a parciálním tlakem kyslíku v tkáni není popsána. K hodnocení jsme použili 40 záznamů od 22 pacientů, což může vést k určitému zkreslení, které je však dle našeho názoru zanedbatelné. Vzhledem k tomu, že každý pacient může mít záznam pořízen v situaci s vazospazmy nebo bez nich, předpokládáme při hodnocení tohoto parametru minimální vliv charakteristiky jednotlivého pacienta a naopak zásadní vliv absence či přítomnosti vazospasmů.

U zkoumaného souboru jsme prokázali korelaci $PbtO_2$ s PI a RI. PI vyjadřuje rozdíl rychlostí toků během systoly a diastoly ($PI = (PSV - EDV)/Vmean$), tento parametr tedy zejména reflektuje nitrolební tlak. RI vystihuje pulzatilní tok krve odpovídající rezistenci mikrovaskulárního řečiště ($RI = (PSV - EDV)/PSV$). Oba parametry charakterizují situaci v periferní oblasti, stejně jako tkáňová oxymetrie, která měří koncentraci kyslíku v tkáni. PI i RI jsou ovlivněny nejen cerebrálním perfuzním tlakem (pro který jsou kritické rychlosti toků na magistralních cévách), ale také dalšími parametry, jako je například intrakraniální tlak. Dříve již bylo popsáno, že vyšší hodnoty PI i RI jsou spojeny s vyšším ICP a horším výsledným stavem [24]. V naší práci jsme prokázali, že vyšší hodnoty indexů PI i RI jsou spojeny s nižšími hladinami $PbtO_2$, což zatím publikováno nebylo.

U zkoumaného souboru jsme neprokázali korelaci mezi $PbtO_2$ s PSV, EDV ani Vmean. Tyto parametry reprezentují toky na magistralních tepnách (v našem případě ACM), zatímco tkáňová oxymetrie měří koncentraci kyslíku v tkáni a absence korelace mezi těmito parametry a $PbtO_2$ naznačuje, že mezi nimi neexistuje jednoduchá souvislost. Avšak dá se předpokládat hlubší souvislost mezi $PbtO_2$ a výše zmíněnými parametry nejen z logiky věci samotné (průtok krve – koncentrace kyslíku), ale i proto, že PI i RI jsou parametry odvozené z těchto veličin; $RI = (PSV - EDV)/PSV$, $PI = (PSV - EDV)/Vmean$. Hodnocení hlubších souvislostí je však za daných okolností obtížné, neboť TCD mo-



Obr. 2. Korelace mezi jednotlivými parametry TCD a průměrnými hodnotami $PbtO_2$ v intervalech ± 10 min a ± 60 min.

Signifikantní je korelace $PbtO_2$ s PI a RI v obou časových intervalech. U ostatních parametrů TCD (PSV, EDV, Vmean) korelace signifikantní není ani v jednom časovém intervalu.

monitoring není schopen poskytnout informaci o době vzniku vazospazmů, informuje nás pouze o jejich přítomnosti nebo nepřítomnosti. Nevíme tedy, zda v okamžiku, kdy jsou vazospazmy pomocí TCD diagnostikovány, tyto teprve vznikají, trvají delší dobu nebo již odeznívají. Takovou informaci by nám poskytl pouze kontinuální TCD monitoring, který by odhalil, kdy vazospazmy reálně vznikají a na tento okamžik by bylo možné centrovat výchozí bod hodnocení PbtO₂. Tak by bylo teoreticky možné zjistit informace o dynamice mezi rychlostí toků na hlavních kmenech mozkových tepen a koncentrací kyslíku v tkáni a také vliv dalších parametrů, jako je např. ICP. Použití kontinuálního TCD monitoringu je však limitováno potenciálním nárůstem teploty tkáně, ke kterému může takto prováděný monitoring vést, a proto je v současnosti doporučováno používat kontinuální monitoring intermitentně [25]. Doporučený časový interval použití kontinuálního TCD monitoringu se pohybuje kolem 30 min [25]. Tento časový interval se zdá být z pohledu hodnocení dynamiky TCD PbtO₂ dostačující, neboť se dá předpokládat, že po změně rychlosti toků na magistralních tepnách dojde ke změně koncentrace kyslíku v tkáni s menší latencí. Je však třeba zdůraznit, že zásadní vliv na hladiny PbtO₂ mohou mít také další faktory, jako například periferní vazospazmy nebo mikroembolizace, které jsou však v klinických podmínkách obtížně definovatelné.

Znalost dynamiky mezi příčinou (vazospazmus), následkem (ischemie reprezentovaná hodnotou PbtO₂) je důležitá klinická informace. Vzhledem ke skutečnosti, že tuto dynamiku zatím nejsme schopni detailně popsat, nelze v současnosti nahradit informaci, kterou nám poskytuje TCD, měřením PbtO₂ a naopak. Proto se v klinické praxi jeví jako vhodné paralelní užití obou metod. Monitoring PbtO₂ umožňuje kontinuální měření, je tedy schopen časně detekovat pokles PbtO₂, který však nemusí být způsoben pouze vazospazmy. TCD vyšet-

ření zase poskytne informaci o tom, zda jsou vazospazmy přítomny, avšak není schopné podat jakoukoliv časovou informaci. Z výše uvedeného také vyplývá, že zavedení kontinuálního TCD monitoringu by mohlo představovat výrazný přínos v léčbě pacientů po SAK.

Závěr

PbtO₂ monitoring poskytuje informace o stavu mozkové tkáně a její mikrovaskulatury. Neexistuje jednoduchá souvislost mezi hodnotami PbtO₂ a rychlostmi toků na magistralních cévách, které charakterizují cévní vazospazmy. Protože neumíme určit dynamiku změn rychlostí toků na magistralních cévách v souvislosti se změnami hodnot PbtO₂, jeví se v klinické praxi u pacientů s těžkým SAK vhodné paralelní užití TCD i PbtO₂ monitoringu.

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3. Pathophysiology of subarachnoid hemorrhage

3.1 Early brain injury

Cerebral vasospasm was traditionally considered the only cause of DCI. Although several promising mechanisms of vasospasm development were proposed, including smooth muscle dysfunction due to Ca^{2+} overload or endothelial dysfunction associated with excessive release of very potent vasoconstrictor endothelin, the research was initially focused mostly on vessels with insufficient respect to brain tissue^{47,48}. Clinical trials focused on vasospasm brought controversial results. In CONSCIOUS clinical trials, a selective antagonist to endothelin receptor clazosentan significantly reduced cerebral vasospasm but failed to improve patient outcomes^{49,50}. Additionally, DCI may develop independently of cerebral vasospasm and cerebral vasospasm is not always associated with DCI^{33,51,52}.

When research dominantly focused on vessels brought limited results, the field of SAH research was redefined in order to understand the pathophysiology of SAH in its complexity and this resulted in the elaboration of early brain injury (EBI) concept⁵³. EBI is a complex of pathologies that occur before vasospasm development, typically in the first 72 hours following SAH^{26,54}. These pathologies may represent interlink among DCI, cerebral vasospasm, and poor outcome^{51,55}. The most important contributors to early brain injury are transient global cerebral ischemia, brain edema, apoptosis in neurons and endothelial cells, oxidative stress, and inflammation^{26,54}.

As mentioned in Chapter 2, SAH following aneurysm rupture is usually severe, because cerebral aneurysms are most often localized on Willis circus and because blood from ruptured aneurysm spreads relatively freely into subarachnoid space. Accumulation of blood in subarachnoid space (mass effect) leads to critical elevation of intracranial pressure (ICP) resulting in a drop of cerebral blood flow (CBF) and temporal intracranial circulatory arrest⁵⁶. Intracranial circulatory arrest occurs when ICP reaches the level of mean arterial pressure, this develops within 1 minute after the onset of bleeding, and then during several minutes ICP drops but remains higher than normal⁵⁶. Intracranial circulatory arrest may be considered a crucial pathophysiological event allowing blood clot formation in a ruptured aneurysm, which is essential for patient survival. However, intracranial circulatory arrest leads to transient global cerebral ischemia which is associated with severe pathophysiological consequences.

Transient global cerebral ischemia is associated with energetical disturbance in neurons and neuroglia resulting in cytotoxic edema formation⁵⁷. Ischemic damage of endothelial cells and associated blood-brain barrier (BBB) disruption leads to vasogenic edema which occurs later than the cytotoxic type of edema⁵⁷. Endothelial damage and BBB disruption are enhanced by ischemic damage of neurons and astrocytes via the HIF-1 α -VEGF mechanism, which promotes endothelial apoptosis²⁶. Cerebral edema formation may represent a vicious circle, because cerebral edema increases ICP compromising CBF, resulting in subsequent ischemia and edema formation.

The effect of ischemia on cells is very complex. Neuronal cells have a high metabolic rate to cover membrane electrical activity and this requires almost exclusively oxidative phosphorylation^{58,59}. SAH-associated transient global cerebral ischemia causes disruption of blood supply to neurons. Lack of oxygen and glucose leads to mitochondrial dysfunction and insufficient ATP production. ATP deficiency leads to the malfunction of ATP-dependent ion pumps, mainly sodium-potassium pumps resulting in the accumulation of sodium intracellularly. Accumulation of sodium is accompanied by water influx forming cytotoxic edema.

Mitochondrial dysfunction and subsequent malfunction of sodium-potassium pumps also lead to membrane depolarization because the membrane cannot be kept repolarized due to ATP insufficiency. Spreading depolarization wave is an important contributor to SAH-associated excitotoxicity, which is however mainly caused by an increase in extracellular levels of glutamate⁶⁰. Excessive levels of glutamate may also result from decreased reuptake of glutamate by neuroglia because this process is also ATP-dependent²⁶.

Accumulation of glutamate is associated with an influx of sodium, calcium, and chloride via AMPA and NMDA receptors. NMDA receptor is highly permeable for calcium playing a crucial role in excitotoxicity⁶¹. Accumulation of calcium in neurons may lead to cellular damage or apoptosis by several calcium-dependent mechanisms including activation of enzymes with proteolytic activity, enzymes generating reactive oxygen species (ROS), or mitochondrial pathway^{58,62}.

Oxidative stress is an important contributor to early brain injury because both ROS and reactive nitrogen species (RNS) have a high potential to cause cellular/tissue damage. RNS is derived from nitric oxide which is overproduced by an inducible form of nitric oxide synthase in response to ischemia⁶³. An important source of ROS is respiratory chain leakage associated with mitochondrial dysfunction, and inflammation represents another significant source of both ROS and RNS^{64–66}. Another significant source of oxidative stress is iron released during the degradation of extravasate hemoglobin⁶⁷. Oxidative stress may lead to damage of crucial cellular macromolecules - lipid peroxidation, protein oxidation, or oxidative damage of DNA^{68–70}. Oxidative stress also promotes BBB disruption and inflammation^{65,71}.

All above mentioned pathophysiological mechanisms as well as inflammation, which will be discussed in the next chapter, are closely interconnected processes that may result in cellular apoptosis. Both neuronal and endothelial apoptosis are important contributors to EBI, while necrosis does not play a major role in the pathophysiology of SAH in survival patients because transient global cerebral ischemia after SAH is not so severe as to cause direct irreversible ischemic damage resulting in necrotic cell death⁵⁴. Massive necrotic cell death is on the contrary associated with SAH which is not compatible with life and which causes critical cerebral ischemia resulting in cerebral death in patients who die immediately due to onset of bleeding⁵⁴. Many apoptosis-associated pathways play a significant role in the pathophysiology of EBI including crucial cellular signaling pathways like PI3K-Akt, MAPK-ERK/JNK/p38, and HIF-1⁵⁴. Prevention of neuronal apoptosis after SAH is an important topic of current research which recently also turns attention to autophagy as well as other ways of neuronal death like necroptosis, ferroptosis, or pyroptosis^{72–74}.

3.1.1 The author's publications related to the topic of early brain injury

3.1.1.1 Sampling of CSF via the Cisterna Magna and Blood Collection via the Heart Affects Brain Water Content in a Rat SAH Model

Duris K, Manaenko A, Suzuki H, Rolland W, Tang J, Zhang JH. Sampling of CSF via the Cisterna Magna and Blood Collection via the Heart Affects Brain Water Content in a Rat SAH Model. *Transl Stroke Res.* 2011;2:232–237.

Type of article: Research article

IF (2011): -

Number of citations without self-citations according to WoS (7/2025): 19

The reduction of the amount of animals used in experiments is an important issue of experimental research. Collecting more samples from one animal is not only desirable from a scientific point of view, but it is also a way, how to reduce the number of animals used in experiments. However, collecting several samples from one living animal may significantly affect crucial physiological parameters.

Simultaneous collection of blood and CSF from one subject is important for the comparison of pathophysiological processes in the brain and periphery. Brain water content reflects cerebral edema and it is a widely used parameter in animal experiments. The goal of this research was to evaluate whether the collection of CSF via cisterna magna and blood via cardiac puncture may affect brain water content in rat SAH model.

We used twenty-nine animals which were divided into four groups according to procedure (SAH or Sham) and sample collection (with or without): Sham S+, Sham S-, SAH S+, and SAH S-. The perforation model of SAH was used for the purpose of this study and the animals were sacrificed 24 hours after the surgery. Samples of CSF and blood were collected in S+ groups before scarification. After scarification brains were collected and cut into four parts (left/right hemisphere, cerebellum, and brain stem). Brain water content calculation was based on the difference between the weight of a particular part of the brain immediately after collection and after drying at 100°C for 72 hours⁷⁵.

Simultaneous collection of the CSF and blood samples did not affect the neurological score, however, brain water content was affected by sample collection. The brain water content of left hemisphere was significantly higher in Sham S+ animals compared to Sham S- animals indicating that simultaneous collection of CSF via cisterna magna and blood via the heart may affect brain edema formation in the experimental model of SAH.

Sampling of CSF via the Cisterna Magna and Blood Collection via the Heart Affects Brain Water Content in a Rat SAH Model

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Abstract The aim of this study was to evaluate whether sampling of cerebrospinal fluid (CSF) via the cisterna magna and of blood via the heart affects brain water content in a rat subarachnoid hemorrhage (SAH) model. Twenty-nine animals were divided into four groups: sham-operated group with sampling of CSF and blood (Sham S+), sham-operated group without sampling of CSF and blood (Sham S-), SAH group with sampling of CSF and blood (SAH S+), and SAH without sampling of CSF and blood (SAH S-). SAH was induced via endovascular perforation of the left internal carotid artery bifurcation. Cerebrospinal fluid via the cisterna magna and blood via cardiac puncture was collected in the Sham S+ and SAH S+ groups before killing the animals for brain water content measurements. Left hemisphere brain water content was significantly higher in the SAH S- group compared with the Sham S- group ($p < 0.05$) and in Sham S+ group compared with the Sham S- group ($p < 0.05$). There was no significant difference in brain water content of the left hemisphere between the SAH S+ and Sham S+ groups ($p = \text{NS}$). There was no significant difference in brain water content in other parts of brains. Sampling of CSF and blood affected brain water content in Sham animals and therefore it is not accurate to use these

values from Sham animals for comparison with SAH animals.

Keywords SAH · Rat · Brain water content · Blood · CSF

Introduction

Subarachnoid hemorrhage (SAH) is a devastating event with an incidence of seven per 100,000 each year [1, 2]. Nearly 50% of patients die within 1 month and approximately 60% of surviving patients have a relevant neurological deficit [1, 2]. SAH research often involves brain edema monitoring as well as cerebrospinal fluid (CSF) and blood collection [3–5]. In experimental animal SAH models, brain water content and CSF/blood sampling are often conducted in the same animal to reduce cost [6–8]. Similarly, brain water content and CSF/blood sampling are conducted in the same animal in experimental intracerebral hemorrhage [9] and brain ischemia [10] animal models.

However, we are not aware of evidence suggesting whether sampling of CSF and blood affects brain water content in these animal models. The aim of this study was to evaluate whether sampling of CSF via the cisterna magna and blood via the heart affects the brain water content in a rat SAH model.

Materials and Methods

General Information and Experimental Design

All experiments were approved by the Institutional Animal Care and Use Committee of Loma Linda University. Adult male Sprague–Dawley rats were purchased from Harlan

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Laboratories (Indianapolis, Indiana). In total, 29 animals were operated in this study, 12 in Sham groups, 17 in SAH groups from which five animals died due to massive subarachnoid hemorrhage. The animals were divided into the following groups: sham-operated group with sampling of CSF and blood (Sham S+, $n=6$), sham-operated group without sampling of CSF and blood (Sham S-, $n=6$), SAH group with sampling of CSF and blood (SAH S+, $n=6$), and SAH without sampling of CSF and blood (SAH S-, $n=6$).

Body weight was measured before surgery and before killing. SAH was induced via endovascular perforation of the left internal carotid artery bifurcation. Sham-operated animals underwent the introduction of the suture into internal carotid artery, but no perforation was performed. Neurological score was evaluated at 23 h after surgery. CSF via cisterna magna and blood via heart was collected in the Sham S+ and SAH S+ groups immediately before killing. Animals were killed 24 h after surgery, brains were collected, quickly photographed for SAH grading evaluation, and then the brains were processed for brain water content evaluation.

Surgery

The endovascular perforation model of SAH in rats was used for this study as previously described [11]. Briefly, rats were anesthetized with 3% isoflurane in 60/40% medical air/oxygen. The animals were intubated and kept on artificial ventilation with 3% isoflurane in 60/40% medical air/oxygen during surgery. Body temperature was monitored by rectal probe and normothermia was maintained by heating lamp during surgery. Left common carotid artery, external carotid artery, and internal carotid artery were isolated. The external carotid artery was ligated, coagulated, cut, and shaped into a 3-mm stump. A sharpened 4–0 monofilament nylon suture was introduced into the internal carotid artery from the external carotid artery stump until resistance was felt (approximately 18 mm from the common carotid bifurcation). The suture was then pushed further to perforate the bifurcation of the anterior and middle cerebral arteries until resistance was overcome. Sham-operated animals underwent the identical procedures, but the suture was not inserted over skull base. The incision was closed, and rats were individually housed in heated cages until recovery from anesthesia.

Neurological Evaluation

Neurological scores were evaluated 23 h after surgery in a blinded fashion with a modification of the scoring system described by Garcia [12]. An 18-point scoring system was used to evaluate the neurological deficits. In S+ groups,

neurological testing was performed before collecting samples.

CSF and Blood Collection

In S+ groups, CSF was collected during terminal anesthesia and blood was collected immediately after completion of terminal anesthesia just before decapitation. CSF was collected as previously described [13]. The animals were terminally anesthetized with 5% isoflurane in 60/40% medical air/oxygen by face mask. The 27 G needle attached on tuberculin syringe was inserted into the cisterna magna through the occipital membrane and 50 μ l of CSF was collected. The syringe was guided through a holder to prevent damage of the brain. Terminal anesthesia was maintained for 5 min while CSF collection procedure was performed. Directly after CSF collection 3 ml of blood was sampled by cardiac puncture as previously described [14]. Animals were decapitated immediately after blood collection and the brains were collected. Only Sham S+ and SAH S+ animals were subjected to CSF and blood collection. Sham S- and SAH S- animals were anesthetized by 5% isoflurane for the same duration and then decapitated.

SAH Grade

After decapitation, brains were photographed and SAH grade was evaluated according to blood clot occurrence (maximum of 18 points) as previously described [15].

Brain Water Content Evaluation

Brain water content was evaluated as previously described [16]. Brains were removed 24 h after surgery and separated into four parts (left hemisphere, right hemisphere, cerebellum, and brain stem). Each part was weighed immediately after removal (wet weight) and after drying in 100°C for 72 h (dry weight). The percentage of water content was calculated as $[(\text{wet weight} - \text{dry weight}) / \text{wet weight}] \times 100\%$.

Statistical Analysis

Data are expressed as a mean and standard error of mean. Brain water content data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's test. Mortality data was analyzed by Fischer exact test. Body weight data were analyzed using two-way ANOVA followed by Bonferroni test. SAH grading data were evaluated by two-tail t test. A p value of <0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism version 5.02 for Windows.

Results

Mortality

The mortality rate in the SAH groups was as follows: 25% (two of eight animals) in the SAH (S+) group and 33% (three of nine animals) in the SAH (S-) group. There was no statistical difference in mortality between SAH groups (Fischer's exact test, $p=NS$). None of the Sham animals died.

Body Weight Before Surgery and Before Killing

There was no significant difference in body weight between groups before surgery ($p=NS$), or 24 h after surgery ($p=NS$). Body weight was significantly decreased in all groups 24 h after surgery compared with body weight before surgery ($p<0.05$). Body weight loss after surgery was between 7% and 10% of body weight before surgery (Fig. 1a, Table 1).

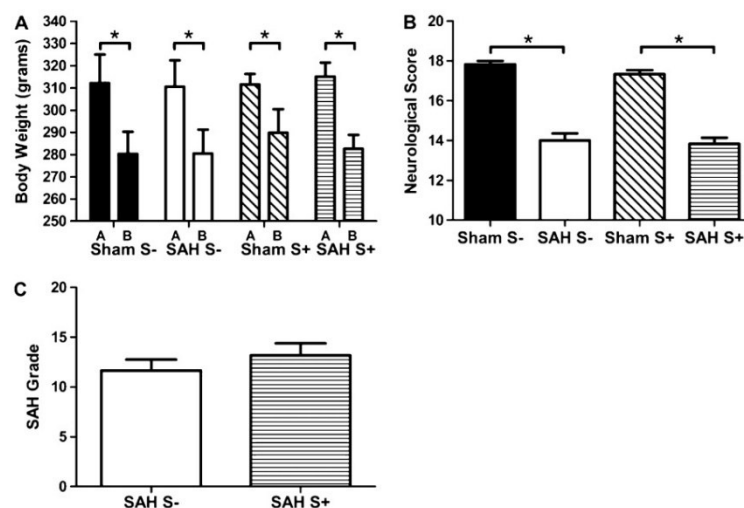
Neurological Score

Neurological score was significantly lower in the SAH groups compared with the Sham groups ($p<0.05$). There was no significant difference between the SAH+ group and SAH S- group ($p=NS$). There was no significant difference between the Sham S+ group and Sham S- group ($p=NS$) (Fig. 1b, Table 1).

SAH Grade

There was no significant difference in SAH grade between the SAH S+ and SAH S- groups ($p=NS$) (Fig. 1c, Table 1).

Fig. 1 Body weight (a), neurological score (b), and SAH grade (c). * $P<0.05$. **a** There was a statistically significant body weight loss after surgery (b) compared with the body weight before surgery (a) in all groups ($p<0.05$). There was no significant difference between groups in body weight, before surgery, nor in the body weight loss after surgery ($p=NS$). **b** There was a significant neurological deficit in SAH animals compared with Sham animals ($p<0.05$). The differences between Sham and SAH animals were comparable between S+ and S- groups. **c** There was no significant difference in SAH grade between SAH S+ and SAH S- animals ($p=NS$)



Brain Water Content

Brain water content of the left hemisphere was significantly higher in the SAH S- group compared with Sham S- group ($p<0.05$). There was no significant difference in brain water content of the left hemisphere between the SAH S+ and Sham S+ groups ($p=NS$). Brain water content was significantly higher in the Sham S+ group compared with Sham S- group ($p<0.05$) (Fig. 2, Table 1).

There was no significant difference between groups either in the right hemisphere, cerebellum nor brain stem ($p=NS$ for all). The right hemisphere showed a similar tendency compared with the left hemisphere, however there was no statistical significance seen.

Discussion

The aim of this study was to evaluate whether collection of CSF via the cisterna magna and of blood via the heart could affect brain water content in a rat SAH model. We used CSF collection via the cisterna magna and blood collection via the heart, because these techniques have been commonly used in rat experiments [17, 18].

All parameters except brain water content were comparable between the SAH S- and SAH S+ groups, same as between the Sham S- and Sham S+ animals. There was 0% mortality in the sham groups, 33% mortality in the SAH S- group and 25% mortality in the SAH S+ group. There is high variation in the mortality of rat SAH perforation model between different researchers from 18% [8] to 46% [19]. Body weight before surgery was similar between all groups,

Table 1 Parameters and results of experiment

	Sham S−	SAH S−	Sham S+	SAH S+
Numbers of operated animals	6	9	6	8
Survived/Dead animals (mortality %)	6/0 (0%)	6/3 (33%)	6/0 (0%)	6/2 (25%)
Average body weight (g) and SEM before surgery	312 (5.25)	311 (4.83)	312 (1.98)	315 (2.55)
Average body weight (g) and SEM after surgery	280 (4.06)	281 (4.39)	290 (4.36)	283 (2.55)
Average body weight loss (%) after surgery	10.3	9.6	7.1	10.2
Average neurological score and SEM after surgery	17.83 (0.17)	14.00 (0.37)	17.33 (0.21)	13.83 (0.31)
Average SAH grade and SEM	0	11.67 (1.12)	0	13.17 (1.22)
BWC in left hemisphere (%) and SEM	78.88 (0.11)	79.52 (0.13)	79.49 (0.15)	79.42 (0.17)
BWC in right hemisphere (%) and SEM	78.93 (0.07)	79.43 (0.25)	79.34 (0.11)	79.34 (0.17)
BWC in cerebellum (%) and SEM	78.26 (0.11)	78.95 (0.52)	79.19 (0.19)	78.95 (0.32)
BWC in brain stem (%) and SEM	73.62 (0.17)	74.33 (0.79)	74.47 (0.26)	73.83 (0.25)

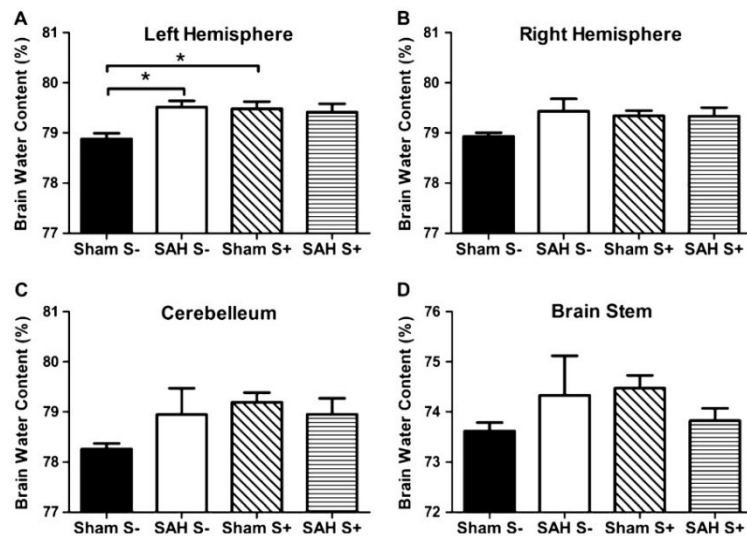
Sham S− sham-operated group without sampling of cerebrospinal fluid (CSF) and blood, SAH S− subarachnoid hemorrhage-operated group without sampling of CSF and blood, Sham S+ sham-operated group with sampling of CSF and blood, SAH S+ subarachnoid hemorrhage-operated group with sampling of CSF and blood, Mortality (number of dead animals/number of operated animals)×100 (%), SEM standard error of mean, BWC brain water content

same as the body weight before killing. Body weight loss after surgery was between 7% and 10% of body weight before surgery, which is similar to that previously described [8]. No significant difference in body weight loss between the Sham and SAH animals implies that body weight decreases more by the surgical procedure than by SAH. The neurological deficits were comparable between the SAH S− and SAH S+ groups, same as the neurological score between the Sham S− and Sham S+ groups. We used a neurologic scoring system proposed by Garcia [12] and modified in our laboratory [11]. There was no significant

difference in SAH grade between the SAH S− and SAH S+ groups. We used SAH scoring system described by Sugawara [15] which is well established in our laboratory.

Brain water content was significantly increased ipsilaterally in SAH S− group compared with Sham S− animals, while the increases of brain water content in the contralateral hemisphere, cerebellum and brain stem were not significant, which is in line with previously published data. In the literature there are references regarding an increase of brain water content in rat perforation model of SAH in the ipsilateral hemisphere only [6], both in ipsilateral and

Fig. 2 Brain water content in the left hemisphere (a), right hemisphere (b), cerebellum (c), and brain stem (d). * $P<0.05$. **a** In the left hemisphere, there was a significant difference in BWC between the Sham S− and SAH S− groups ($p<0.05$). There was a significant difference in BWC between the Sham S− and Sham S+ groups ($p<0.05$). There was no difference between Sham S+ and SAH S+ ($p=NS$). **b** In the right hemisphere, there was no statistically significant difference ($p=NS$); however, there was a similar tendency compared with the left hemisphere. **c** In the cerebellum, there was no statistically significant difference ($p=NS$); however, there was a similar tendency compared with the left hemisphere. **d** In the brain stem, there was no statistically significant difference ($p=NS$)



contralateral hemisphere [8] and some authors report an increase of brain water content in hemispheres, cerebellum and brain stem [7]. In S+ groups there was no significant difference in brain water content between Sham and SAH animals in the left hemisphere or in any other part of the brain. The brain water content of the left hemisphere in SAH S+ group was comparable with SAH S– group, while the brain water content in Sham S+ animals was significantly higher than in the Sham S– group. Thus brain water content was affected by simultaneous collection of CSF and blood selectively in Sham animals, which suggests that preexisting edema (SAH animals) was not affected by simultaneous collection of CSF and blood while this procedure caused a rise in brain water content in animals without preexisting edema (Sham animals).

The mechanism of changes in brain water content in the Sham animals after CSF and blood sampling is not clear. Animals were decapitated shortly after sampling (approximately 2 min after CSF collection and seconds after blood collection) and this time is likely not long enough for added brain edema to form. Therefore a possible mechanism could be increased cerebral blood volume (CBV), due to vascular engorgement. Both CSF and blood collection procedures can theoretically increase CBV. CSF sampling can cause dilation predominantly in the venous system [20], while blood collection can cause dilation in the arterial system [21].

CSF collection could affect CBV mainly via the venous system. Decreased CSF pressure without decrease in mean arterial pressure (MAP) leads to venodistension and accumulation of blood in the brain, because veins, with a thin muscle layer, cannot resist pressure differences across their walls [20]. In SAH animals, preformed brain edema could attenuate the venodistension. Decreased CSF pressure could be compensated more by brain tissue expansion than by blood compartment expansion. Additionally, the accumulation of blood in the brain can be reduced by arterial vasoconstriction. CSF collection could also result in decreased ICP, which would lead to an increased cerebral perfusion pressure (CPP). An increased CPP results in autoregulatory vasoconstriction of cerebral arteries reducing CPP back to normal [21]. Vasoconstrictor mechanisms are augmented after SAH [22] thus lowering blood volume in cerebral arteries in SAH animals compared with Sham animals, regardless of CSF collection. Therefore in summary, CSF collection could have a larger effect on brain water content in Sham animals, because of higher venodistension and a lower level of vasoconstriction, compared with SAH animals.

While there are complex mechanisms following CSF collection that can possibly affect brain water content, the effect of blood collection could be more straightforward. Hypovolemia caused by blood sampling, leads to decreased MAP and CPP which results in compensatory vasodilation

of cerebral arteries to bring the CPP back up [21]. After SAH vasodilation is impaired [22], so vascular engorgement and resulting effects on brain water content could be more pronounced in Sham animals than in SAH.

Comparing Sham S– animals to Sham S+ animals, brain water content could be higher in Sham S+ because of vascular engorgement resulting from venodistension and vasodilation due to collection of CSF and blood. However, in SAH S+ animals the increase in CBV after sample collection can possibly be reduced by the presence of preformed brain edema, attenuating the degree of venodistension. Additionally, impairment in vasodilation associated with SAH can reduce CBV. Therefore theoretically the brain water content of SAH S+ may not be higher than in SAH S– animals.

Our study was focused on brain water content and simultaneous collection of CSF and blood. Direct comparison of CSF and blood parameters is important from a clinical point of view and the relationship of these parameters to brain edema could provide interesting information. We realize that from a pathophysiological point of view our study is limited and to further clarify which of these procedures mainly affected brain water content, additional experiments would be needed using separate groups collecting only CSF and blood.

Our results suggest that simultaneous collection CSF via the cisterna magna and of blood via the heart affected brain water content in Sham animals and for that reason it is not suitable to simultaneously collect the CSF and blood in Sham animals and then measure brain water content for comparison with SAH animals.

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3.1.1.2 Oxidative Stress in the Brain: Basic Concepts and Treatment Strategies in Stroke

Jelinek M, Jurajda M, Duris K. Oxidative Stress in the Brain: Basic Concepts and Treatment Strategies in Stroke. *Antioxidants (Basel)*. 2021;10:1886.

Type of article: Review article

IF (2021): 7,675

Number of citations without self-citations according to WoS (7/2025): 121

In this review article, we sketch the general concept of oxidative stress in the brain and its role in stroke. In the first part, we describe the physiology and pathophysiology of free radicals and oxidative stress in general as well as in the brain. The main part of the article is dedicated to treatment strategies targeted to oxidative stress in both ischemic and hemorrhagic stroke. Finally, the use of several antioxidants in combination and the limitations of antioxidant therapy in stroke patients are discussed.



Review

Oxidative Stress in the Brain: Basic Concepts and Treatment Strategies in Stroke

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Abstract: The production of free radicals is inevitably associated with metabolism and other enzymatic processes. Under physiological conditions, however, free radicals are effectively eliminated by numerous antioxidant mechanisms. Oxidative stress occurs due to an imbalance between the production and elimination of free radicals under pathological conditions. Oxidative stress is also associated with ageing. The brain is prone to oxidative damage because of its high metabolic activity and high vulnerability to ischemic damage. Oxidative stress, thus, plays a major role in the pathophysiology of both acute and chronic pathologies in the brain, such as stroke, traumatic brain injury or neurodegenerative diseases. The goal of this article is to summarize the basic concepts of oxidative stress and its significance in brain pathologies, as well as to discuss treatment strategies for dealing with oxidative stress in stroke.

Keywords: oxidative stress; free radicals; ROS; RNS; brain; stroke; antioxidants; scavengers



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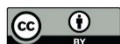
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1. Introduction to Physiology of Free Radicals

Free radicals are highly reactive molecules because they contain unpaired valence electrons [1]. The most important radicals in biological systems are reactive oxygen species (ROS) and reactive nitrogen species (RNS). The production of free radicals is inevitably associated with metabolism and other enzymatic processes. The major sources of free radicals are potentially mitochondria (intracellular) and inflammation (extracellular). Under physiological conditions these processes are highly regulated. When imbalance with anti-oxidative mechanisms occurs these reactive molecules can cause DNA damage and protein oxidation and lipid peroxidation, leading to cell toxicity and tissue damage [2].

Some of the most relevant types of ROS and RNS are superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\bullet}$), hypochlorous acid (HOCl), nitric oxide (NO) and peroxynitrite ($ONOO^{\bullet}$). Superoxide is the most important ROS in the central nervous system (CNS). Intracellularly, it is generated as a side-product of metabolism, mainly the mitochondrial respiratory chain [3]. Extracellularly, it is generated as a part of the immune response, primarily by NADPH oxidases (NOX), myeloperoxidase (MPO) and xanthine oxidase (XO) [4,5]. Superoxide quickly disintegrates into oxygen and hydrogen peroxide. However, in the presence of redox-active iron, highly reactive hydroxyl radicals are generated from superoxide and hydrogen peroxide in the Fenton and Haber–Weiss reactions.

Nitric oxide (NO) is generated by nitric oxide synthase (NOS). It plays an important role in the immune response, where large amounts of NO are generated by phagocytes [6]. Furthermore, through interaction with soluble guanylyl cyclase, it dilates blood vessels, thus raising blood supply and lowering blood pressure [7]. It is also functions as a retrograde neurotransmitter [8].

There are three isotypes, endothelial NOS (eNOS), inducible NOS (iNOS) and neuronal NOS (nNOS). The two most important isotypes involved in stroke are eNOS and iNOS. In the presence of superoxide highly oxidative peroxynitrite can form.

Although an excessive production of free radicals is considered harmful, regulated production is a normal physiological process that plays a vital role in metabolic processes and in the immune response. Therefore, there must also be some anti-oxidative mechanisms to counter the production of ROS and regulate the oxidative state of the cell (Figure 1).

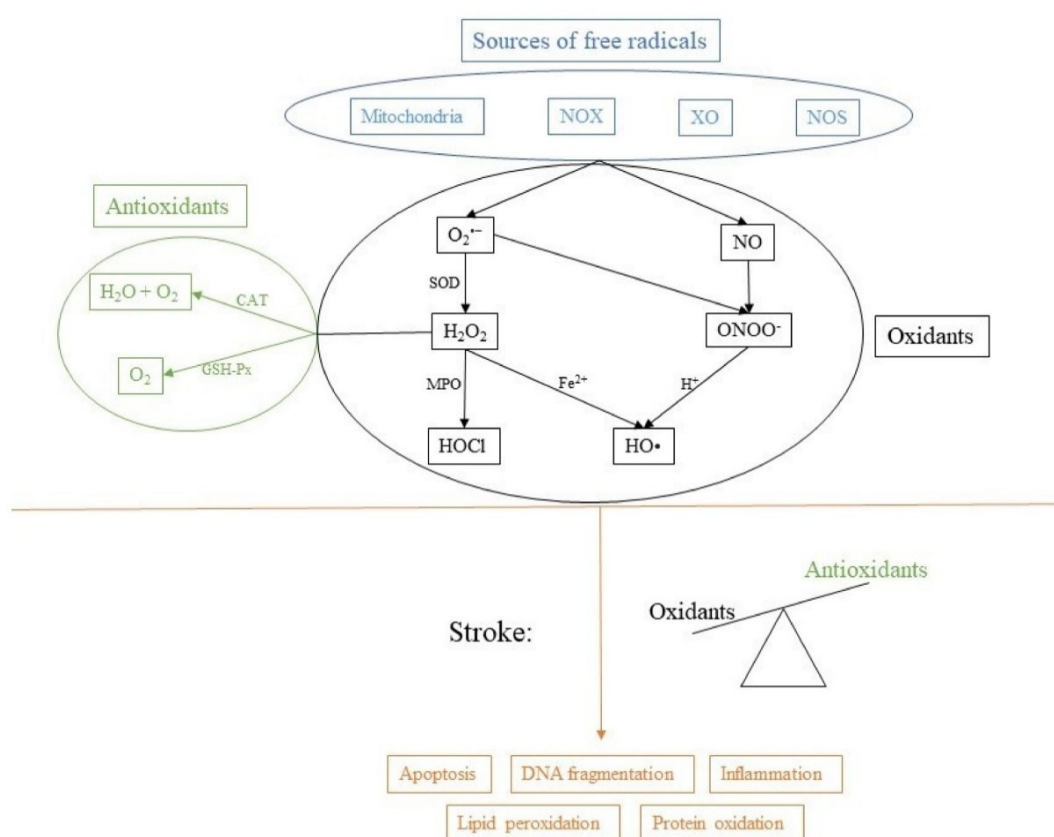


Figure 1. Sources of free radicals and consequences of imbalance. NADPH oxidase (NOX), xanthine oxidase (XO), nitric oxide synthase (NOS), superoxide dismutase (SOD), myeloperoxidase (MPO), catalase (CAT) and glutathione peroxidase (GSH-Px).

There are many antioxidant mechanisms in the cell to prevent generation of ROS or to remove them. They can be of internal, or external (nutritional) origin. Some of the most important antioxidants include glutathione (GSH), vitamin C and E, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), heme oxygenase (HO) and glutathione reductase (GR). They contribute to the antioxidant potential of the cell and work as an important cellular defense against ROS and RNS. SOD generates hydrogen peroxide, which is converted by CAT to oxygen and water. GSH-Px uses GSH to reduce hydrogen peroxide to water. HO-1 has antioxidant effects, maintains microcirculation in ischemic injury and has modulatory effects on the cell cycle [9].

2. Oxidative Stress

Oxidative stress is described as a state of imbalance between the production of free radicals and their elimination by an organism's anti-oxidative mechanisms. The base of oxidative phosphorylation (and other catabolic processes) is the transfer of electrons, which is essential for energy release. Electrons travel from one protein complex to the next in the inner mitochondria membrane. Therefore, intermediates in this reaction are, in nature, radicals [10]. However, these intermediates are broken down by subsequent reactions. At the end of the electron transport chain, the final electron acceptor is oxygen, and ultimately water is formed, which is not radical in nature [11]. Thus, it is important these reactions take place from their beginnings to their ends. Mitochondrial oxidative stress arises when these reactions stop halfway in, for example, due to lack of oxygen. In that case, antioxidant mechanisms kick in to scavenge or eliminate free radicals. However, they do not have unlimited capacity and their recycling may be limited by the same factors as mitochondrial dysfunction [12].

The other major physiological source of free radicals is inflammation. Inflammation is part of a complex biological response to harmful stimuli, such as bacteria. Upon contact with pathogens (e.g., bacteria), phagocytes try to entrap and destroy them by complex set of digestive enzymes, ROS and RNS [13]. Oxidative stress plays a dual role in an infection. Free radicals are released from immune cells protect against invading pathogens, but they can also cause tissue damage during inflammation [13]. Superoxide can be produced by neutrophils in phagosomes or in the cell membrane surface through the function of an oxidase enzyme (NADPH oxidase and myeloperoxidase) [14,15]. The excessive production of various ROS and RNS overwhelms endogenous antioxidant defense mechanisms and promotes the development of oxidative state.

Given the immense variety and extent of pro-oxidative and anti-oxidative compounds, attempts to categorize oxidative stress have been made, ranging from physiological oxidative stress to excessive and toxic oxidative overload [16,17].

Oxidative stress plays an important role in a number of pathologies and diseases. One of the basic but often overlooked mechanisms that reinforces oxidative stress is a decrease of antioxidant mechanisms due to a lack of vital nutrients [18,19]. The opposite process would be an increase in the generation of free radicals, which can happen through intracellular mitochondrial dysfunction, or through an extracellular source such as inflammation, as discussed above. The time-course of oxidative stress is initially mitochondria-mediated, for example, due to oxygen and glucose deprivation and following mitochondrial depolarization, and is then xanthine oxidase-mediated, NOS-induced and later NADPH oxidase-mediated [20].

Furthermore, oxidative stress is also involved in aging [21]. According to the oxidative stress theory of aging, age-associated functional losses can be attributed to the accumulation of oxidative damage to DNA, proteins and lipids by free radicals [22].

The consequences of oxidative stress are extensive and affect many cellular processes. Oxidative stress damages all important macromolecules. Lipid peroxidation, protein oxidation and DNA fragmentation can lead to multiple cell signaling effects that can result in the initiation of apoptosis [23]. As already mentioned, the primary site for ROS generation is the mitochondria. They can initiate cell death via cytochrome C release leading to activation of the intrinsic apoptotic pathway [24]. Furthermore, oxidative stress plays an important role in cancer development [25,26].

3. Oxidative Stress in the Brain

The brain is especially sensitive to oxidative damage because of its high and specific metabolic activity. High consumption of oxygen, almost exclusive oxidative phosphorylation, no reserves of energy, high concentrations of lipids prone to peroxidation, and high levels of iron, all acting as a pro-oxidant [27]. Neuronal cells are, therefore, highly susceptible to metabolic/ischemic damage and associated oxidative stress.

Lipid peroxidation is the main mechanism of oxidative damage by ROS [23]. Furthermore, initiation of lipid peroxidation develops a positive feedback loop [23]. Reactive species are unstable and react quickly with surrounding molecules. Oxidative stress is, therefore, a very rapid pathology, and it is hard to predict the type of damage.

In stroke, the results of ROS imbalance are considerable and include apoptosis, blood-brain barrier (BBB) disruption, inflammation, edema formation, autophagy and other pathophysiological events [20]. ROS and RNS are generated mainly by microglia and astrocytes [28].

4. Antioxidant Treatment Strategies of Oxidative Stress in Stroke

Antioxidant treatment, in general, is not very effective because the consequences of oxidative stress are severe. Associated apoptosis is connected with tissue damage and potentially with an impairment of function, which may be irreversible. The oncological consequences are even more severe.

In physiological conditions in the brain, the continuous production of ROS in various physiological processes is balanced by endogenous antioxidant mechanisms. However, after stroke free radicals' production is massively increased and overwhelms the natural antioxidant systems. There are three main mechanisms by which antioxidants function (Table 1): (1) inhibition of production of free radicals, (2) scavenging of free radicals and (3) increasing free radicals degradation [29].

4.1. Inhibition of ROS-Producing Enzymes

This approach targets the source of ROS by utilizing specific inhibitors of ROS-generating enzymes. The two enzymes that are most often targeted in stroke are NADPH oxidase (NOX) and xanthine oxidase (XO).

NADPH oxidase produces superoxide thus inhibition of NOX has been used as a target for stroke treatment. There are several subtypes of NOX; NOX2 and NOX4 make a major contribution to oxidative stress following stroke [30]. Apocynin has been shown to attenuate brain injury after experimental ischemic stroke and to reduce infarct size and levels of the apoptosis-inducing enzymes Bax and Bcl-2 [31,32]. It seems that apocynin functions as a nonspecific antioxidant, because it also inhibits Rho kinase inhibitor, thus the p47phox subunit cannot migrate to the membrane and the NOX complex cannot assemble [33–35]. VAS2870 is a pharmacological inhibitor of NOX and has been shown to decrease infarct volume, oxidative stress, neuronal apoptosis and BBB leakage [36]. Several new small-molecule NOX inhibitors have been recently identified, such as VAS2970, M13, GKT136901, ML090 and MLM171 [37–40]. They have the potential to reduce ROS production and inhibit brain tissue damage [40]. The NOX inhibitors are non-specific and not isoform selective, although it may not be particularly important in stroke treatment, the development of selective NOX inhibitors could provide information about the precise role of various NOX isoforms.

During ischemia, xanthine dehydrogenase undergoes proteolytical convection to xanthine oxidase (XO) [41]. Ischemia also induces intracellular depletion of ATP, which leads to the accumulation of hypoxanthine and xanthine [41]. They serve as substrates for the ROS-producing XO. Allopurinol and its metabolite oxypurinol have been suggested as scavengers of hydroxyl radicals [42]. Allopurinol was found to reduce the infarct size by approximately 35%, and to have protective effects against neurological impairment and mortality [43,44]. Newly used XO inhibitors with ROS scavenging properties include caffeic acid phenethyl ester (CAPE) [45].

Myeloperoxidase (MPO) is a critical enzyme in oxidative stress and neuroinflammation in pathologies such as stroke. MPO activation catalyzes reaction of chloride and H_2O_2 creating hypochlorous acid (HOCl) [46]. Targeting MPO with inhibitors (quercetin, eriodictyol, resveratrol, curcumin, trigonelline, dauricine and many others) reduces brain infatuation and improves neurological outcome [46–52].

4.2. Free Radical Scavengers

Compounds capable of scavenging free radicals have been in development for a long time, however the translation from pre-clinical to clinical trials have, so far, been disappointing.

Tirilazad, an inhibitor of lipid peroxidation, was reported in a pre-clinical study to reduce size of lesions and improve neurological score; nevertheless, the clinical trials have not been successful [53]. Edaravone, a compound known to scavenge peroxy, hydroxyl and superoxide radicals, was approved for use in Japan in 2001. Pre-clinical studies showed promising results; however in human clinical trials, the positive results are less clear [54].

4.3. Free Radical Degradation

Another important strategy that aims at reducing oxidative stress is to increase levels of the antioxidant superoxide dismutase (SOD). SOD catalyzes the conversion of superoxide to less potent H_2O_2 and O_2 . Other enzymes, catalase (CAT) and glutathione peroxidase (GSH-Px), help to eliminate H_2O_2 , thus improving the effectiveness of SOD.

Of the three SOD isoforms, the SOD1 has been the most studied in experimental stroke treatment. Transgenic mice and rats with SOD1 overexpression shown reduced apoptosis [55,56].

Nitric oxide (NO) is an important vasoactive molecule with broad physiological functions. It is produced by NO synthase (NOS) of which there are three isoforms, inducible NOS (iNOS), endothelial NOS (eNOS) and neuronal NOS (nNOS). NO derived from eNOS can have a neuroprotective effect because it can terminate the chain reactions within lipid peroxidation, while NO derived from iNOS acts as pro-oxidant because it can react with superoxide, forming more potent $ONOO^-$ [23,57]. L-betulinol reduces NO levels and, therefore, also $ONOO^-$ levels. Pre-clinical results showed promise; nonetheless, the primary clinical trial was terminated early due to unexplained mortality in a higher-dose group of patients [58,59].

4.4. Mitochondrial Targeted Antioxidants

The mitochondrial matrix acts as an important endogenous source of free radicals in ischemic conditions. Oxidative damage in mitochondria can lead to increased ROS production, decreased ATP production and the release of pro-apoptotic signals. The inhibition of mitochondrial respiratory chain complex I has been found to be beneficial in stroke treatment [60].

The use of antioxidants targeting mitochondrial ROS generation is complicated due to the difficulty in achieving the high concentrations of antioxidants necessary in the intracellular space [61]. This problem can be solved by conjugating an antioxidant with a lipophilic cation to promote diffusion and accumulation in the mitochondria. Mitoquinone, a derivative of ubiquinone, is reduced to ubiquinol and has been shown to protect mitochondria from the oxidative damage caused by H_2O_2 [62].

4.5. Antioxidant Supplementation to Scavenge ROS

Vitamin C (ascorbate, ascorbic acid) is a potent antioxidant. Ascorbate directly scavenges ROS and nitrogen-based radicals. It also upregulates eNOS and downregulates NOX. Its antioxidant properties are intensified in combination with other antioxidants, such as vitamin E. Vitamin C plasma concentrations high enough to be able to effectively scavenge ROS can only be achieved by intravenous supplementation [28]. Higher vitamin C serum concentration was associated with a reduced risk of incidence of ischemic and hemorrhagic stroke [63]. The oral supplementation was shown to slightly reduce lipid peroxidation markers [28].

Vitamin E (α -tocopherol) is also a potent antioxidant with the ability to prevent the propagation of ROS chain reactions, mainly in membrane lipids. Vitamin E (isoforms α -tocopherol and γ -tocopherol) has been shown to reduce infarct volume by 45–55% in transient and permanent models of cerebral ischemia [64,65].

An association between vitamin C and vitamin E plasma concentrations has been shown. Vitamin C has the ability to “recycle” α -tocopherol in lipid bilayers and erythrocytes, thus increasing the vitamin E antioxidant capacity [66]. The brain is a lipid-rich environment, so recycling vitamin E may be an important function of ascorbic acid. When stroke patients were given both vitamin C and vitamin 3 supplements, there was a reduction of lipid peroxidation and exerted anti-inflammatory effects [66].

Resveratrol, a polyphenol that is a major component of red wine, has been found to prevent lipid peroxidation and scavenge ROS [67]. Resveratrol-pretreated mice showed significantly reduced infarct size [68]. Resveratrol-treated mice experienced decreased neuronal death, lower glial activation, lower matrix metalloproteinase 9 (MMP9) and induction to neuroprotective HO-1 [67,69].

N-Acetylcysteine (NAC) has been shown to scavenge ROS and increase the rate of endogenous GSH synthesis [70]. NAC administered prior or after reperfusion was shown to reduce lesion size by 49% and 29% and to have anti-inflammatory effects [71].

Other compounds, such as the vitamin B group, extract from Ginkgo biloba, lazaroids, Edaravone and quercetin, have also been shown to have ROS-scavenging, neuroprotective, anti-inflammatory and antioxidant properties in experimentally induced stroke [28,47,72–74].

4.6. Antioxidant Treatment of Oxidative Stress in Hemorrhagic Stroke

In the treatment of intracerebral hemorrhage (ICH), several natural compounds have been used (such as pyrroloquinoline quinone, melatonin, sulforaphane and ursolic acid) [75–79]. In ICH animal studies, these compounds exhibited neuroprotective effects by inhibiting oxidative stress, upregulating antioxidant levels and reducing secondary brain injury [75–79]. Furthermore, treatment of ICH using hydrogen and active hydrogen compounds, targeted therapy against oxidative stress signaling pathways, synthetic antioxidant drugs (Edaravone) and targeted gene therapy have also been used [75]. Molecules with antioxidant capacity for alleviating oxidative stress were also used in an experimental treatment of subarachnoid hemorrhage [80,81].

Table 1. A list of compounds used in antioxidant stroke treatment that were discussed in the article.

Compound	Origin	Stroke Type	Target	Outcome	References
allopurinol + ** $\pm$	synthetic	ischemic stroke	XO	stroke volume and cerebral edema reduction	[44]
ascorbate (vitamin C) ++ * $\pm$	natural	ischemic stroke, hemorrhagic stroke	NOS, NOX, vitamin E, free radical scavenger	reduced risk of stroke; lower levels of peroxidation markers; reduced infarct size	[63]
CAPE + ** $\pm$	synthetic	ischemic stroke	XO	XO inhibition	[45]
curcumin ++ * $\pm$	natural	ischemic stroke	MPO, cytokines	inhibition of NF- κ B; reduced inflammation and brain damage	[50]
dauricine + ** $\pm$	natural	ischemic stroke	MPO, cytokines	reduced activity of MPO; reduced inflammation	[51]
Edaravone ++ ** $\pm$	synthetic	ischemic stroke	free radical scavenger	improvement of functional outcome	[54]
GKT136901 + ** $\pm$	synthetic	ischemic stroke	NOX1, NOX2, NOX4, NOX5	NOX inhibition	[37]
lubeluzole ++ * $\pm$	synthetic	ischemic stroke	NOS	reduced infarct volume	[58,59]
M13 + ** $\pm$	synthetic	ischemic stroke	NOX1, NOX4	NOX inhibition	[39]
melatonin + ** $\pm$	natural	ischemic stroke, hemorrhagic stroke	HO-1	increased HO-1 expression; amelioration of brain edema, BBB impairment, apoptosis and neurological deficits	[78]
mitoquinone + ** $\pm$	synthetic	ischemic stroke	mitochondria	recovery of O ₂ consumption and complex I activity	[62]
ML090 + ** $\pm$	synthetic	ischemic stroke	NOX1, NOX4, NOX5	NOX inhibition	[38]
ML171 + ** $\pm$	synthetic	ischemic stroke	NOX1, NOX4, NOX5	NOX inhibition	[38]
N-Acetylcysteine ++ ** $\pm$	natural	ischemic stroke	free radical scavenger, GSH	reduction of infarct volume; reduction of expression of pro-inflammatory cytokines; reduced cell death	[71]

leakage [90,91]. MSCs also regulate imbalances in autophagy, thus conferring neuroprotection against cerebral ischemic injuries [92]. In general, MSCs seem to be a promising treatment option for stroke due to their pleiotropic effects.

6. Limitations of Current Antioxidant Therapy in Stroke

A trend has emerged, in which compounds with promising results in pre-clinical trials fail in clinical trials. There are several potential reasons for this. An obvious reason could be that an invalid compound or combination of compounds have been studied [93]. Another potential explanation could be that, although appropriate compounds were tested, they were tested in inadequate doses or durations [93]. The optimal dose for antioxidant therapy remains controversial, because the most effective doses are not known. For example, 800 units of vitamin E and 500 mg of vitamin C have been suggested to be the effective threshold dose; however, doses greater than 400 units a day have been suggested to increase mortality [94]. Another possible reason is the lack of measures of oxidation, without which it is not known whether sufficient amounts of antioxidants are absorbed or biologically active [95]. Finally, most trials were conducted with patients with established pathology, and since oxidation can occur through multiple pathways, different antioxidants with different targets can be combined to study the effects on clinical outcomes [95].

The lack of positive results in clinical trials does not necessarily disprove the important role of treating oxidative stress using antioxidants. Rather, these trials challenge us to better design antioxidant trials in the future and focus on using the best antioxidants at the right doses, in the right population and for the optimal duration.

7. Conclusions

Both mitochondrial dysfunction and massive inflammatory response are strongly associated with cerebrovascular diseases. Oxidative stress thus plays a major role in the pathophysiology of stroke and other brain-related pathologies. Highly reactive species have short half-lives; therefore, they react with the adjacent structures with no specific preference to any kind of macromolecules. Damage caused by oxidative stress is, thus, hard to predict. Targeting oxidative stress for the treatment of stroke is a challenge for the current research. Multimodal therapy, as well as innovative techniques such as therapy using nanoparticles or stem cells, are potential ways to minimize the harmful effect of oxidative stress.

This review was written using published articles that examined various antioxidant treatment strategies in stroke. An electronic database search of SpringerLink, ScienceDirect and PubMed/Medline was conducted with the search terms Stroke AND Antioxidants, (Stroke OR “Ischemic Stroke” OR “Hemorrhagic Stroke”) AND (Antioxidants OR “Antioxidant Therapy” OR “Antioxidant Treatment”), “Intracerebral Hemorrhage” AND (Antioxidants OR “Antioxidant Therapy” OR “Antioxidant Treatment”), and “Subarachnoid Hemorrhage” AND (Antioxidants OR “Antioxidant Therapy” OR “Antioxidant Treatment”). We excluded all non-English papers. Articles were excluded if they were unrelated to the review’s topic. Our goal was not to include all compounds used in stroke antioxidant treatment, but, rather, to provide a comprehensive review of the types of therapeutic approaches available, with examples of compounds used for each given type.

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3.1.1.3 The Role of Oxidative Stress in Early Brain Injury after Subarachnoid Hemorrhage

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This review article is focused on the role of oxidative stress in early brain injury. In the first part, we describe the pathophysiology of oxidative stress associated with SAH, i.e. sources of free radicals in EBI and disruption of antioxidant mechanisms after SAH. In the main part, we cover the topic of molecular signaling in oxidative stress and molecular mechanisms of EBI related to oxidative stress.

Review Article

The Role of Oxidative Stress in Early Brain Injury after Subarachnoid Hemorrhage

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This review focuses on the problem of oxidative stress in early brain injury (EBI) after spontaneous subarachnoid hemorrhage (SAH). EBI involves complex pathophysiological mechanisms, including oxidative stress. In the first section, we describe the main sources of free radicals in EBI. There are several sources of excessive generation of free radicals from mitochondrial free radicals' generation and endoplasmic reticulum stress, to hemoglobin and enzymatic free radicals' generation. The second part focuses on the disruption of antioxidant mechanisms in EBI. The third section describes some newly found molecular mechanisms and pathway involved in oxidative stress after EBI. The last section is dedicated to the pathophysiological mechanisms through which free radicals mediate early brain injury.

1. Introduction

Spontaneous subarachnoid hemorrhage (SAH) is a serious form of hemorrhagic stroke with high morbidity and mortality. SAH patients suffer from an initial mortality of 15–20%, 40% die within one month, and around one-third of survivors have serious morbidity in the form of neurological deficits [1]. The majority of SAH cases are caused by a nontraumatic rupture of a cerebral aneurysm. For decades, research concerning SAH has focused on delayed cerebral ischemia (DCI) and delayed cerebral vasospasms (CVS). However, controversial results of the studies with clazosentan (the CONSCIOUS studies) and lack of prevention against DCI are why the trend in SAH research has been shifting from DCI and delayed CVS to early brain injury [2].

Early brain injury (EBI) encompasses complex pathophysiological changes in the central nervous system (CNS) in the first 72 hours after the onset of bleeding and before the development of CVS. It has been suggested that EBI mechanisms strongly determine the morbidity and mortality

of SAH patients and is important in the development of impairments like CVS, DCI, and associated delayed cerebral ischemic deficit (DIND) [2]. The range of pathophysiological impairments that get triggered during EBI includes ionic, biochemical, molecular, and vascular changes, oxidative stress, inflammation, blood-brain barrier (BBB) dysfunction, apoptosis, and necrosis [2].

Oxidative stress is strongly associated with pathophysiological mechanisms of EBI after SAH [3]. Cellular respiration impairment results in production of free radicals, which are molecules with an unpaired valence electron and are highly chemically reactive. Disruption of metabolism of neurons due to hypoxia and the resultant changes in cellular oxidative state are associated with oxidative stress [4]. Therefore, the brain, with its high metabolic demands, is especially sensitive to free radicals' injury whenever there is a disruption in the cellular respiration. In EBI, an imbalance is struck between production of reactive oxygen species (ROS) and intrinsic antioxidant system's ability to counteract or repair the damage. This imbalance favors ROS and leads to damage to the

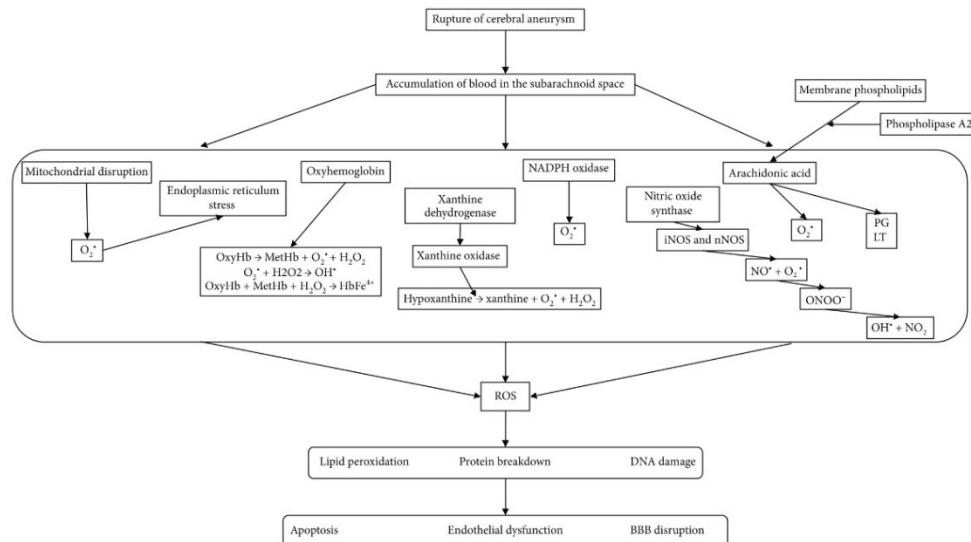


FIGURE 1: Diagram of major sources of oxidative stress in the pathophysiology of EBI after SAH. BBB: blood-brain barrier; iNOS: inducible nitric oxide synthase; LT: leukotriene; MetHb: methemoglobin; nNOS: neuronal nitric oxide synthase; OxyHb: oxyhemoglobin; PG: prostaglandin; ROS: reactive oxygen species.

brain. The most common free radicals involved in oxidative stress after SAH are superoxide anion, hydroxyl radical, nitric oxide, and peroxynitrite [5].

This review discusses known mechanisms of excessive free radicals' production, a disruption of antioxidant mechanisms, basic molecular signaling pathways, and brain injury associated with oxidative stress in EBI after SAH (Figure 1).

2. Free Radicals Mediated Early Brain Injury

ROS are key mediators of pathophysiological mechanisms involved in EBI after SAH. There is an early generation of ROS in the form of superoxide anion, hydroxyl radical, hydrogen peroxide, NO, and peroxynitrate after SAH [6]. There are also several antioxidant systems that try to deal with ROS. However, their levels are inadequate to deal with such an excess of ROS formation, leading to an imbalance between ROS production and antioxidant systems in the brain after SAH.

There are several mechanisms of ROS-associated injury after SAH. ROS damage the vascular smooth muscle and endothelium [2]. This involves damage to membrane-bound phospholipids, which leads to production of second messengers like inositol-3-phosphate (IP3) and diacylglycerol (DAG) and derivatives of arachidonic acid such as leukotriene C4 and prostaglandin D2 [5]. Their presence leads to vasoconstriction and subsequently to delayed cerebral vasospasms [2, 5]. These pathophysiological changes lead to the disruption the blood-brain barrier (BBB) and consequently to cerebral

edema [2]. ROS induce enzymes of apoptotic pathways such as p53 and caspase-3 and caspase-9 leading to cell death [7].

Oxidative stress in EBI could be linked to cerebral vasospasms and delayed cerebral ischemia (DCI). The changed levels of vasodilators (NO) and vasoconstrictors (endothelin 1) or tissue unresponsiveness can lead to microvascular dysfunction and to cerebral vasospasms [8, 9]. Furthermore, microvascular dysfunction can lead to parenchymal hypoperfusion [8]. Cerebral vasospasms and parenchymal hypoperfusion can be linked to cell death (apoptosis, necrosis, and autophagy) in neurons [8]. Other pathophysiological mechanisms like microthrombosis and neuroinflammation, disruption of the BBB, and the subsequent cerebral edema also contribute to the neuronal cell death [2, 8]. Neuronal cell death along with other complications (like hydrocephalus and systemic inflammatory response syndrome) can lead to DCI.

3. Main Sources of Free Radicals in EBI after SAH

There are several sources that contribute to excessive free radicals' production in EBI after SAH. The two main sites that can tip the scales in free radicals' favor are mitochondrial overproduction, hemoglobin autooxidation, and inflammation. Additionally, endoplasmic reticulum stress can also contribute to high free radicals' production and consequently oxidative stress in EBI. Finally, there are also several enzymatic pathways that can generate free radicals.

3.1. Mitochondrial Free Radicals' Generation. The main source of free radicals in EBI is the leakage of superoxide anions from mitochondria. It is caused by the ischemic disruption of the electron transfer chain [3]. In normal (physiological) conditions, electrons leak from the transport chain and react with oxygen (O_2) producing superoxide anion ($O_2^{\cdot-}$). Superoxide is then cleared by superoxide dismutase, which converts superoxide radicals to molecular oxygen and hydrogen peroxide [10]. However, after a period of ischemia that occurs in EBI pathophysiology, the mitochondria produce excessive amounts of free radicals that can no longer be cleared by antioxidants (such as superoxide dismutase) [2]. The overload of free radicals that cannot be cleared by antioxidants then have the potential to cause damage to lipids, proteins, and DNA [3]. In general, the production of ROS is highest when components of the electron transfer chain are maximally reduced [11].

Under physiological conditions, mitochondria display a high electrochemical gradient which is generated by the proton pumping components of the respiratory electron transport chain. This gradient is the driving force for phosphorylation of adenosine diphosphate (ADP) to adenosine triphosphate (ATP) and closing off Ca^{2+} from the cytosol. However, EBI after SAH and the subsequent ischemia cause an exaggerated Ca^{2+} accumulation in the mitochondria [12]. This accumulation leads to disruption of the mitochondrial membrane potential and opening of membrane permeability transition pores (MPTPs) [13]. MPTPs are multiprotein channel complexes that span the inner and outer mitochondrial membranes, allowing solutes of less than 1500 Daltons (Da) to cross the membrane from the matrix to the cytosol [12, 13]. Mitochondria tries to reestablish an electrochemical gradient by increasing substrate oxidation and O_2 consumption, which leads to more free electrons interaction with O_2 and the creation of superoxide [3]. To support this, state 4 mitochondrial respiration and decreased respiratory control ratios that are associated with increased ROS production has been recorded after SAH [14]. Discussion continues about the possible pros and cons of targeting the MPTPs as a therapeutic strategy after SAH [12]. Specific molecules had been used for the inhibition of MPTPs, such as immunosuppressant cyclosporin A and NIM811, reducing oxidative damage [12].

Mitochondrial dysfunction is also thought to contribute to oxidative stress and the pathophysiological mechanisms of EBI. Mitochondrial dynamics, such as mitochondrial fission and fusion imbalance, have been recently recognized as some of the key components of mitochondrial dysfunction [15]. Their mechanisms in attenuating oxidative stress and apoptosis in EBI after SAH have been recently explored by Wu et al. [15] and Zhang et al. [16].

The protein Drp1 plays an important role in the mitochondrial dysfunction [15]. Mdivi-1 is a selective inhibitor of Drp1, a mitochondrial fission protein [15]. It has the ability to penetrate the BBB, giving it importance in CNS diseases [15]. Mdivi-1 treatment alleviated oxidative stress during EBI elevation of superoxide dismutase (SOD) activity and reduced markers of lipid, protein, and DNA oxidative injury

malondialdehyde (MDA), 3-nitrotyrosine (3-NT), and 8-hydroxy-2'-deoxyguanosine (8-OHdG) [15].

Zhang et al. [16] researched docosahexaenoic acid (DHA) effects on EBI after SAH. DHA is an omega-3 fatty acid. In in vitro study using OxyHb to simulate the effects of hemorrhagic stroke [16–18], DHA attenuated mitochondrial disorders (fission and fusion imbalance), which possibly contributes to its antioxidative and antiapoptotic effects [16]. The mechanism of DHA neuroprotective properties has to do with decreased mitochondrial fragmentation in after SAH as well as the expression of Drp1 [16]. Furthermore, DHA was able to improve neurological deficits and attenuate brain edema, as well as significantly limit the increase of SAH-induced oxidative stress and apoptosis [16]. Taken together, oxidative stress induced by an imbalance of mitochondrial dynamic was improved by DHA in both *in vitro* and *in vivo* conditions [16]. Suggesting that improved mitochondrial dynamics may be responsible for the antioxidative effects of DHA.

3.2. Endoplasmic Reticulum Stress. Another important mechanism that is linked with oxidative stress is an endoplasmic reticulum (ER) stress. Their interaction has been found in acute central nervous system injuries, such as EBI after SAH [19]. EBI causes ROS overproduction, leading to the disorder of ER redox and cell apoptosis [19]. ER stress leads to ROS overproduction making it a vicious cycle and further worsening the oxidative damage [20]. Combination of therapies that would alleviate both stresses could be of potential therapeutic interest. Resveratrol has been shown to reduce both oxidative and ER stresses [21]. ER stress was also shown to be alleviated by apelin-13 through inhibition of activating transcription factor 6 (ATF6) in EBI after SAH [22], thus, alleviating ER stress-mediated neuronal apoptosis [22]. Similarly, apelin-13 has been shown to alleviate ER stress via the apelin receptor (APJ) through the AMPK/TXNIP/NLRP3 signaling pathway, which downstream signaling results in inflammation and cellular apoptosis [23–25].

3.3. Hemoglobin Free Radicals' Generation. Another major source of free radicals and oxidative stress in the pathophysiology of EBI after SAH is autooxidation of hemoglobin. The introduction of oxyhemoglobin (oxyHb) into the subarachnoid space and consequently to the cerebrospinal fluid (CSF) is a major producer of superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) as it goes through autooxidation to methemoglobin [3]. The iron ion liberated from oxyHb propels the generation of hydroxyl radical ($OH^{\cdot}$) from $O_2^{\cdot-}$ and H_2O_2 (both from metHb) [26]. Interaction between metHb and oxyHb with H_2O_2 also generates ferryl hemoglobins ($HbFe^{4+}$), which are strong oxidizing agents [26].

Many studies mention the oxidizing effects of hemoglobin on lipids, proteins, and DNA [3, 26, 27]. Products of hemolysis in the subarachnoid space also increase cytochrome c-mediated DNA fragmentation [27]. A study by [26] found an increased nonheme iron and iron-handling proteins during EBI. They also introduced iron chelator DFX into the system that helped reduce SAH-induced cell damage and changes related to hemoglobin-related oxidative

stress, making it a possible therapeutic target for patients with SAH [26].

3.4. Enzymatic Free Radicals' Generation. Besides free radicals generated by mitochondria and hemoglobin autooxidation, there are several other enzymatic pathways that can produce free radicals. The intracellular accumulation Ca^{2+} in neurons (due to extracellular hemoglobin and ischemia) can produce free radicals through activation of several prooxidative mechanisms like phospholipases, nitric oxide synthase, NADPH oxidase, and xanthine oxidoreductase.

3.4.1. Arachidonic Acid Derivates. Arachidonic acid (AA) is released from phospholipids in the cytoplasmic membrane by phospholipase A2 (PLA2). AA is then processed by cyclooxygenase, lipoxygenase, or cytochrome P450, where each of these pathways can produce superoxide anion as their byproduct [28]. These mechanisms are considered to be an important source of free radicals in brain injury and ischemia [28]. Increased PLA2 activity, as well as cyclooxygenase and lipoxygenase, has been found to follow SAH [3, 29–31]. Inhibition of these pathways had a positive clinical impact (on vasospasms), but it is unclear if it was through reduction of oxidative stress. A study by [32] focused on inhibition of leukotriene B4 (LTB4). LTB4 is a highly potent chemoattractant for leukocytes (mainly neutrophils). LTB4 is generated from arachidonic acid (AA) by cytosolic phospholipase A2, 5-lipoxygenase, and leukotriene A4 hydrolase [33]. Activated neutrophils are a source of oxidative stress because of their respiratory burst and are drawn to the subarachnoid space by, among others, LTB4 [34]. Reduced LTB4-driven neutrophil infiltration decreases ROS and MDA and increases levels of SOD and glutathione peroxidase (GSH-Px) during EBI [32]. Therefore, inhibition of LTB4 synthesis may decrease the neutrophil-generated oxidative stress. Furthermore, blockage to LTB4 synthesis reduces translocation of nuclear factor kappa B (NF- κ B) and its DNA-binding activity [32]. It leads to the alleviation of the NF- κ B-mediated inflammation during EBI after SAH [32]. Furthermore, the reduction of synthesis of LTB4 could decrease neutrophil recruitment to the brain parenchyma after SAH. The specific mechanism of LTB4/NF- κ B-mediated inflammation remains unknown.

3.4.2. Nitric Oxide in Early Brain Injury. Generally, there are two main systems that play crucial role in oxidative stress after EBI. One of them is the oxygen-based free radicals and the other is nitrogen-based free radicals. Nitric oxide has a versatile function in the CNS. It is an important regulator of the cerebral blood flow (CBF); it inhibits platelet adherence, reduces adherence of leukocytes, suppresses vessel injury, acts as a neurotransmitter and finally a free radical [9].

Nitric oxide (NO) as a free radical ($\text{NO}^{\bullet}$) is generated by nitric oxide synthase (NOS). NOS has three isoforms: endothelial (eNOS), inducible (iNOS), and neuronal (nNOS) [35]. Two main types involved in EBI are iNOS and nNOS [35]. $\text{NO}^{\bullet}$ levels in the first 24 hours after SAH decrease, recover, and increase, making it a very dynamic process [9]. Cerebral ischemia increases NOS activity and

gene expression [36]. In EBI, around hour 6 after the initial bleeding, the major producer of $\text{NO}^{\bullet}$ is iNOS [9].

There are three main phases of the NO activity that are described in EBI. Information about phase I and II come from experimental animal studies. Phase III has been studied in both animals and humans [37]. Phase I describes the first hours after SAH and is characterized by an acute decrease in cerebral NO level [38]. Cerebral vessels are constricted and CBF is reduced [39]. NO-dependent vasodilation is impaired in this early stage of EBI after SAH [39]. However, NO donor increases the reduced CBF at this stage [39]. Interestingly, in occlusive ischemic stroke, there is an increase in cerebral NO in phase I. Phase II defines hours 1–6 in which NO levels recover to their baseline [9]. The CBF remains reduced, which suggests that NO produced during phase II is unable to recover the initial CBF [40]. Also, NO donor has little effect on CBF [9]. Phase III encompasses the rest of the EBI period (hour 6–72) [9]. Arterial response to NO is reduced at this stage, possibly due to alterations in cGMP pathways [41]. Increase in NO levels in phase III has been linked to oxidative damage in the brain [9].

$\text{NO}^{\bullet}$ can interact with $\text{O}_2^{\bullet -}$ to form peroxynitrite ($\text{ONOO}^{\bullet -}$), which can then turn into $\text{OH}^{\bullet}$ [42]. $\text{O}_2^{\bullet -}$, $\text{ONOO}^{\bullet -}$, and $\text{OH}^{\bullet}$ are neurotoxic free radicals, damaging the cell and leading to cell death [42]. The presumed mechanisms of NO oxidative damage involve lipid peroxidation of the cell membranes and DNA and mitochondrial damage, leading to apoptosis [9].

On the other hand, $\text{NO}^{\bullet}$ could have beneficial effects on the cerebral blood flow [39, 43]. In addition, it has been hypothesized that $\text{NO}^{\bullet}$ may also possess neuroprotective properties due to its ability to modify the NMDA receptor response and reduce toxicity [43].

3.4.3. NADPH Oxidase. NADPH oxidase (NOX) is a membrane-bound enzyme complex found on the extracellular side of the cytoplasmic membrane and may produce $\text{O}_2^{\bullet -}$ [44]. NOX has been found to contribute to oxidative stress in *in vitro* studies [45] and increased expression of NOX in SAH could be associated with elevated oxidative stress [46]. Reduction of NOX-associated free radicals' generation was associated with reductions in mortality, neurological deficits, and neuronal cell death [46]. Direct inhibition of NOX in EBI after SAH has not yet been studied in SAH.

3.4.4. Xanthine Dehydrogenase. Xanthine dehydrogenase (XDH) is an enzyme homodimer found among others in the endothelium [47]. It is involved in the oxidative metabolism of purines creating uric acid [47]. XDH does not produce free radicals directly, rather it is converted to xanthine oxidase (XO) during ischemia, hypoxia, and excitotoxicity [47]. XO then catalyzes the oxidation of hypoxanthine to xanthine creating uric acid, $\text{O}_2^{\bullet -}$ and H_2O_2 [3]. The general impact of XO inhibition during SAH is being discussed because of other more significant pathways that generate free radicals.

4. Disruption of Antioxidant Mechanisms

There are several protective systems that try to balance out the production of free radicals. Superoxide dismutase

(SOD), glutathione peroxidase (GSH-Px), and catalase (CAT) are important enzymatic scavengers during normal cellular respiration [3]. However, in EBI after SAH, these systems become downregulated, overwhelmed, or modulated leading to reduction of their antioxidative capacities [14].

Under physiological conditions, SOD produces H_2O_2 from O_2^- ; H_2O_2 is then scavenged by GSH-Px to prevent formation of the more dangerous $OH^\cdot$ [48]. However, in SAH, there is an increased activity of Cu-SOD and Zn-SOD and an increase in the SOD/GSH-Px ratio [14]. This leads to excess production of $OH^\cdot$ which cannot be scavenged by GSH-Px, meaning increased SOD/GSH-Px ratio has the potential to be a significant source of free radicals.

Several molecules have been studied due to their antioxidant capacity for alleviating oxidative stress in EBI after SAH [16, 21, 49–52]. Resveratrol was studied as one such molecule by Xie et al. [21]. Resveratrol is a natural phenol with broad bioactive capacity. Resveratrol treatment decreased the excessive production of ROS and MDA, which are indicators of oxidative stress [21]. Resveratrol has been reported to inhibit protein carbonylation and nitration and lipid peroxidation, attenuating oxidative stress [21]. Furthermore, resveratrol has an antioxidant capacity of activating endogenous mechanisms, such as upregulation of Nrf2 and HO-1 in EBI after SAH [21]. Under pathological conditions, such as oxidative stress during EBI, the Nrf2 pathway gets activated and triggers the antioxidant enzyme expression, including HO-1, attenuating cellular oxidative stress [53, 54]. HO-1 exerts its antioxidant properties by catalyzing the degradation of heme [55]. Therefore, induction of the Nrf2 and HO-1 systems result in protective mechanisms against cellular oxidative stress. Another molecule, cysteamine, was studied by Zhang et al. [51]. Cysteamine is a product of coenzyme A degradation. Intraperitoneal administration of cysteamine was shown to have positive antioxidative and antiapoptotic effects in EBI after SAH [56]. Similar to other molecules discussed in this review, cysteamine reduced ROS, MDA, 3-NT, and 8-OHdG levels and increased GSH-Px activity [56]. The mechanism of effect of cysteamine is not yet known.

5. Molecular Signaling

Molecular signaling, different intracellular pathways, their interactions, and effects are important for our understanding not only of the mechanisms of oxidative stress after EBI but also generally the pathophysiology of SAH. Although our knowledge in this field is still limited, there are some known mechanisms that may help understand the underlying mechanisms of what is happening inside the cell because of oxidative stress during EBI.

An important signaling pathway that gets activated in EBI after SAH is proapoptotic (and proinflammatory) mitogen-activated protein kinase (MAPK) [51, 57]. Oxidative stress is a strong inducer of MAPK signaling [50]. Antioxidants have been shown to attenuate the MAPK pathway signaling [58]. Antioxidant naringin was found to restore the decreasing levels of SOD, CAT, and GSH-Px [59]. Naringin has also reduced levels of phosphorylated ERK1/2, JNK,

and p38, which are downstream molecules of MAPK signaling [59]. This indicated that naringin could have potential antiapoptotic and antioxidative effects due to its targeting of MAPK signaling.

In addition, Dou et al. [60] have studied the involvement of tumor necrosis factor receptor-associated factor 6 (TRAF6) on oxidative stress. TRAF6 is a protein with ubiquitin ligase activity that mediates signal transduction from members of the TNF receptor superfamily [60]. Toll-like receptor 4 (TLR4) has also been suggested as an upstream molecule (receptor) of the TRAF6 signaling pathway [60]. TRAF6 plays a possible role as an intersection between activating NF- κ B pathway, which is an important regulator in immune development, immune response, inflammation, and cancer, and MAPK/ERK pathway, which controls adaptive responses to intracellular and extracellular stresses [60–63]. The final downstream molecules NF- κ B and AP-1 then can add to a great number of pathophysiological processes. TRAF6 levels increase during EBI (peaking at 24 hours) [60]. The connection between TRAF6 and oxidative stress in EBI after SAH may lie in its capacity to mediate the production of ROS and ability to translocate to mitochondria and ubiquitinate ECSIT, which is important for assembly and stabilization of respiratory complex I, and consequently to further increasing production of ROS in mitochondria [60]. Thus, administration of TRAF6 inhibitors could be of a potential interest in the reduction of oxidative stress in EBI. Liu et al. [64] has shown the TRAF6 inhibitor (sophoridine) potential in reducing the size of infarctions in ischemic injury.

In addition, another pathway, possibly involved in signaling due to oxidative stress in EBI, is the Mas/PKA/CREB/UCP-2 pathway [65]. Mo et al. [65] studied AVE 0991 which through this pathway reduced oxidative stress and neuronal apoptosis in EBI around hour 24 after the onset of bleeding. AVE 0991 (AVE) is a nonpeptide analog of angiotensin (1–7) [65]. It signals through receptor Mas, which is a G-protein coupled receptor and has been identified as a component of the renin-angiotensin system in the brain [65]. The suggested mechanism of reduced oxidative stress and neuronal apoptosis involves upregulation of PKA-C alpha, phosphorylated CREB, a transcription factor, UCP-2, which is a controller of mitochondria-derived reactive oxygen species, and Bcl-2, apoptosis regulatory protein [65], as well as the downregulation of Bax, which is an apoptotic activator, and Romo-1, which can induce production of reactive oxygen species during EBI [65].

6. Molecular Mechanisms of Free Radicals Mediated Early Brain Injury

Oxidative stress or consequently free radicals lead to neuronal damage by promoting lipid peroxidation, protein breakdown, DNA damage which in turn contributes to apoptosis, endothelial dysfunction, and blood-brain barrier (BBB) disruption [3]. The effects of oxidative stress known as lipid peroxidation alter membrane fluidity and permeability [27]. Oxidation of proteins can change the functions of enzymes and receptors.

Free radicals' damage can also initiate apoptotic cascades or shift them into necrosis [66, 67]. Apoptosis has been shown to be induced by oxidative stress, either by increasing p53, induction of cytochrome c release, and activation of caspase-9 and caspase-3, or through p38 mitogen-activated protein kinase (MAPK) and signal-regulated kinase- (ERK-) mediated apoptosis [66, 67]. Some studies [50, 68] focus on molecules (apigenin, paeoniflorin) that could be of potential therapeutic interest because of their antioxidative and antiapoptotic effects. Apigenin (API) is a flavone naturally occurring in plants (it is mainly isolated from *Hypericum perforatum*) [50]. It has been found that API can reduce the concentration of ROS and elevate levels of GSH-Px and SOD during EBI after SAH [59], although the underlying mechanism has not been described. It was also reported that API could have antiapoptotic effects due to its ability to reverse the upregulation of Bax and caspase-3 [59]. Bax-dependent caspase-3 has been reported as the initiation molecule of neuronal apoptosis [69]. Oxidative stress upregulates Bax and caspase-3 during EBI after SAH [59]. API also has anti-inflammatory effects through the inhibition of the TLR4-mediated inflammatory pathway [70]. Taken together, API is a molecule of great potential in EBI after SAH due to its antioxidative, anti-inflammatory, and antiapoptotic properties, nevertheless further research is still needed. Similar results as with API were reached with paeoniflorin. Paeoniflorin is a natural monoterpene glucoside from the root of *Paeonia lactiflora* [68]. Paeoniflorin was able to decrease levels of proinflammatory cytokines IL-1 β , IL-6, and TNF α , which are upregulated during EBI [68]. Similar to API, the concentration of ROS has decreased and levels of GSH-Px and SOD have increased during EBI after SAH [68]. Antiapoptotic effects through Bax-dependent caspase-3 were also observed [68].

Blood-brain barrier (BBB) disruption is an important part of the SAH pathophysiological mechanisms. Microvessels and their tight junctions help to constitute the BBB and have been shown to be disrupted in EBI, because their endothelial cells are susceptible to injury by oxidative stress [71]. This effectively leads to BBB dysfunction and subsequent cerebral edema. Antioxidants (edaravone and curcumin) have been shown to prevent MMP-9 expression and activation [49, 52]. Subsequently blocking MMP-9 mediated degradation of claudin-5 and occludin, damaging tight junctions [49, 52]. The specific regulatory signal of MMP-9 is still unknown, but possible candidates and interleukin- (IL-) 1 β , IL-6, tumor necrosis factor- α (TNF α), and NF- κ B [49].

7. Clinical Implications and Future Directions

As mentioned above, there are many sources of free radicals' production in the EBI after SAH. There is also promising potential for using free radicals' scavengers, antioxidants, and reinforcing the innate antioxidative mechanisms that already exist in the brain. Many of these compounds, like DHA [16], resveratrol [21], API [50], paeoniflorin [68], and ebselen [72], have been described in this review. These antioxidants have been used in clinical and experimental trials with promising results.

Some clinical research has also been focused on the use of antioxidant micronutrients like vitamins C and E, copper, selenium, and zinc [73]. The overall results of Berger et al.'s [73] study did not find significant all-around improvement of SAH patient's conditions, which is understandable due to the many pathophysiological mechanisms of SAH. However, the administration antioxidant micronutrients significantly blunted the associated inflammation [73].

The suggested therapeutic window is difficult to pinpoint because of the lack of a larger clinical research. However, based on the article by Bergen et al. [73] article, the micronutrient antioxidant intervention started at 24 hours after the initial bleeding. For the first 2 days, they used double dosage, and for the last 3 days, they used therapeutic dose as described in their article.

Other pharmacological research has focused on the use of lipid peroxidation inhibitor tirilazad and the superoxide scavenger PEG-SOD [74]. The therapeutic window for PEG-SOD is around 4 hours after the initial bleeding and tirilazad has the therapeutic window of 1 to 4 hours after the initial bleeding [74]. The results for both antioxidant drugs failed to produce the desired results [74]. However, tirilazad produced statistically significant increase in survival [74]. Therefore, further research with an LP-inhibiting antioxidant might be more relevant to the treatment of SAH.

Although the practical applicability of antioxidative treatment is still limited, the potential for therapeutic use exists and is worth investigating. Currently, there is no antioxidative treatment being routinely used in SAH patients. It can be contributed to the fact that oxidative stress is merely one parameter of a boarder EBI problematic. However, it is possible that targeted antioxidant therapy could be effective as one component of a larger treatment regime that would address the many components and mechanisms that are involved in EBI after SAH.

8. Conclusion

The content of this article could be summarized as a review of various aspects of oxidative stress associated with early brain injury (EBI) after subarachnoid hemorrhage (SAH). Mechanisms of oxidative stress after EBI are a subject of an extensive research. EBI and oxidative stress, as a part of its pathophysiology, are of great importance in understanding the complex mechanisms of SAH. Mechanisms that can lead to severe complications (like cerebral vasospasms (CVS), delayed cerebral ischemia (DCI), and delayed ischemic neurological deficit (DIND)) get activated within minutes after the onset of bleeding. That is why the focus of SAH research has been shifting towards EBI.

Under physiological conditions the brain is able to deal with the generation of ROS through different antioxidative mechanisms. However, when there is such an excessive production of free radicals like in SAH, the antioxidative capacities are overused. It has serious negative effects and worsens the conditions and general outcome.

Oxidative stress comes from several sources in EBI after SAH. Mitochondrial disruption and subsequent endoplasmic reticulum stress play an important role in excessive production

of ROS. Hemoglobin autooxidation and various interactions of OxyHb and MetHb also lead ROS overproduction. Unregulated enzymatic pathways like xanthine dehydrogenase, NADPH oxidase, NOS, and derivatives of arachidonic acid processing can also be a major source of ROS.

The importance of studying oxidative stress after EBI lies in its multisource nature. Oxidative stress is among others involved in apoptosis, endothelial dysfunction, and BBB disruption. This is due to the ability of ROS to cause lipid peroxidation, protein breakdown, and DNA damage.

Oxidative stress after EBI poses as a potential therapeutic target and needs to be examined and understood in more detail. It would be helpful to integrate basic oxidative stress research of molecular signaling with SAH-specific characteristics. Further research in this field could provide a better care for SAH patients.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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3.1.1.4 $\alpha 7$ nicotinic acetylcholine receptor agonist PNU-282987 attenuates early brain injury in a perforation model of subarachnoid hemorrhage in rats

Duris K, Manaenko A, Suzuki H, Rolland WB, Krafft PR, Zhang JH. $\alpha 7$ nicotinic acetylcholine receptor agonist PNU-282987 attenuates early brain injury in a perforation model of subarachnoid hemorrhage in rats. *Stroke*. 2011;42:3530–3536.

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In this article, we describe the beneficial effect of $\alpha 7$ agonist PNU-282987 on early brain injury in an experimental model of SAH. $\alpha 7$ receptor is a subtype of the nicotinic receptor, which is specific for the cholinergic anti-inflammatory pathway (this pathway will be described in Chapter 4). In this research, we studied the anti-apoptotic properties of $\alpha 7$ signaling because the $\alpha 7$ receptor was described to be associated with the PI3K-Akt pathway, which plays, among others, an important role in the downregulation of apoptosis-related processes.

A perforation model of SAH in rats was used for this study. Parameters of early brain injury were studied at the time points of 24 and 72 hours after SAH. PNU-282987 improved neurological scores at both time points and reduced brain water content in the left hemisphere 24 hours after the SAH. On the molecular level, PNU-282987 significantly increased levels of phosphorylated Akt and decreased levels of cleaved caspase 3 which is a crucial proapoptotic factor. The beneficial effect of PNU-282987 was reversed by receptor antagonist methylcaconitine as well as by PI3K inhibitor wortmanin proving the mechanism of PNU-282987 action. Using immunohistochemistry, we demonstrated, that phosphorylated AKT was mainly localized in non-apoptotic neurons and that the treatment decreased the overall level of neuronal apoptosis.

This study was, according to our best knowledge, the first study describing the beneficial effect of CHAIP stimulation after subarachnoid hemorrhage.

$\alpha 7$ Nicotinic Acetylcholine Receptor Agonist PNU-282987 Attenuates Early Brain Injury in a Perforation Model of Subarachnoid Hemorrhage in Rats

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Background and Purpose—Early brain injury is an important pathological process after subarachnoid hemorrhage (SAH). The goal of this study was to evaluate whether the $\alpha 7$ nicotinic acetylcholine receptor ($\alpha 7$ nAChR) agonist PNU-282987 attenuates early brain injury after SAH and whether $\alpha 7$ nAChR stimulation is associated with down-regulation of caspase activity via phosphatidylinositol 3-kinase-Akt signaling.

Methods—The perforation model of SAH was performed, and neurological score, body weight loss, and brain water content were evaluated 24 and 72 hours after surgery. Western blot and immunohistochemistry were used for quantification and localization of phosphorylated Akt and cleaved caspase 3. Neuronal cell death was quantified with TUNEL staining. $\alpha 7$ nAChR antagonist methylcaconitine and phosphatidylinositol 3-kinase inhibitor wortmannin were used to manipulate the proposed pathway, and results were quantified with Western blot.

Results—PNU-282987 improved neurological deficits both 24 and 72 hours after surgery and reduced brain water content in left hemispheres 24 hours after surgery. PNU-282987 significantly increased phosphorylated Akt levels and significantly decreased cleaved caspase 3 levels in ipsilateral hemispheres after SAH. Methylcaconitine and wortmannin reversed effects of treatment. Phosphorylated Akt and cleaved caspase 3 were colocalized to neurons in the ipsilateral basal cortex. Phosphorylated Akt was mainly localized in TUNEL-negative cells. PNU-282987 significantly reduced neuronal cell death in the ipsilateral basal cortex.

Conclusions— $\alpha 7$ nAChR stimulation decreased neuronal cell death and brain edema and improved neurological status in a rat perforation model of SAH. $\alpha 7$ nAChR stimulation is associated with increasing phosphorylation of Akt and decreasing cleaved caspase 3 levels in neurons. (*Stroke*. 2011;42:3530-3536.)

Key Words: $\alpha 7$ nicotinic acetylcholine receptor ■ PNU-282987 ■ subarachnoid hemorrhage ■ early brain injury ■ apoptosis ■ Akt ■ cleaved caspase 3

Subarachnoid hemorrhage (SAH), with an annual incidence of 7 per 100 000 and a case fatality rate of $\approx 50\%$, is a severe condition caused in $\approx 85\%$ by rupture of intracranial aneurysm.¹ Early brain injury is a major contributor to the high mortality and morbidity after SAH, and apoptosis is a significant element of early brain injury.² The phosphatidylinositol 3-kinase (PI3K)-Akt pathway is an important pathway in neuronal cells, decreasing activation of proapoptotic caspases.³

$\alpha 7$ nicotinic acetylcholine receptors ($\alpha 7$ nAChRs) are ion channels expressed mainly in neurons and immune and endothelial cells, where they are responsible for providing antiapoptotic and anti-inflammatory effects.^{4,5} The antiapoptotic effect of $\alpha 7$ nAChR activation in neuronal cells was reported to involve PI3K-Akt.⁵ The protective role of the PI3K-Akt pathway in reducing cleaved caspase 3 (CC3) after SAH was described previously⁶; however, references regarding $\alpha 7$ nAChR and SAH are still missing.

In this study we tested 2 hypotheses. First, the $\alpha 7$ nAChR agonist PNU-282987 attenuates early brain injury after SAH. Second, the $\alpha 7$ nAChR stimulation is associated with the PI3K-Akt signaling pathway, which decreases proapoptotic caspase activation.

Materials and Methods

Animals and Drugs

All of the experiments were approved by the Loma Linda University Institutional Animal Care and Use Committee. Adult male Sprague-Dawley rats (277–320 g) were purchased from Harlan Laboratories (Indianapolis, IN). Animals were fed ad libitum. We used 134 animals in this study, assigned to the following groups, naïve (n=4), sham (n=24), vehicle (n=39), PNU-12 (n=36), PNU-4 (n=10), methylcaconitine (MLA; n=12), and wortmannin (WOR; n=9).

All of the drugs were purchased from Sigma-Aldrich. Drugs were dissolved and administered according to data published previously.^{7–10}

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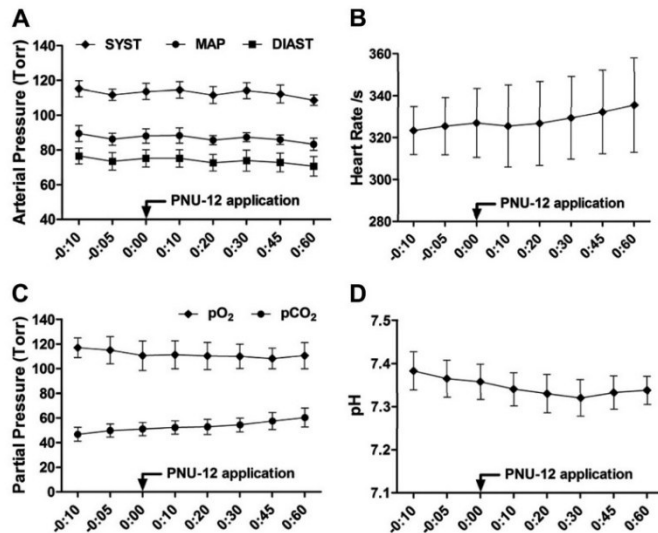


Figure 1. Arterial pressure (A), heart rate (B), P_{O_2} , P_{CO_2} (C), and pH (D) from 10 minutes before to 60 minutes after application of PNU-282987 in naïve animals (N=4). Data are expressed as a mean and SEM. SYST indicates systolic pressure; MAP, mean arterial pressure; DIAST, diastolic pressure.

$\alpha 7nAChR$ agonist PNU-282987 was dissolved in saline and was injected IP 1 hour after surgery in doses of 12 mg/kg (PNU-12, MLA, and WOR groups) or 4 mg/kg (PNU-4 group). In the naïve group, 12 mg/kg of PNU-282987 was injected IP 15 minutes after initiation of physiological parameter monitoring. $\alpha 7nAChR$ antagonist methylscopolamine was dissolved in saline and injected IP at a dose of 6 mg/kg 15 minutes before administration of PNU-282987 (MLA group). PI3K inhibitor wortmannin (Sigma-Aldrich) was dissolved in saline and injected IV at a dose of 15 μ g/kg 90 minutes before PNU-282987 administration. Sham or vehicle animals received equivalent volumes of saline given to animals in other groups (500 μ L).

Physiological Parameters

Blood pressure/heart rate measurements and analysis of blood samples were performed in naïve animals. Animals were anesthetized, intubated, and kept on artificial ventilation using isoflurane 3% in 60/40% medical gas/oxygen mixture. The right femoral artery was cannulated for invasive blood pressure/heart rate monitoring (DIGI-MED Blood Pressure Analyzer) and collection of blood samples for subsequent analysis (IL GEM 4000 Premiere automatic analyzer). The following parameters were evaluated from the blood samples: pH, P_{O_2} , P_{CO_2} , Na^+ , K^+ , Cl^- , and glucose. Monitoring was performed from 10 minutes before (3 measurements) and ≤ 60 minutes after (5 measurements) application of PNU-282987 (12 mg/kg). Mean values of physiological parameters before PNU-282987 administration were compared with the mean values after administration. Selected data are shown in Figure 1 at each time point.

Surgery

The endovascular perforation model of SAH was performed as described previously.¹¹ Briefly, rats were anesthetized, intubated, and kept on artificial ventilation during surgery with 3% isoflurane in 60%/40% medical-air/oxygen. Body temperature was monitored by rectal probe, and normothermia was maintained by a heating lamp. A sharpened 4-0 nylon suture was introduced into the left internal carotid artery until resistance was felt (≈ 18 mm from the common carotid bifurcation). The suture was then pushed further to perforate the bifurcation of the anterior and middle cerebral arteries until resistance was overcome and withdrawn immediately after perforation. In sham-operated animals, the suture was inserted into the left carotid artery; however, no perforation was performed. After

suture removal, the incision was closed, and rats were individually housed in heated cages until recovery.

Body Weight and SAH Grade

Body weight was measured after induction of anesthesia before intubation and before euthanization. Results are expressed as a ratio of body weight after surgery/body weight before surgery.

SAH grade was evaluated as described previously.¹² Briefly, after removing the brain, a picture of the underside of the brain was taken, and pictures were divided into 6 parts (left and right frontal, left and right temporal, and upper and lower brain stem). Each part was subscored according to occurrences of blood in the subarachnoid space and a total score (maximum: 18) was calculated as the sum of all of the subscores. Animals with an SAH score of ≤ 5 were excluded from the study for low SAH grade.^{10,12}

Neurological Score

Neurological scores were evaluated before euthanization in a blinded fashion using a Garcia scoring system.¹³ The following were evaluated: spontaneous activity, symmetrical movements of limbs, forelimbs outstretching, climbing a wall of a wire cage, axillary touch response, and vibrissae touch response. Minimal score was 0 (worst) and maximal 3 (the best) for each subtest, and total score was calculated as a sum of all of the subtests.

Brain Water Content

Brains were removed 24 or 72 hours after surgery and separated into left hemisphere, right hemisphere, cerebellum, and brain stem. Each part was weighed immediately after removal (wet weight) and after drying in 100°C for 72 hours (dry weight). The percentage of water content was calculated as [(wet weight–dry weight)/wet weight]*100%.

Western Blot

Animals were euthanized 24 hours after surgery, and ipsilateral hemispheres were processed for Western blot as described previously.¹¹ Equal amounts (50 μ g) of total protein were separated in 12% SDS-PAGE and transferred onto nitrocellulose membranes. As primary antibodies we used anti-phospho-Akt (Ser 473; 1:1000; Cell Signaling, No. 9271), anti-CC3 (1:1000; Cell Signaling, No. 9661), and anti- β -actin (1:1000; Santa Cruz Biotechnology, SC-1616), and appropriate secondary antibodies (Santa Cruz Biotechnology) were

Table. Physiological Parameters Before and After Application of PNU-282987 in Naïve Animals (n=4)

Variable	MAP, mm Hg	SYST, mm Hg	DIAST, mm Hg	HR, /s	Po ₂ , mm Hg	Pco ₂ , mm Hg	pH	Na ⁺ , mmol/L	K ⁺ , mmol/L	Cl ⁻ , mmol/L	Glucose, mg/dL
Before PNU	87.9 (±2.90)	113.5 (±3.87)	75.1 (±3.19)	325.3 (±13.76)	114.2 (±10.11)	49.17 (±5.17)	7.36 (±0.042)	132.8 (±0.854)	4.89 (±0.038)	103.4 (±0.84)	185.3 (±12.53)
After PNU	86.1 (±2.91)	112.2 (±3.70)	73.0 (±5.07)	329.9 (±20.26)	110.1 (±10.03)	55.40 (±6.237)	7.33 (±0.038)	134.3 (±0.919)	5.03 (±0.043)	102.6 (±0.56)	183.7 (±10.47)

Data are expressed as mean (±SEM).

MAP indicates mean arterial pressure; SYST, systolic pressure; DIAST, diastolic pressure; HR, heart rate.

used in dilution 1:1000. Bands were visualized using the enhanced chemiluminescence reagent kit (Amersham ECL plus kit, GE Healthcare UK Limited), and quantification was performed by optical density methods using ImageJ software (National Institutes of Health). Results are expressed as a relative density to β -actin subsequently normalized to the mean value of the sham group.

Immunohistochemistry and TUNEL Staining

Animals were euthanized 24 hours after surgery and brains were processed as described previously.¹⁴ Double-fluorescence labeling for NeuN (1:100; Millipore, MAB377) and CC3 (1:1000; Cell Signaling, No. 9661) or NeuN and phosphorylated Akt (p-Akt; Ser473; 1:25; Cell Signaling, No. 9271) was performed as described previously.¹⁴ TUNEL staining (In Situ Cell Death Detection kit, Roche Inc) either with NeuN and p-Akt for neuronal localization or with NeuN alone for cell counting was performed as described previously.³ For negative controls, the same staining without primary antibody was performed. The stained sections were processed using a fluorescent microscope and Magna Fire SP system (Olympus). The TUNEL-positive cells were counted in the ipsilateral basal cortex in 3 fields per animal at $\times 400$ magnification, and data were expressed as the number of TUNEL-positive neurons per millimeter squared.¹⁵

Statistical Analysis

Data are expressed as a mean and SEM. Mortality data were analyzed by Fisher exact test. SAH grading data in the 72-hour study were evaluated by unpaired *t* test. Physiological parameters were evaluated by paired *t* test. All of the other data were analyzed by 1-way ANOVA followed by Tukey post-test. A *P* value of <0.05 was considered statistically significant. All of the statistical analyses were performed using GraphPad Prism version 5.02 for Windows.

Results

We performed 134 surgeries, 29 animals died because of severe SAH, and 11 animals were excluded because of low SAH grade, 5 animals in the vehicle group, 3 in the PNU-12 group, 1 in the PNU-4 group, and 2 in the MLA group. Mortality rates were as follows: sham 0% (0 of 24 animals), naïve + PNU-12 0% (0 of 4 animals), vehicle 29% (10 of 34 animals), PNU-12 27% (9 of 33 animals), PNU-4 33% (3 of 9 animals), MLA 40% (4 of 10 animals), and WOR 33% (3 of 9 animals). Mortality occurred within 6 hours after surgery, and there were no statistically significant differences between the vehicle and PNU-12 groups (*P* value not significant [NS], Fisher exact test).

Effect of PNU-282987 Administration on Physiological Parameters

Blood pressure/heart rate monitoring and blood sample analysis (pH, Po₂, Pco₂, Na⁺, K⁺, Cl⁻, and glucose) were performed in naïve animals (n=4) from 10 minutes before and ≤ 60 minutes after application of PNU-282987 (12 mg/kg).

There were no significant differences in any measured parameters between mean value before application and mean value after PNU application (*P*=NS; Table). Selected data

(blood pressure, heart rate, pH, Po₂, and Pco₂) are also shown in Figure 1 at each time point.

Outcome Study

Neurological Score, Body Weight, and Brain Water Content 24 Hours After Surgery

For the 24-hour outcome study we used sham, vehicle, PNU-12, and PNU-4 groups (n=6 in each group). There were no significant differences in SAH grade among vehicle (12.33±0.71), PNU-12 (11.83±1.01), and PNU-4 (11.67±1.09) groups (*P*=NS). Neurological score (Figure 2A) was significantly higher in sham group (17.50±0.22) compared with vehicle (13.67±0.71), PNU-12 (15.83±0.31), and PNU-4 (15.00±0.45) groups 24 hours after surgery

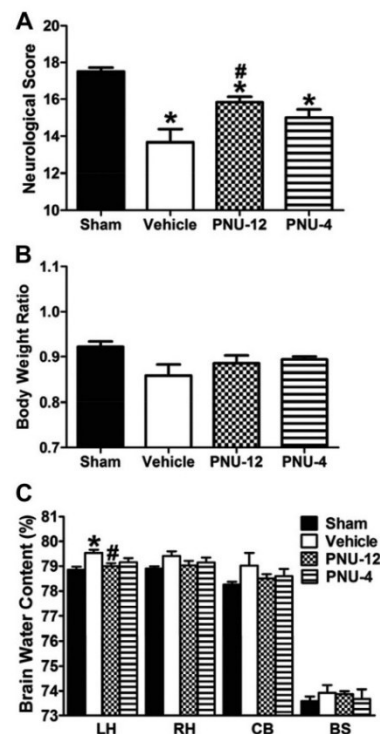


Figure 2. Neurological score (A), body weight ratio (B), and brain water content (C) 24 hours after surgery. Data are expressed as a mean and SEM. **P*<0.05 vs sham, #*P*<0.05 vs vehicle. LH indicates left hemisphere; RH, right hemisphere; CB, cerebellum; BS, brain stem.

($P<0.05$). Compared with vehicle, neurological score was significantly higher in the PNU-12 group ($P<0.05$); however, there was no significant difference between the vehicle and PNU-4 group ($P=NS$). Body weight ratio 24 hours after surgery (Figure 2B) was not significantly different between groups ($P=NS$). Brain water content (Figure 2C) was significantly higher in the left hemisphere of vehicle animals (79.54 ± 0.12) compared with the sham (78.86 ± 0.11) and PNU-12 (79.00 ± 0.12) groups ($P<0.05$). There was no significant difference in brain water content in the left hemisphere between the PNU-4 group (79.16 ± 0.17) and vehicle group ($P=NS$). There were no significant differences between groups in brain water content in the right hemisphere, cerebellum, and brain stem ($P=NS$).

Neurological Score, Body Weight, and Brain Water Content 72 Hours After Surgery

At 72 hours we evaluated the same parameters as in the 24-hour outcome study in sham, vehicle, and PNU-12 groups ($n=6$ in each group). We did not use the 4-mg/kg dose of PNU-282987, because this dosage did not show significant effects after 24 hours.

There was no significant difference in SAH grade between vehicle (10.33 ± 0.92) and PNU-12 (11.50 ± 0.99) groups ($P=NS$). Neurological score (Figure 3A) was significantly lower in the vehicle group (14.50 ± 0.43) compared with the sham (17.67 ± 0.21) and PNU-12 (16.33 ± 0.33) groups ($P<0.05$). There was a significant difference in neurological score between the sham and PNU-12 groups ($P=NS$). Body weight ratio (Figure 3B) was significantly lower in the vehicle (0.87 ± 0.014) compared with the sham (0.97 ± 0.022) group ($P<0.05$). There were no significant differences in body weight ratio of PNU-12 (0.92 ± 0.012) compared with the sham or vehicle groups ($P=NS$). Brain water content (Figure 3C) was not significantly different between groups in any other part of the brain 72 hours after surgery ($P=NS$).

Mechanism Study

Quantification of p-Akt and CC3 at 24 Hours After Surgery

Western Blot analysis was used for quantification of p-Akt and CC3 levels in ipsilateral hemispheres 24 hours after surgery in sham, vehicle, PNU-12, MLA, and WOR animals ($n=6$ in each group). There was no significant difference in SAH grade among vehicle (12.67 ± 0.56), PNU-12 (12.00 ± 0.73), MLA (11.33 ± 0.61), and WOR (11.17 ± 0.48) groups ($P=NS$). The levels of p-Akt (Figure 4A and 4B) in the ipsilateral hemisphere were significantly higher in the PNU-12 group (1.95 ± 0.31) compared with both vehicle (0.58 ± 0.11) and sham (1.00 ± 0.17) groups ($P<0.05$), whereas there was no significant difference between sham and vehicle groups ($P=NS$). P-Akt level was significantly lower in both MLA (1.08 ± 0.19) and WOR (0.95 ± 0.16) groups compared with the PNU-12 group ($P<0.05$). CC3 levels (Figure 4A and 4C) were significantly higher in the vehicle (2.62 ± 0.31) compared with the sham (1.00 ± 0.10) and PNU-12 (1.41 ± 0.14) groups ($P<0.05$), whereas there was no significant difference between sham and PNU-12 groups ($P=NS$). CC3 levels in both MLA (2.16 ± 0.26) and

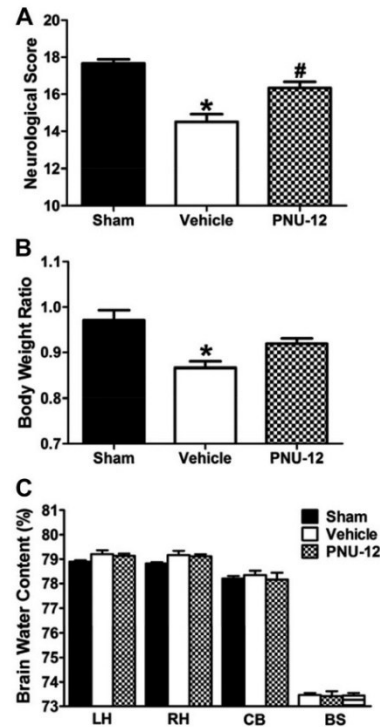


Figure 3. Neurological score (A), body weight ratio (B), and brain water content (C) 72 hours after surgery. Data are expressed as a mean and SEM. * $P<0.05$ vs sham, # $P<0.05$ vs vehicle. LH indicates left hemisphere; RH, right hemisphere; CB, cerebellum; BS, brain stem.

WOR (2.41 ± 0.33) groups were significantly higher compared with sham ($P<0.05$); however, there was no significant difference between these and the PNU-12 group ($P=NS$).

Neuronal Colocalization of p-Akt and CC3

Immunoreactivity and Neuronal Cell

Death Quantification

Localization of previously described changes was performed by immunohistochemistry in sham, vehicle, and PNU-12 groups ($n=2$ in each group). Neuronal cell death was quantified by TUNEL cell counting in the above groups ($n=6$ in each group). Ipsilateral basal cortex was the area of interest in all of the cases.

CC3 immunoreactivity (Figure 5A) was enhanced in the vehicle group compared with both PNU-12 and sham groups with good colocalization to neurons. P-Akt immunoreactivity (Figure 5B) in the PNU-12 group tended to be higher than the sham group and even higher when compared with the vehicle group. P-Akt signal was mainly colocalized to neurons, and TUNEL-positive cells did not generally colocalize with p-Akt-positive cells. The number of TUNEL-positive neurons in the ipsilateral basal cortex was significantly lower in sham (29.67 ± 7.2) compared with both the vehicle (383.79 ± 50.6) and PNU-12 (222.51 ± 29.6) groups. There was statistically a signif-

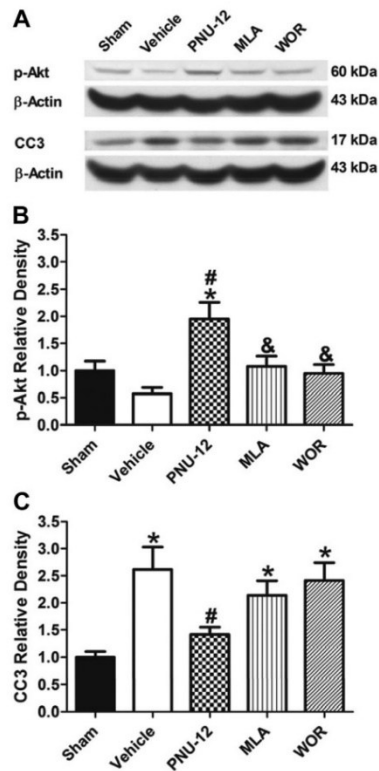


Figure 4. Representative Western blots of phosphorylated (p)-Akt, cleaved caspase 3 (CC3), and β -actin at 24 hours after surgery (A) and quantitative analysis of p-Akt (B) and CC3 (C). Data are expressed as a mean and SEM. * $P < 0.05$ vs sham, # $P < 0.05$ vs vehicle, & $P < 0.05$ vs treatment.

ificant difference in TUNEL-positive neuron counts between the vehicle and PNU-12 groups ($P < 0.05$; Figure 5C).

Discussion

In this study we tested 2 hypotheses. First, the $\alpha 7$ nAChR agonist PNU-282987 attenuates early brain injury after SAH. Second, the $\alpha 7$ nAChR stimulation is associated with the PI3K-Akt signaling pathway, which decreases proapoptotic caspase activation. In the descriptive part (the first part), we focused on brain water content, neurological score, and body weight loss; whereas in the mechanistic part (the second part), we focused on the involvement of p-Akt-mediated decrease in caspase activation and neuronal cell death after $\alpha 7$ nAChR stimulation.

SAH grade, at each time point, was comparable among all of the groups. This precaution was taken to allow comparison of measured parameters. All of the cases of mortality experienced severe SAH. The mortality rate was not improved by treatment with PNU-282987 compared with vehicle possibly because of SAH grade.

Basic physiological parameters were not affected by the application of PNU-282987 in our study. We performed monitoring for 60 minutes after PNU-282987, because this procedure is invasive, and a longer exposure to anesthesia could affect physiological parameters. Physiological parameters during our experiment were comparable with previous work from our laboratory.^{10,14} PNU-282987 was reported previously to inhibit human ERG potassium channels; however, this adverse effect is associated with the structure of this drug, not with specific $\alpha 7$ nAChR stimulation. It should be noted that a newer $\alpha 7$ nAChR agonist with reduced human ERG has been developed.¹⁶

Neurological dysfunction is an important clinical and experimental outcome present after SAH. Neurological deficits follow the peak of brain edema after SAH.¹⁷ The Garcia test is widely used in animal models of SAH.^{10–12} In our experiment, PNU-282987 improved neurological deficit at 24 hours in a dose-dependent manner and significantly improved neurological function 72 hours after surgery.

Body weight can be considered as a nonspecific marker, providing information about the animal's overall condition. In our experiment, body weight was not significantly different between groups 24 hours after surgery. Body weight at 72 hours was significantly lower in vehicle compared with sham, and PNU-282987 groups showed a tendency to decrease body weight loss. Given these results, we believe that body weight is influenced more by surgery than by SAH at 24 hours, and this effect is not present at 72 hours.

Brain edema is an important element of early brain injury, the etiology of which is cytotoxic and vasogenic.² Brain edema peaks 24 hours after surgery in the rat perforation model of SAH.¹⁷ Previous studies show increased brain water content after SAH in the left hemisphere only,¹⁷ as well as in all parts of the brain.¹⁴ In our study, PNU-282987 reduced brain water content in left hemispheres 24 hours after surgery in a dose-dependent manner, whereas no significant differences in brain water content were found 72 hours after surgery.

The PI3K-Akt signaling pathway plays important roles in preventing apoptosis.¹⁸ Akt is a member of serine/threonine kinase family, which is activated directly by PI3K-mediated phosphorylation. p-Akt activates several antiapoptotic mechanisms; among them is the regulation of BCL-2 family proteins, which control proapoptotic caspase activation.^{3,18} The antiapoptotic role of the PI3K-Akt pathway in neurons after SAH was reported previously,⁶ and the neuroprotective effects of $\alpha 7$ nAChR stimulation via the PI3K-Akt pathway was also reported.⁵ In our study we evaluated p-Akt and CC3 levels 24 hours after surgery in ipsilateral hemispheres, and we used methylcaconitine and wortmannin to manipulate this antiapoptotic pathway. Methylcaconitine is a potent competitive antagonist of $\alpha 7$ nAChR,⁸ whereas wortmannin is an irreversible inhibitor of PI3K, which is widely used to inhibit downstream Akt phosphorylation.¹⁰ We did not evaluate total levels of Akt, because expression of Akt was reported not to be changed after SAH⁶ or after $\alpha 7$ nAChR stimulation/inhibition.^{19,20} In our study, p-Akt levels were increased in ipsilateral hemispheres after PNU-282987 treatment compared with vehicle, and both methylcaconitine and wortmannin reversed the effect of treatment. CC3 was increased in ipsilateral hemispheres in the

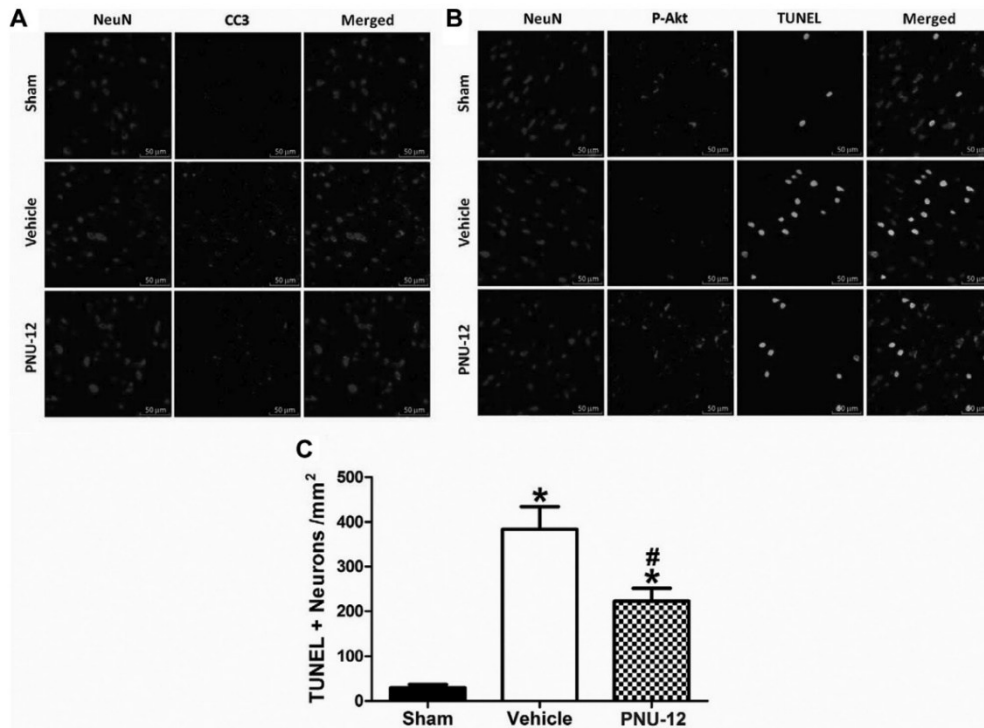


Figure 5. Representative immunofluorescence of NeuN and cleaved caspase 3 (CC3) in the ipsilateral basal cortex (A), NeuN and phosphorylated (p)-Akt combined with TUNEL staining (B), and neuronal cell death quantification (C) in the ipsilateral basal cortex at 24 hours after surgery. Data are expressed as mean and SEM. * $P < 0.05$ vs sham, # $P < 0.05$ vs vehicle.

vehicle group compared with the PNU-282987-treated group, and both methylcaconitine and wortmannin showed tendencies to reverse the effect of PNU-282987 treatment.

$\alpha 7$ nAChR is reported in endothelial cells and immune cells,⁴ as well as neurons.⁵ Our focus centered around neuronal apoptosis. Therefore, we used immunohistochemistry to localize our findings and found that both p-Akt and CC3 were colocalized to neurons in the ipsilateral basal cortex. Additionally, we used TUNEL staining, a marker for cells undergoing DNA fragmentation in the final phase of apoptotic cell death.²¹

Apoptosis is an important component of early brain injury after SAH, which occurs both in neurons and endothelial cells.²² In our study we used TUNEL staining for quantification of neuronal cell death in the ipsilateral basal cortex,²² and PNU-282987 significantly reduced neuronal cell death in this area 24 hours after SAH.

In this study we focused on PI3K-Akt signaling, which plays a significant role in apoptosis, a major contributor to early brain injury after SAH. However, there are also other mechanisms of $\alpha 7$ nAChR stimulation that could have a beneficial effect after SAH. $\alpha 7$ nAChR is a part of the cholinergic anti-inflammatory pathway²³ that attenuates the inflammatory response by decreasing both endothelial cell and

macrophage activation.⁴ Inflammation is reported to be involved after SAH, both in early brain injury²⁴ and vasospasm.²⁵ The cholinergic anti-inflammatory pathway is mostly associated with the parasympathetic nervous system, but $\alpha 7$ nAChR stimulation is also reported to induce NO-dependent dilation of the basilar artery associated with the sympathetic nervous system.²⁶ Taken together, the antiapoptotic effect of $\alpha 7$ nAChR stimulation in combination with the anti-inflammatory and antivasospastic effects make this a promising approach for treatment after SAH; however, further studies are required to elucidate those beneficial mechanisms.

Conclusions

$\alpha 7$ nAChR stimulation decreased neuronal cell death, brain edema, and improved neurological status in a rat perforation model of SAH. $\alpha 7$ nAChR stimulation is associated with increasing phosphorylation of Akt and decreasing CC3 levels in neurons. These results indicate that $\alpha 7$ nAChR agonists are promising treatments after SAH.

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Disclosures

None.

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3.1.1.5 Role of microRNA in Ischemic and Hemorrhagic Stroke

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MicroRNAs represent the most flexible network of gene regulation and thus miRNAs seem to be a promising target for therapeutic interventions focused on apoptosis and inflammation. This review article is focused on the description of the role of microRNA in stroke. After a brief summarization of microRNA signalization and microRNA functions in the central nervous system, the main part of the article reviews the involvement of microRNA in the pathophysiology of both ischemic and hemorrhagic stroke. Several promising candidate microRNAs are discussed more in detail. These include microRNAs which are mainly involved in apoptosis (miR-124, mir-29), inflammation (miR-181 family, mir-29, miR-21, let-7 family) and microRNAs associated with hypoxia/ischemia (miR-210, miR-21).



REVIEW ARTICLE

The Role of microRNA in Ischemic and Hemorrhagic Stroke

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Abstract: Background: Stroke is the second leading cause of death worldwide. Understanding of gene expression dynamics could bring new approaches in diagnostics and therapy of stroke. Small noncoding molecules termed “microRNA” represent the most flexible network of gene expression regulators.

Method: The aim of this review was to briefly describe the structure and function of microRNA and summarize the current knowledge about the involvement of microRNAs in the pathophysiology of ischemic and hemorrhagic stroke based on both experimental and clinical studies.

Results: Numerous profiling studies identified candidate microRNAs and partially described dynamics of their expression after the stroke. However, complex associations of specific microRNAs expression with main clinical characteristics and deeper insight into mechanisms of their regulatory functions are still missing. In this review, we put special emphasis on several microRNA clusters involved in neuroprotection (miR-124, miR-181, miR-21, miR-29, miR-210 and let7). Potential application of microRNAs as biomarkers and diagnostic or therapeutic targets was also discussed.

Conclusion: Full understanding of the regulatory mechanisms of the microRNA networks represents a novel direction for stroke research. To date, we do not have effective tools to control pathophysiological processes associated with stroke. Thus the microRNAs have to be considered as a very promising target for future stroke therapies.

Keywords: Central nervous system, embolectomies, hemorrhagic stroke, ischemic stroke, microRNA, thrombolysis.

1. INTRODUCTION

Stroke is a devastating event associated with a high mortality and morbidity. Stroke is number two leading cause of death worldwide, 6.7 million people died due to stroke in 2012 [1]. One of the major targets of WHO in the recent period is to improve coverage of treatment and prevention of heart attacks and stroke [2]. We have quite complex information about pathophysiological mechanism associated with stroke, due to intensive effort of researchers in the past decades [3-5]. More research on this field is, however, required for both medical and economic reasons [6]. Studies focused on protein expression and their mRNAs brought us important information about the role and dynamics of key molecules involved in pathophysiology of stroke. Another level of information brings us studies focused on microRNA molecules, which represents the most flexible gene expression regulatory system. The goal of this article is to describe

recent findings about microRNAs and their role in ischemic and hemorrhagic stroke.

2. NOVEL INSIGHT INTO A STRUCTURE AND FUNCTION OF microRNA

Substantial progress has been made in the research of genome regulation during the past decade. Class of gene-regulatory molecules termed “microRNA” (microRNA, miR) represents one of the rapidly developing areas. MicroRNAs belong to a group of short (20-23-nucleotide) endogenous non-coding single-stranded RNA molecules [7]. The regulatory mechanisms of the microRNA pathways, the principles of their expression and activity are just beginning to emerge. It has been known so far, that small RNAs can bind to complementary area of the messenger RNAs (mRNA) and inhibit the translation and/or stability of the target molecule [8, 9]. Although the most common binding site is 3' untranslated regions of mRNA, the growing number of experiments reported ability of microRNA to bind to the 5' untranslated regions [10]. Novel computational approaches to predict potential target places revealed that microRNA can bind also to DNA sequence (coding areas

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and promotor sequences) [11-13]. In contrast to traditional scheme of negative regulation by gene silencing, new studies revealed that microRNAs have also the capability to activate gene expression [14, 15]. Distinct microRNAs can thus regulate from tens to thousands of targets in different intracellular compartments, providing unique regulatory flexibility [16]. Consistent with this notion, microRNA-modulated gene expression is nowadays considered as a basal, widespread and dynamic regulatory system [17].

3. INTERCELLULAR SIGNALIZATION MEDIATED BY MicroRNA

The number of newly discovered microRNA grows rapidly, more than two thousand microRNAs have been identified in human so far [18, 19]. The range of microRNA network can be further supported by the existence of extracellular and circulating microRNA molecules discovered in various body fluids [20-22]. Although the precise mechanism of microRNA transportation, activity and stability is still unclear, many molecules have been described to stabilize the microRNA. Those "cell-free microRNAs" are transferred enclosed in microvesicles [23], exosomes [24], bound to HDL (High Density Lipoprotein) or other carriers [25, 26]. Beside those formations, microRNA can be freely present as "naked" microRNAs [27]. Extracellular microRNAs participate in intercellular communication system, acting as bioactive signaling molecules and one of the potential mechanisms of genetic exchange between cells [24, 28, 29].

4. MicroRNA IN CENTRAL NERVOUS SYSTEM

MicroRNAs have a wide range of functions in central nervous system, varying from neuronal development to mediating plasticity [30-32]. Knowledge of microRNA expression in distinct neuronal subtypes is, however, very limited, due to high heterogeneity of cells in the neural circuits. Other limitations are methods of microRNA detection, because they may harm the cells and thus alter the gene expression [33]. Non-coding RNA has been connected to various processes in brain including neural patterning [34], neural stem cell differentiation [35], cell type specification [36] or axonal path finding and synaptic plasticity [37]. Expression studies determined a subset of brain-specific microRNAs which can dynamically regulate neurobiological functions by post-transcriptional gene silencing within the cell or in intercellular signalization. This feature is particularly important for the synaptic function, where protein expression is rapidly modulated in response to changing requirements [38, 39]. Involvement of microRNA in a variety of neural processes suggests that disbalance in microRNA regulation or function might contribute to the pathogenesis of cerebral injuries as well as other brain diseases [40]. Aberrant expression (both up- and downregulation) of some tissue specific microRNA has been determined in many disease-specific patterns including neurodegenerative [41], neuropsychiatric [42] or cerebrovascular disorders [43]. Generally, studying of transcriptome and proteome dynamics brings deeper insight into the understanding of stroke pathophysiology. Many biological pathways activated during ischemic and hemorrhagic stroke are similar despite different etiology of these pathologies. On the other hand, remarkable changes on various levels

of gene regulation including mRNA, alternative spliced mRNA isoforms, microRNA or mature protein have been reported [44-46]. The role of microRNAs in the pathophysiology of ischemic and hemorrhagic stroke, with a respect to relationships between vessels, neurons and glia, will be described in the following text. Additional information about role of individual microRNA in pathophysiology of stroke is summarized in tabular form.

5. ISCHEMIC STROKE

Ischemic stroke, which represents about 80% of all the strokes, is caused by inadequate cerebral blood flow due to vessel occlusion [47]. Loss of blood supply initiates ischemic cascade, which may result in a tissue injury [48]. Irreversibly damaged neuronal cells form ischemic core, whereas surrounding area, penumbra, contains neurons affected by ischemia reversibly [49]. In the core of infarction, failure of energy-dependent processes leads immediately to neuronal necrosis and this is called primary brain injury. In penumbra, ischemia induces several pathological mechanisms of secondary brain injury which may promote neuronal or endothelial apoptosis. The most important contributors to secondary brain injury are excitotoxicity, oxidative stress and inflammation [50-52]. Fast intervention is required to minimize development of secondary brain injury. Thrombolysis and less frequent mechanical embolectomies are the only causal therapeutic strategies for acute ischemic stroke [53]. Finally, new therapeutic approaches using novel routes of administration are tested in both experimental and clinical conditions [54, 55].

5.1. MicroRNA Profiling Studies in Acute Ischemic Stroke

It has been shown, that alterations in molecular processes during ischemic condition are actively regulated by microRNAs. Large-scale profiling, which allows us to detect hundreds of miRNAs in parallel, provide the background of microRNA research under pathophysiological condition. Candidate microRNAs are evaluated for specific regulatory mechanism and function is finally confirmed by targeted manipulation of microRNA expression. MicroRNA screening studies in experimentally induced middle cerebral artery occlusion (MCAO) in rodents determined a large number of microRNA dysregulated in both brain tissue and circulation [56]. Persistent change in expression of many microRNAs was also described during reperfusion period (3 h- 3 days) [57, 58]. Beside acute phase of cerebral ischemia, microRNAs are also important in regulation of neuronal repair after ischemic stroke [59]. Some of the ischemia-induced microRNA showed correlation to targets (mRNA, promoters) involved in transcription, inflammation, neuroprotection, receptors function, ionic homeostasis or apoptosis [57, 60]. Few studies proved that microRNAs during early ischemic/hypoxic preconditioning can be directly responsible for the neuroprotection [61-64]. Interestingly, expression of some post-stroke microRNAs in rat brain and circulation seems to be affected by age and sex. MicroRNA profiles were associated with age at an early time points and sex at the later time points [65]. Moreover, microRNA expression patterns following ischemic injury were cell-specific. Both astrocytes

and neurons subjected to 60 minutes of ischemia showed a significant alteration in microRNA expression. Of particular interest was a set of hypoxia-regulated microRNAs: miR-29b, miR-21, miR-30b, 107, and 137 with unique cell-specific temporal expression patterns [66].

In human, first profiling studies conducted by Tan *et al.* [67, 68], showed not only case-control difference, but also a correlation of blood microRNA with stroke subtypes (cardioembolic, large artery stroke, small artery stroke), clinical outcome (6 months after stroke) and pre-existing risk factors (arterial hypertension, type 2 diabetes, dyslipidemia). Beside stroke progression, blood microRNA profiles can also differentiate stroke etiologies during acute phase [69]. In addition, few studies have shown that some of circulating microRNA can serve as a potential biomarker for early detection (up to 24 h) of acute ischemic stroke [56, 57, 70-72]. Deeper insight into the role of blood microRNA in stroke was provided by Jickling *et al.* by measuring expression directly from blood mononuclear cells. The authors described that leukocytic microRNAs dysregulated due to stroke are involved in activation of the immune system, leukocyte extravasation and thrombus formation [73]. Kim *et al.* focused on relation between microRNAs profile and stroke prognosis. Several circulating microRNAs related to atherosclerosis were measured in 83 stroke patients and 37 control patients with vascular risk factors without incident stroke. The levels of microRNAs correlated to the presence of acute stroke, stroke subtypes, vascular risk factors and stroke recurrence. Especially miR-17 was elevated in acute stroke patients and associated with future stroke recurrence (shorter event-free survival) [74]. In comparison to blood-based arrays, cerebrospinal fluid (CSF) assessment provides unique signatures of microRNA expression associated with various brain pathologies. So far, only one study analyzed microRNA expression profiles in CSF in acute stroke patients [75]. The expression pattern differed between CSF and blood, with overall 183 microRNA detected in CSF and 287 in blood. From those, particularly miR-523-3p, miR-221-3p and let-7c was upregulated or more often in stroke patients [75]. Although microRNA research in stroke is still in its infancy, there are few candidate microRNAs which play significant role in pathophysiology of acute ischemic stroke (Fig. 1).

5.2. Candidate microRNA Biomarkers and Therapeutic Targets in Acute Ischemic Stroke miR-124 (miR-124a) and Neuroprotection Against Apoptosis

One of the best-studied microRNAs in brain is miR-124, which is >100 times more expressed in CNS than in other organs [76-78]. This neuron-specific microRNA occurs in embryonic and adult cortical tissues and participates in neural development and differentiation [30, 79, 80]. It has been shown that miR-124 participates in the control of cell cycle and DNA repair [81] and miR-124 upregulation can inhibit cell cycle progression [82]. Interestingly, miR-124 expression is regulated by RE1 silencing transcription factor (REST) [83], which is repressor of many neural-specific genes and its upregulation was detected in ischemic neurons destined to die [84]. While the exact functions of miR-124 in brain is still poorly defined, it has been shown that brain-specific miR-124 is expressed in microglia but not in peripheral monocytes or macrophages. MiR-124 is considered as a

key regulator of microglia quiescence in the central nervous system and modulator of monocyte and macrophage activation in circulation [85]. With respect to ischemia-related brain injury the results of distinct studies are discrepant, reporting both upregulation and downregulation of miR-124. Such a discrepant report may be caused by differences in methodology or different timing of sample collection. In rat MCAO model Jeyaseelan and colleagues detected elevated level of miR-124 in brain at 24 h after the occlusion, but not at 48 h or in blood [58]. Laterza *et al.* described increased levels of plasma miR-124 at 8 h and maximum at 24 h after experimental artery occlusion in rats [86]. Early increase in miR-124 was only seen in the transient occlusion/ischemia, whereas large elevation was apparent after 24 h in case of both transient and permanent occlusion. Elevated blood levels were measured by Weng *et al.*, who detected elevated miR-124 in serum 6 h and also 48 h after the MCAO [80]. Upregulation of miR-124 in brain tissue and cell cultures during ischemic conditions was published by Doeppner *et al.* [87]. The authors described that miR-124 can indirectly increase levels of REST via negative feedback loop and prevent neuronal apoptosis. MiR-124 promotes neuronal survival under ischemic conditions and brain recovery after stroke. Experimental delivery of miR-124 led to increased resistance of cultured oxygen-glucose deprived (OGD) neurons, and decreased infarct volume size and functional impairment in rats [87]. Another study [88] reported similar miR-124 upregulation in ischemic penumbra in mice and OGD neurons. In brain tissue, the overexpression/knockdown manipulation with miR-124 expression levels led to reduction/increase of infarct zone size, whereas in OGD cell led to reduced/increased cell death and late apoptosis, respectively. Taken together, higher levels of miR-124 may protect against neuronal apoptosis probably mediated via anti-apoptotic proteins Bcl-2 and Bcl-xl [87]. Nevertheless, the most recent study demonstrates significantly increased levels of miR-124 in the penumbra (24 h after ischemia) but any changes in ischemic core at the same interval. Moreover, it has been shown that miR-124 can strongly suppress ASPP protein family which is a central set of apoptosis-stimulating proteins for the p53 pathway [89]. On the other hand, Liu and colleagues described miR-124 downregulation 7 days after the MCAO, correlating with proliferation of neuronal progenitor cells. Delivery of miR-124 to the cells caused a decrease in cell proliferation and increase in differentiation suggesting miR-124 as a potential therapeutic target to amplify neurogenesis after stroke [90]. Contradictory to previous results, Zhu *et al.* reported that miR-124 knockdown protects against I/R induced neuronal death and apoptosis by upregulating Ku70, protein involved in DNA repair and apoptosis. It can also reduce brain infarct size and improve neurological outcomes suggesting that rather miR-124 downregulation works as a protective mechanism [91]. MiR-124 have multiple and complex roles in pathophysiology of cerebral ischemia such as regulation of cell cycle, neuronal cell death, apoptosis and proliferation to inactivation of macrophages and inflammation. Hence, miR-124 may represent a therapeutic target for neuroprotection rather than potential biomarker of ischemic stroke.

5.3. miR-181 Family, Neuroinflammation and Stress Response

MiR-181 family contains four highly conserved members miR-181a, miR-181b, miR-181c and miR-181d which are involved especially in hematopoiesis and inflammation [92, 93]. From those, especially miR-181a and miR-181b are highly abundant in brain, with predominant expression in astrocytes [94]. Members of miR-181 are considered as important players in neuroinflammatory responses, where miR-181a has a strong anti-inflammatory effect and shows transient increase at the early stage of inflammatory response [94]. This suggests a role of miR-181a in homeostatic response to inflammatory stimuli, which may be useful in protecting tissues from excessive injury [93]. Another member, miR-181b directly targets IL-6 [95], a key player in early inflammatory response after cerebral ischemia, and down-regulates its expression [96]. MiR-181b has also been considered as an essential regulator of nuclear factor-kappaB (NF- κ B)-mediated vascular inflammation and endothelial cell activation. Conversely, inflammatory stimuli can influence both miR-181a and miR-181b and modulate their expression [97]. The anti-inflammatory effect in astrocytes exposed to lipopolysaccharide (LPS) was also described in the case of miR-181c. Furthermore, the family of miR-181 can target several important cell death/survival pathways, including apoptotic proteins (Bcl-2 pathway) [98], proteins involved in responses to stress (heat-shock protein 70), proteins involved in controlling mitochondrial function, redox state and proteins involved in tissue protection (e.g. protective proteins of endoplasmic reticulum) [99, 100]. It has been recently shown, that all the ischemia related-processes are interconnected; disrupting of Bcl-2 pathway can trigger autophagy and reduce mitochondrial damage in rats after ischemic stroke [101]. Dynamics of miR-181 cluster expression were studied by various authors in models of transient and permanent cerebral ischemia. Both upregulation and downregulation of miR-181 members were described in selected time-points [56-58]. In a study conducted by Ouyang and colleagues, remarkable changes in miR-181a levels were observed between ischemic core (upregulation) and penumbra (downregulation), with subsequent exacerbation of injury by experimentally induced overexpression of miR-181a. Opposite changes in miR-181a levels in core and penumbra can be explained by differences between dying cells in ischemic core and the cells in penumbra, which may survive. These results were further supported by correlation of low levels of miR-181a with increased levels of molecular chaperone GRP78. It has been previously reported, that cells destined to survive can produce new heat shock proteins [99]. Generally, heat shock proteins belong to a group of stress induced proteins overexpressed during cerebral ischemia [102]. Thus, inhibition of selected heat shock proteins can inhibit inflammation and protects blood-brain barrier integrity [103]. Assuming deleterious effects of miR-181 on stroke outcome, reducing miR-181a levels before and after stroke onset by different approaches has been continuously studied. Other studies reported that decreased miR-181a brain levels by application of antagomir, specific microRNA inhibitor, the day prior to MCAO caused an infarct size reduction in focal ischemia model [104] and reduced neuronal loss in global cerebral ischemia model [105]. Using primary

culture transfection with miR-181a inhibitor led to protection of astrocytes from ischemia-like stresses, but not neurons [99, 105]. Treatment with miR-181a antagomir preserved astrocyte mitochondrial function, reduced ROS ("Reactive Oxygen Species") and protected astrocytic glutamate transporter GLT-1, which is responsible for removing of glutamate from synaptic sites and reducing excitotoxicity [106]. Recently, extended possibilities in ischemia treatment was presented by Xu and colleagues, who tested the effect of post-ischemic miR-181a antagomir applied intravenously (1 h after the MCAO) and intracerebroventricularly (2 h after the MCAO). Both ways of administration reduced infarct size, improved survival and long-term neurobehavioral recovery. Additionally, the reduction in the induction of inflammation, including decreased NF- κ B activation and decreased leukocytic infiltration were observed. Hence, miR-181 based treatment, particularly intravenous route, represents innovative therapeutic applications in the early post-stroke treatment with long lasting effect [107].

5.4. miR-21, Ischemia and Immunity

MiR-21 is one of the first identified mammalian microRNAs characterized by the ubiquitous role in biological processes. The complexity of its regulation is due to an own promoter that is target for many transcription factors and hormones. MiR-21 has been found to be upregulated in many pathological conditions including cancer, pulmonary disease or cardiovascular diseases [108]. In both tumorigenesis and myocardial infarction miR-21 is particularly involved in apoptosis with the strong anti-apoptotic effect [109, 110]. MiR-21 regulates cell cycle [111], promote cell differentiation, cell proliferation or prevent extracellular matrix (ECM) remodeling [112] by targeting matrix-metalloproteinases or their regulators [113, 114]. Apart from developmental processes, miR-21 is involved in ischemia, hypoxia and immunological processes including inflammation. [115, 116], which are common pathophysiological changes after arterial occlusion. Regarding the role of miR-21 in the immune system and inflammation, miR-21 is considered to be important in regulation of Th1 and Th17 immune responses and cytokine production, targeting IL-12 [117], IFN- γ [118], TNF- α and IL-17A [119, 120] or IL-2 [121]. Conversely, it has been reported that miR-21 gene transcription could be controlled by IL-6 [122] and TGF- β "Tumor Growing Factor- β " *via* feed-forward loop [112]. Consistent with this notion, miR-21 probably promotes tissue inflammation and autoimmunity [123]. In pathophysiology of ischemia and related processes, miR-21 was described to be upregulated by ischemic preconditioning of the heart and its inhibition attenuates cardiac protection from next injury *via* anti-apoptotic function [63]. In case of cerebral ischemia miR-21 showed a peak of expression at 24 h after preconditioning in spontaneously hypertensive rats [61]. In MCAO animal study a significant elevation of miR-21 was detected in neurons isolated from ischemic boundary zone, but not in contralateral area [124]. Although no changes of expression have been measured in cultured OGD neurons, attenuation of endogenous miR-21 improved survival by apoptosis reduction *via* targeting Fas-Ligand, tumor necrosis factor- α (TNF- α) family member involved in ischemia-induced apoptosis [124]. Furthermore, it has been described that global miR-21 upregulation occurs

in response to traumatic brain injury [125]. Taken together, upregulation of miR-21 due to brain tissue injury may be protective and participate in tissue reparation. Interestingly, in ischemic stroke patients the levels of miR-21 in peripheral blood were reported to be both decreased [126] and increased (especially in small artery stroke) [67]. Taking into account the discrepancy of plasma levels, due to the critical functions of its target proteins in various signaling pathways miR-21 represents an attractive target for stroke treatment.

5.5. miR-29 Family, Differential Function in Apoptotic Pathway

The human miR-29 family consists of three mature members, miR-29a, miR-29b (b-1, b-2), and miR-29c. They exhibit differential regulation and subcellular distribution, and target expression of diverse proteins like collagens, transcription factors, methyltransferases and others [127]. It has been shown, that miR-29 family regulates extracellular matrix by targeting at least 16 extracellular matrix genes [128]. Expression of miR-29 family can be activated also by interferon signaling and/or reduced by TGF- β , which suggests immunomodulating properties [129, 130]. The cellular effect of miR-29 members includes mainly regulation of proliferation, differentiation and apoptosis [131]. With respect to the heterogeneity of miR-29 family targeting and regulations, both pro- and anti-apoptotic targets have been described with particular role of Bcl-2 pathway [132]. Due to modulation of opposing pathways, members of miR-29 family have controversial function. During neuron maturation, upregulation of miR-29b act as a survival factor interfering with apoptosis, whereas in myocardial ischemic/reperfusion injury, overexpressing of miR-29 family promoted apoptosis [132, 133]. Shi *et al.* reported that, miR-29b levels were significantly increased after ischemia and upregulation was associated with the neuronal cell death [134]. Similarly, overexpression of miR-29c resulted in increased sensitivity to I/R injury, while downregulation of endogenous miR-29c was neuroprotective [135]. On the other hand, downregulation or loss of miR-29b and miR-29c in ischemic area was associated with neuronal cell death and increase in infarct size [136, 137]. Beside necrotic core, expression profile was determined also in salvageable apoptotic penumbra with significant upregulation of miR-29b-2 [138]. Despite ambiguous results in above mentioned studies, oral supplementation of α -tocotrienol was referred to restore stroke-induced loss of both miR-29b and miR-29c and minimize infarct size in the brain [136].

5.6. miR-210 and Hypoxia

MiR-210, termed as a “master hypoxamir”, belongs to the spectrum of microRNA induced in response to low oxygen in wide range of cells [139]. During hypoxic conditions miR-210 is induced by the key regulators of the cellular hypoxic response HIF-1 α and HIF-2 α [140, 141]. In hypoxic environment, miR-210 shows anti-apoptotic functions and ability to modulate cellular metabolism by repressing mitochondrial respiration [142]. In human endothelial cell exposed to hypoxia, expression of miR-210 shows rapid and persistent increase. Hence, its overexpression stimulates the formation of capillary-like structures and increases cell migration in normoxic conditions [143]. In addition, miR-210

can support cell differentiation and inhibit cell proliferation by targeting proteins that are crucial for cell cycle progression [139, 144]. Furthermore, Crosby *et al.* reported that miR-210 can silence the DNA repair system, which might be critical for energy conservation for cell preservation during acute hypoxic condition [145]. During ischemic brain injury miR-210 plays multiple roles and increased miR-210 levels have protective function. In patients with acute ischemic stroke, circulating miR-210 gradually decreased with the negative peak at the day 7 and 14 after onset of stroke and the levels were higher in patients with better clinical outcome [146]. In rat MCAO model, both brain and blood miR-210 levels were elevated at 1 day after induction of ischemia, and gradually decreased at the day 7 and 14. This parallel expression changes suggest that blood levels can significantly reflect ischemic brain tissue injury [146]. In later studies, miR-210 expression was found to be markedly higher in rat ischemic cortex between 1 day and 7 days after transient MCAO [147]. Considering strong pro-angiogenic effect, miR-210 overexpression by lentiviral vector promoted neovascularization in ischemic cortex [147] and increased endothelial cell proliferation in subventricular zone [148]. These data suggest that higher levels of miR-210 promote angiogenesis, neurogenesis and helps to prevent ischemia-induced injury. Given the fact that miR-210 is aberrantly expressed during ischemic conditions and participates in hypoxia, angiogenesis and apoptosis. Therefore it represents to be potential candidate for therapeutic purposes.

5.7. let-7 Family and Neuroprotection

Another abundantly expressed microRNA in the brain is let-7 family (lethal 7a-e), which contains groups of microRNA with different functions. Let-7 belongs to the firstly discovered group of microRNA essential for early development timing in *C. elegans* [149]. In this context, let-7 family is crucial for neurogenesis and neuronal maturation [150], with increasing levels during tissue differentiation [151]. Many members of let-7 family act as a tumor suppressor in a variety of cancers, so the loss of let-7 expression was linked to the development of poorly differentiated aggressive cancers [152]. Interestingly, let-7 family was found to be secreted not only to serum but also to saliva [24]. Using microvesicles transport, some members of let-7 family have been described to participate in inflammation process by targeting pro-inflammatory cytokine IL-6 in macrophages [153]. Considering secretion into body fluids, let-7c belong to group of most frequently detected and upregulated microRNA in CSF of stroke patients. The elevation of let-7 (c, d and e) in blood of young stroke patients was reported by Tan *et al.*, [67] similarly to Jeyaseelan *et al.*, who found let-7 (a, b, c and e) upregulated in blood and in rat brain after artery occlusion [58]. In other studies focused on different stroke subtypes, expression of let-7 members was found to be rather upregulated [60, 65, 154]. Recent study has focused on expression levels (CSF, serum) and clinical significance of let-7e at different stages following ischemic stroke. Levels of let-7e in CSF and serum were markedly upregulated in ischemic stroke patients at the acute stage. Although, let-7e was not significantly correlated with parameters describing stroke severity (NIHSS scores), it was positively correlated with the serum levels of C-reactive protein, a marker of

inflammation. Serum let-7e levels showed both high specificity and sensitivity, thus may serve as a useful circulating biomarker for the acute stage of ischemic stroke [155]. Apart from the role of biomarker for identifying and evaluating ischemic stroke in human CSF and blood, some let-7 family members are potential therapeutic candidates for neuroprotection following stroke. For instance, specific blockade of let-7f can promote neuroprotection *via* insulin like growth factor (IGF-1) signaling pathway in rat model of ischemic stroke [156]. Additional microRNA involved in pathophysiology of ischemic stroke is summarized in Table 1.

6. HEMORRHAGIC STROKE

Hemorrhagic stroke is a bleeding into the brain parenchyma (intracerebral hemorrhage- ICH) or subarachnoid space (subarachnoid hemorrhage-SAH). ICH is caused by the rupture of cerebral vessel, while the classification of SAH is based on underlying pathology from the more common rupture of the aneurysm to less common hemorrhage caused by an arteriovenous malformation or others [187, 188]. Although hemorrhagic stroke represents only 15-20% of strokes, it is responsible for about 40 percent of all stroke deaths [189]. Despite the fact that ICH and SAH are different pathologies due to different localization of hematoma, there are a couple of similar mechanisms which play major role in the pathophysiology of both ICH and SAH. Accumulation of blood in extravascular space causes transient ischemization of brain tissue [4, 5, 190]. Extravasated blood which has direct toxic effects due to cytotoxic plasma components (complement molecules, coagulation factors, immunoglobulins) as well as oxidative stress and inflammation play an important role in neuronal and endothelial injury [5, 190]. Recently, research focused on diagnostic and therapeutic interventions in early stage of hemorrhagic stroke is emerging [191, 192]. However, unlike ischemic stroke, which have been intensely studied so far, little is known about involvement of microRNAs in pathophysiology of hemorrhagic stroke (Fig. 1).

6.1. MicroRNA profiling studies in intracerebral hemorrhage

First attempt to study specific roles of microRNAs in hemorrhagic stroke was performed by Liu *et al.* [56]. In this study authors compared microRNA expression following different brain injuries including brain ischemia, intracerebral hemorrhage (lysed blood, fresh blood, thrombin) or kainate seizures in rats. In case of intracerebral hemorrhage (lysed blood) 17 microRNAs were upregulated and 12 down-regulated in brain and 21 microRNAs were detected in blood. The results showed unique microRNA profiles for the brain, blood, and for each experimental condition, with few microRNAs (miR-298, miR-155, miR-362-3p) dysregulated in both brain and blood after almost all experimental conditions. Guo *et al.* studied circulating microRNA expression in 15 patients with spontaneous intracerebral hemorrhage (ICH) and 8 healthy controls [193]. To clarify unique profile and eliminate possible effects of confounding factors, ischemic stroke group with comparable clinical parameters (16 patients) were recruited. In patients with intracerebral hemorrhage a subset of 30 microRNAs were selectively upregulated in a sex-independent manner. Network analysis suggested the number of upregulated microRNAs and inflamma-

tory cell-derived microRNAs, including miR-150, miR-365, miR-30c, miR-27a, miR-574-5p, miR-130a and miR-423. Interestingly, in stroke patients the above mentioned microRNAs were detectable in plasmatic microvesicles and supernatant fraction, while in controls, microRNAs were detected only in microvesicles. This suggests that disease condition has impact not only on the total concentration of microRNAs, but also on difference in plasma compartmentalization. Although the origin of upregulated plasma microRNAs is still under debate, they can be released from inflammatory cells, mechanically destructed blood vessel cells and/or injured neural cells [194]. Slightly different approach was chosen by Zheng *et al.*, whose group focused on circulating markers predicting secondary hematoma enlargement (HE), which is the most devastating complication after ICH [195]. Microarray method identified over 30 differentially expressed microRNA between 79 patients with HE and 30 control. Predicted targets participate in apoptosis and cell proliferation, immune and hypoxia response, collagen biosynthesis and blood vessel development, water homeostasis and electric ion channel activity, energy metabolic process and coagulation, extracellular matrix constituent and cell surface binding, and blood pressure control. Most recently, Zhu *et al.* described microRNA changes in other secondary brain damage after ICH- perihematomal edema (PHE) [196]. Whole-genome microRNA profiles of ICH patients revealed 54 downregulated microRNAs. Among the altered microRNAs, only miR-126 was positively correlated with the extent of PHE, suggesting pathogenic role in the development of PHE after ICH.

6.2. Profiling in Subarachnoid Hemorrhage

Later, studies were focused on microRNA expression in cerebral aneurysm formation or in pathophysiology of aneurysmal subarachnoid hemorrhage. So far only one study from 2013 evaluated expression patterns of microRNAs in experimentally induced formation of cerebral aneurysm (CA) in rats [197]. Compared to normal cerebral arteries in the CA tissues 14 microRNAs were over-expressed above 200% (miR-1, miR-21, miR-22-5p, miR-24-1-5p, miR-26b, miR-29a, miR-29b, miR-29c, miR-101b, miR-140, miR-147, miR-181c, miR-223, miR-451) and 6 microRNAs were down-expressed under 50% (miR-92b, miR-138, miR-181d, miR-433, miR-489, miR-551b). It has been previously described, that those microRNAs play role in vascular remodeling, apoptosis, proliferation of vascular smooth muscle cells and vascular inflammation [197]. In 2013 Jiang and colleagues compared different microRNA expression patterns in cerebral aneurysm tissue between 14 patients and 14 controls [198]. They identified 30 differentially expressed microRNAs from which 18 microRNAs were robustly downregulated. Predicted functions showed involvement in dysfunction and remodeling of vascular endothelial cells, vascular smooth muscle cell and involvement of inflammatory/immune processes. In a second study by Liu *et al.*, 157 differentially expressed microRNAs (72 upregulated/85 down-regulated) were detected in the aneurysmal tissue of 6 patients [199]. Following microRNAs participate in regulation of smooth muscle cell proliferation and apoptosis, extracellular matrix organization, inflammation (TGF- β signaling pathway) and response to oxidative stress: miR-1, let-7a,

Table 1. Overview of other microRNA involved in ischemic stroke.

microRNA	Target	Function in Ischemic Stroke	References
miR-15a	Bcl-2	Gain/loss led to reduced/increased oxygen-glucose deprivation induced cerebral vascular endothelial cell death. Is regulated by PPAR δ , which overexpression can reduce miR-15a expression, leading to decreased BBB disruption and reduced cerebral infarction in mice.	[157]
miR-17-92	PTEN	Increased level in brain after stroke and in ischemic cells causes proliferation and survival of neural progenitor cells.	[158]
miR-103	NCX	Regulates Na/Ca exchange. Antagomir downregulation reduced brain damage and neurological deficit.	[159]
miR-107	GLT-1	Level was elevated in both brain tissue and plasma in a cerebral ischemia/reperfusion (I/R) injury rat model. Positive correlation between plasma levels and glutamate level in patients with acute ischemic stroke (biomarker of monitoring excitotoxicity).	[160]
miR-125b	CDKN2A IL-6R Mcl-1 Bcl-w p53	Potential mediator of post-stroke astrogliosis induced by IL-6 (increased 125b levels were found in IL-6 stressed normal human astrocytes). <i>Via</i> multiple targeting inhibits glial cell proliferation, promotes cell apoptosis (<i>via</i> p53) and neuronal differentiation.	[161] [162] [163] [164]
miR-126	VCAM1	Endothelial specific microRNA which governs vascular integrity, angiogenesis and wound repair. Targeted deletion causes leaky vessels, hemorrhaging, loss of vascular integrity and defects in endothelial cell proliferation, migration and angiogenesis. Decreased expression of miR-126 enhances leukocyte adherence to the endothelium <i>via</i> VCAM1. Circulating levels are markedly downregulated in patients with ischemic stroke and represents useful biomarker. Serum levels differ in a rat model of permanent versus transient ischemia and may help to distinguish severe permanent ischemia from milder injury after transient ischemia.	[165] [166] [154] [167]
miR-130	Aquaporin 4	Antagomir blockade led to reduction in infarct size and increased neuronal recovery.	[168]
miR-145	SOD2 myocardin	Upregulated in brain after ischemia. Post-ischemic infusion of antagomir-145 into the lateral ventricles decreased the area of infarction (<i>via</i> SOD2). Regulated by p53 in microglia. Modulator of smooth muscle cell phenotype able of controlling vascular neointimal lesion formation.	[57] [169] [170]
miR-146a	eNOS TRAF6 IRAK1	Key regulator of astrocyte-mediated inflammatory response induced in endothelial cells upon exposure to proinflammatory cytokines (negatively regulate the interleukin-1 β). Represses endothelial activation by inhibiting pro-inflammatory pathways. Regulate dendritic cell apoptosis (<i>via</i> TRAF6 and IRAK1). Polymorphisms in miR-146a are associated with risk of ischemic stroke. Circulating levels were downregulated in acute phase of ischemic stroke but upregulated in subacute phase. Promotes stroke-induced oligodendrogenesis.	[171] [172] [173] [174] [175] [176]
miR-200c	Reelin	Antagomir inhibition resulted in reduction in infarct volume, neurological deficit and promotion of neuronal migration and synaptogenesis (<i>via</i> Reelin).	[177]
miR-223	Aquaporin GluR2 NRB2	Secreted by platelets promotes endothelial cells apoptosis. Role in systemic cholesterol regulation. Transferred by lipoprotein particles and regulates ICAM-1 expression in endothelial cells. Regulate multiple inflammatory genes in monocytes and macrophages. Protect brain from glutamate excitotoxicity.	[178] [179] [180] [181] [182]

(Table 1) Contd....

microRNA	Target	Function in Ischemic Stroke	References
miR-320a	Aquaporin1 Aquaporin4	Antagomir blockade increased aquaporin expression leading to reduction of infarct volume.	[183]
miR-424	CCND1 DC25A CDK6 Nr2 SOD	Decreased levels in brain and blood tissue of ischemic stroke patients and experimental animals. Experimental overexpression led to decrease in infarct volume and neuronal cell death through suppressing microglia activation and reduced oxidative stress.	[58] [184] [185]
miR-497	Bcl-2	Induced in mouse brain after ischemic insult. Knockdown of cerebral miR-497 attenuates ischemic brain infarction and improves neurological outcomes.	[186]

BBB - blood brain barrier; Bcl - anti-apoptotic regulator; CCND1 - cell cycle activator; CDK6 - cell cycle activator;

CDKN2A - cyclin-dependent kinase inhibitor 2A; DC25A - cell cycle activator; eNOS - endothelial nitric oxide synthase; GLT-1 glutamate transporter-1; GluR2 - the glutamate receptor subunit; IL-6R - interleukin 6 receptor;

IRAK1/2 - IL-1 receptor-associated kinase 1 (adaptor protein of the NF- κ B signaling cascade); Mcl-1 - anti-apoptotic regulator; NCX - Na⁺/Ca²⁺ exchanger; NR2B - glutamate receptor subunit; Nr2f - nuclear factor erythroid 2-related factor; PPAR δ - peroxisome proliferator-activated receptor; PTEN - phosphatase and tensin homolog; SOD - superoxide dismutase; TRAF6 - TNF receptor-associated factor 6 (adaptor protein of the NF- κ B signaling cascade); VCAM1 - vascular cell adhesion molecule-1.

miR-10a, miR-17, miR-23b, and miR-26a, miR-24-1, miR-125b, miR-126, miR-143 and miR-145 and miR-222 [199].

Circulating microRNAs were studied with regard to aneurysm location and characteristics (aneurysm with/ without daughter sac and ruptured aneurysm). 86 microRNAs (69 upregulated/17 downregulated) differed significantly between all 3 types of aneurysm and control group in total of 24 subjects. In the group of patients with aneurysms with daughter sac, all serum microRNAs were upregulated [200]. In comparison, Li *et al.* examined plasma from 40 patients with intracranial aneurysm (20 ruptured/20 unruptured) and 20 healthy controls examined by digital subtraction angiography due to neurological symptoms [201]. 119 microRNAs were significantly changed in patients with unruptured aneurysms and 23 microRNAs in patients with ruptured aneurysms (20 of them in both ruptured and unruptured aneurysms). Because of the significantly higher abundance of miR-16 and miR-25 in patients with IA, these were chosen for further validation for candidate microRNA. Logistic regression analysis demonstrated that levels of both were independent factors for IA occurrence and together with hypertension may serve as an independent predictor for the presence of IAs. Changes in microRNA levels were measured also in human cerebrospinal fluid (CSF) in patients after aneurysmal SAH. Profiling of 8 patients showed two specific clusters of microRNA differentially regulated over time (daily/10 days). The levels of microRNA in first cluster (miR-92a and let-7b) gradually decreased over ten days, while second cluster (miR-491) showed rather increased tendency [202]. Unfortunately, none of the profiling studies associated microRNA levels with clinical data (severity of SAH, outcome or occurrence of cerebral vasospasm). However, there are few candidate microRNAs (miR-29, miR-30a, miR-143, miR-145), which were described to be upregulated in cerebral vessels after experimental SAH and these could play role in cerebral vasospasm development [203]. Information about biomarkers, including microRNA, in other /rare sources of bleeding such as arteriovenous and cavernous malformation is missing.

So far, only miR-18a, a member of the miR-17/92 cluster, which exhibits strong antiangiogenic properties, was described to be directly associated with arteriovenous malformation (AVM) [204]. Previous studies revealed, that miR-18a can promote angiogenesis by regulating key antiangiogenic factors thrombospondin-1 (TSP-1) and connective tissue growth factor (CTGF) [205, 206]. In human cerebral arteriovenous malformation microRNA-18a normalized the aberrant phenotype of AVM-derived brain endothelial cells by restoring the levels of key angiogenic factors [207]. MicroRNA-18a (miR-18a) shows great potential for treating neurovascular diseases including AVM by using specific miRNA-delivery platform [208]. Additional microRNAs involved in the pathophysiology of hemorrhagic stroke are summarized in Table 2.

7. FUTURE PERSPECTIVES AND POTENTIAL THERAPEUTIC USE OF microRNA

MicroRNA molecules, the most flexible network of gene regulation, represent one of promising directions for stroke therapy. Full understanding of the regulatory mechanisms of the microRNA network in its complexity is a prerequisite for the development and successful application of microRNAs for diagnostic and therapeutic purposes. Firstly, the presence of microRNAs in body fluids may represent a source of promising diagnostic and prognostic markers due to their high stability [27]. Brain-specific microRNAs in circulation or in cerebrospinal fluid to some extent reflect the events that occur at the site of injury, and can be potentially used as biomarkers of stroke development. Secondly, targeted manipulations with the levels of stroke-specific microRNAs can serve as a potential therapeutic option, because of their involvement in the regulation of apoptosis, oxidative stress and inflammation. To date, we do not have effective tools to control these pathophysiological processes once they are deregulated and thus the microRNAs have to be considered as a very promising target for future therapies. Nevertheless, there are several obstacles in the therapeutic use of microRNA, particularly tissue and cell-specific delivery in the

Table 2. Overview of microRNA involved in hemorrhagic stroke.

microRNA	Target	Function in Hemorrhagic Stroke	References
let7c	IGF1R	Upregulated after experimental intracerebral hemorrhage in rats. Antagomir inhibition reduced perihematomal edema, apoptotic cell death, inflammation around hematoma and enhanced neurological recovery.	[209]
miR-18a	TSP-1 CTGF VEGF-A VEGF-D	In AVM-derived brain endothelial cells miR-18a increased aberrant TSP-1 levels (antiangiogenic factor), decreased vascular endothelial growth factor VEGF-A and VEGF-D secretion and regulated cell proliferation and improved tubule formation efficiency.	[207] [208]
miR-21		Increased level in blood of patients with IA. Increased expression (protective) in the development of AAA, miR-21 blockade caused aneurysm enlargement. Key modulator of proliferation and apoptosis of vascular wall smooth muscle cells during development of AAA.	[201] [210]
miR-26	Smad1 Smad4	Decreased in AAA model, involved in apoptosis, response to oxidative stress, TGF- β signaling pathway, smooth muscle cell proliferation, phenotypic switch of VSMCs. Increased levels in CA tissue in rats and in circulation in humans with IA.	[211] [197] [201]
miR-29a,b,c		Associated with downregulation of numerous extracellular matrix components in aorta, silencing inhibited dilation of the aorta in mice. Mediates TGF- β 1-induced extracellular matrix synthesis. Inhibition increased collagen expression, reduced AAA development and progression. Involved in fibrotic processes.	[212] [213] [214]
miR-126	VCAM-1	Decreased in serum in patients with acute ICH, correlated with perihematomal edema volume. Promotes angiogenesis and inhibits inflammation (repressing VCAM-1) by reducing the number of immune cells infiltrating the site of inflammation.	[196]
miR-130	caveolin-1 MMP2 MMP9	Elevated in the serum of ICH patients and positively associated with perihematomal edema volume within the first 3 days after onset. Increased in rat serum and perihematomal tissues Inhibitors reduced brain edema, blood-brain barrier (BBB) permeability and increased neurological deficit scores.	[215]
miR-132		Increased in circulation of patients after acute aSAH. Involved in angiogenesis by suppressing endothelial cell proliferation and vessel growth. Enhance post-ischemic arteriogenesis and regulates dendritic growth.	[216] [217] [218]
miR-143		Decreased levels in patients IA tissue/increased level in cerebral artery after experimental SAH hemorrhage in rats. Decreased in vascular stress, downregulation of miR143/145 cluster can promote VSMC phenotypic switch to "proliferative" phenotype (aneurysm formation).	[198, 203] [219]
miR-145		Decreased levels in patients IA tissue. Decreased in vascular stress, downregulation of miR143/145 cluster can promote VSMC phenotypic switch to "proliferative" phenotype (aneurysm formation).	[198] [219]
miR-223	NLRP3	Crucial regulator of microglial activation, inflammation and neuron injury after intracerebral hemorrhage (via NLRP3).	[220]

AAA - abdominal aortic aneurysm; aSAH - aneurysmal subarachnoid hemorrhage; AVM - arteriovenous malformation; CA - cerebral artery; CTGF - connective tissue growth factor; IA - intracranial aneurysm; ICH - intracerebral hemorrhage; IGF1R - insulin like growth factor receptor 1; MMP - matrix metalloproteinase; NLRP3 - inflammasome involves in inflammation after intracerebral hemorrhage; Smad - Smad family member (TGF- β superfamily signaling cascade); TGF- β - tumor growing factor β ; TSP-1 - thrombospondin; VEGF-A - vascular endothelial growth factor A; VEGF-D - vascular endothelial growth factor D; VCAM-1 - vascular cell adhesion molecule; VSMC - vascular smooth muscle cell.

brain, degradation avoidance, and target specificity. Hence, one of the latest approaches uses locked nucleic acid-modified oligonucleotide antagomir (LNA-antimiR) con-

struct with enhanced *in vivo* stability by increased thermal stability and resistance to exo- and endonucleases [221]. Numerous studies demonstrated neuroprotective potential of

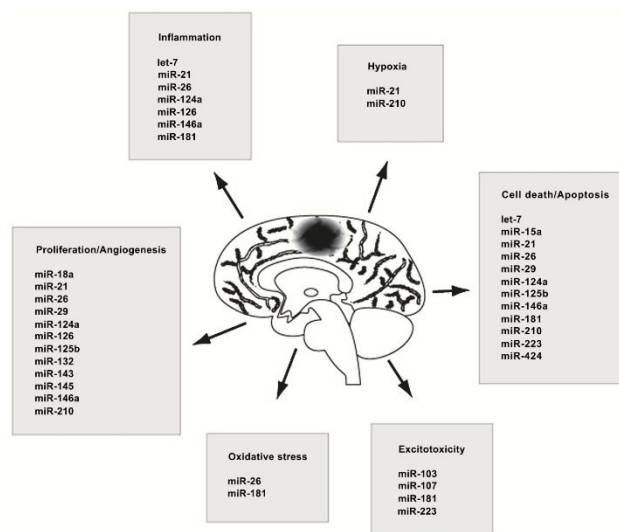


Fig. (1). Role of microRNA in stroke.

targeted microRNA modulations in stroke animal models. In contrast to experimental studies, clinical research is at the very early stage. So far, profiling studies identified candidate microRNAs and partially described dynamics of their expression after the stroke. However complex associations of specific microRNAs expression with main clinical characteristics and deeper insight into mechanisms their regulatory functions are still missing.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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PATIENT CONSENT

Declared none.

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3.2 Inflammation - a crucial pathophysiological aspect of early brain injury

Inflammation is a crucial contributor to EBI; inflammatory response is exaggerated after SAH and it represents a potential cross-link among all EBI-associated pathologies, DCI, and vasospasms^{33,76–78}. Inflammatory response associated with SAH is thus an important topic of research in both clinical and experimental conditions.

The immune system in the brain is mainly represented by macrophages, which are localized in the parenchyma – microglia, as well as outside the parenchyma, alongside barriers – perivascular macrophages, meningeal macrophages, circumventricular organs associated macrophages and choroid plexus associated macrophages⁷⁹. Individual immune cells may traffic between CSF and parenchyma, while trafficking between CSF and regional lymph nodes is possible via nasal and dural lymphatics, which drains CSF to cervical lymph nodes^{80–82}.

Microglia is the most frequent myeloid population in the brain and have several crucial functions, like synaptic pruning, synthesis of neurotrophic factors, immune surveillance, or phagocytosis^{83–86}. In response to pathology, microglia is activated shifting to proinflammatory M1 or anti-inflammatory M2 phenotype⁸⁷.

Inflammatory response after SAH develops in two steps, it is initially a local reaction which is subsequently generalized to systemic response^{88,89}. Local reaction peaks between 4 and 6 hours after SAH and subsequent inflammatory response may persist for approximately two weeks^{90–92}. Inflammatory response after SAH is triggered in reaction to tissue damage and transient global cerebral ischemia^{90,93,94}. These are strong signals mainly for microglia activation, which starts to release proinflammatory cytokines, activating endothelial cells and peripheral leukocytes^{95,96}.

Microglia is also a source of chemokines, which attract peripheral leukocytes to the affected area, while endothelial cells produce adhesive molecules allowing diapedesis of peripheral leukocytes, which are mainly represented by neutrophils and monocytes/macrophages^{96,97,95}. However, diapedesis is complicated, because BBB is permeable for single leukocytes, however, it is not permeable for the trafficking of large leukocyte populations⁹⁸. When leukocytes are stuck in perivascular space, they release proteolytic enzymes which cause BBB disruption and allow them to penetrate BBB^{99,98}. This is an important mechanism of BBB disruption in EBI^{100,101}.

Besides BBB disruption, infiltrating immune cells cause additional tissue damage by overproduction of ROS and proinflammatory cytokines¹⁰². Neutrophils represent a major source of ROS, which are mainly produced by NADPH oxidase and myeloperoxidase¹⁰³. Besides superoxide and hypochlorous acid, the neutrophils produce a significant amount of other ROS such as hydrogen peroxide, chloramine, or hypothiocynite¹⁰³. As mentioned above, oxidative stress may damage lipids, proteins as well as DNA and promotes further inflammatory response^{68–70,65}.

Cytokines represent a complex net of molecules that regulate key homeostatic processes¹⁰⁴. Inflammatory response after SAH is associated with increased levels of TNF α , IL-1 β , IL-6, and

IL-8 which may reflect patient outcome^{105,106}. Probably the most extensively investigated pro-inflammatory cytokine in stroke research is IL-6^{105–110}. High levels of IL-6 in CSF and serum are associated with DCI and patient outcome^{109,110}. IL-6 is involved in several crucial pathophysiological processes such as oxidative stress, apoptosis, and BBB disruption¹⁰².

3.2.1 The author's publications related to the topic of SAH associated inflammation

3.2.1.1 The Role of Inflammatory Response in Stroke Associated Programmed Cell Death

Duris K, Splichal Z, Jurajda M. The Role of Inflammatory Response in Stroke Associated Programmed Cell Death. *Curr Neuroparmacol*. 2018;16:1365–1374.

Type of article: Review article

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Number of citations without self-citations according to WoS (7/2025): 58

In this review article, we discuss the contribution of inflammation to cell death after stroke. At the beginning, we outline the pathophysiology of stroke-associated primary and secondary brain injury and describe the activation of inflammatory response after stroke. The main section is dedicated to the characterization of various types of cell death after stroke with respect to inflammation. Particularly we cover necrosis, apoptosis, necroptosis, pyroptosis, and autophagy. Finally, we discuss inflammation as a target for therapy after stroke.

REVIEW ARTICLE

The Role of Inflammatory Response in Stroke Associated Programmed Cell Death

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Abstract: Stroke represents devastating pathology which is associated with a high morbidity and mortality. Initial damage caused directly by the onset of stroke, primary injury, may be eclipsed by secondary injury which may have a much more devastating effect on the brain. Primary injury is predominantly associated with necrotic cell death due to fatal insufficiency of oxygen and glucose. Secondary injury may on the contrary, lead apoptotic cell death due to structural damage which is not compatible with cellular functions or which may even represent the danger of malign transformation. The immune system is responsible for surveillance, defense and healing processes and the immune system plays a major role in triggering programmed cell death. Severe pathologies, such as stroke, are often associated with deregulation of the immune system, resulting in aggravation of secondary brain injury. The goal of this article is to overview the current knowledge about the role of immune system in the pathophysiology of stroke with respect to programmed neuronal cell death as well as to discuss current therapeutic strategies targeting inflammation after stroke.

Keywords: Inflammation, stroke, necrosis, apoptosis, necroptosis, pyroptosis, autophagy.

1. INTRODUCTION

Stroke is a sudden neurological dysfunction caused by an interruption of blood supply to the brain. Stroke is a major source of long term disability and mortality, it is the number two cause of death worldwide [1]. Each year, approximately 15 million people worldwide suffer from a stroke, from which around one third die and another one third stay permanently disabled [2]. Interruption of blood supply to the brain may be caused by cerebral vessel occlusion causing ischemic stroke (around 70% of strokes) or by vessel rupture leading to hemorrhagic stroke (around 30% of strokes) [3]. Rupture of intraparenchymal artery results in intracerebral hemorrhage (ICH), while the rupture of an extraparenchymal cerebral artery (localized in subarachnoid space) results in subarachnoid hemorrhage (SAH). A cerebral aneurysm is the most common source of spontaneous (non-traumatic) subarachnoid hemorrhage (SAH). Although the hemorrhagic stroke is less frequent than ischemic, hemorrhagic stroke is associated with a higher mortality and altogether it accounts for around same number of deaths as ischemic stroke [3]. Pathological changes similar to stroke may occur also in "a small scale" as a part of small vessel disease which may have a very complex pathophysiological background [4]. In

this article, we will outline main pathophysiological processes associated with stroke with an accent to the inflammation and cell death after stroke. Stroke research is a very dynamic process, new information about pathophysiology of stroke allow researchers to view the problematic from new perspectives and to create new models for research which in turn may bring new information [5, 6].

2. PATHOPHYSIOLOGY OF STROKE

As mentioned above, stroke is a sudden loss of neural functions due to interruption of blood supply to the brain due to ischemic or hemorrhagic event. Neurons are highly metabolically active and almost completely dependent on oxidative phosphorylation [7]. The neurons use the majority of energy to keep the membrane electrical activity and neuronal cells are very sensitive to restriction of oxygen and glucose [8]. Blood supply interruption causes quick development of brain damage which may be reversible or irreversible according to severity of stroke.

Primary brain injury is a direct damage which develops immediately after the onset of stroke due to critical ischemia or due to tissue disruption by hematoma. Critical ischemia results in irreversible neuronal damage forming the core of infarction. In the core of infarction absolute insufficiency of ATP leads to depolarization and bioenergetic failure, the ATP dependent ion pumps are not able to keep minimal required membrane potential, osmotically active ions, such as

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Na^+ , accumulates inside the cell, followed by water influx which result in cytotoxic edema and potentially cell death [7]. Disruption of brain tissue by hematoma is a direct mechanical damage caused by mass effect of extravasated blood [9].

Secondary injury develops in a delayed manner and the main contributors to secondary injury are excitotoxicity, oxidative stress and inflammation [7, 10, 11]. The most severe consequences of secondary brain injury are endothelial damage (and subsequent vasogenic edema) or neuronal cell death [7, 10, 11]. In ischemic stroke, the secondary damage occurs mainly in the penumbra, the area surrounding the core of infarction, where the neuronal cells are affected by ischemia reversibly [12]. Insufficiency of ATP due to decreased blood supply leads to neuronal depolarization, but there is enough ATP to avoid complete bioenergetic failure as occurs in ischemic core. In experimental conditions, ATP levels in the penumbra decreased to 62% of basal production, while in the core of infarction ATP levels reached 22% of basal production [13]. Delayed ischemic cerebral damage, similar to penumbral, has been described after both types of hemorrhagic stroke, although there are still some controversies about this topic in the pathophysiology of ICH [14-16]. Mass effect associated with large intracerebral hemorrhage cause ischemia in perihematomal area due to increased intracerebral pressure [9, 17]. However, increased intracerebral pressure may not be associated with cerebral ischemia in all the cases and this was described in experimental conditions [16]. Increased intracerebral pressure in patients with ICH together with decreased blood pressure and preexisting cerebral microangiopathy due to atherosclerosis was thus hypothesized to be major pathophysiological factors for ischemic cerebral damage after ICH [14]. If there are some controversies about cerebral ischemia after ICH, there seems to be no controversies in case of SAH. It is generally accepted that the mass effect of blood in subarachnoid space after SAH cause a massive increase in intracranial pressure, resulting in transient cerebral ischemia, which play an important role in the pathophysiology of SAH [11, 18].

Massive extracellular accumulation of excitatory neurotransmitters, excitotoxicity, is due to increased release from depolarized neurons and due to decreased reuptake which is ATP dependent. Excitotoxicity amplifies depolarization of surrounding neurons, leading to influx and accumulation of Na^+ , Cl^- and Ca^{2+} [19]. Ionic imbalance and especially high intracellular level of Ca^{2+} may result in excessive activation of proteolytic and reactive oxygen species (ROS) producing enzymes and potentiate secondary neuronal damage [7, 19].

Excessive production of reactive oxygen species, oxidative stress, is a significant contributor to secondary injury in stroke [20]. Stroke associated oxidative stress is a very complex process involving several pathophysiological processes including excessive accumulation of Ca^{2+} in the cytoplasm, mitochondrial dysfunction, decreased activity of anti-oxidant enzymes and inflammation [21-23]. Neurons are vulnerable to oxidative stress because of high metabolic activity, relatively low amount of antioxidant enzymes and high levels of polyunsaturated fatty acids which are prone to oxidative damage [24]. High level of oxidative stress occurs mainly

during reperfusion [21]. Reperfusion injury is a severe complication following restoration of blood supply and in most severe cases, hemorrhagic transformation of the ischemic area may occur [7].

Following intracerebral hemorrhage, or hemorrhagic transformation, several cytotoxic blood components are in contact with brain parenchyma, which may contribute to secondary injury. These include complement molecules, coagulation factors, immunoglobulin molecules and mainly iron from depredated hemoglobin [10, 25]. Subarachnoid hematoma is localized extraparenchymally and the blood is in contact with main stems of cerebral vessels. The cytotoxic blood components may contribute to cerebral vasospasm development, however, other etiological factors, including NO, endothelial cell damage or oxidative stress may be involved in cerebral vasospasm development [26-28]. Cerebral vasospasm represents the most severe late complication after SAH, which is associated with high mortality and morbidity [29]. Unclear pathophysiology of cerebral vasospasm and the absence of effective treatment led to the identification and further research of early brain injury, pathological processes which precede and which may contribute to cerebral vasospasm development [11, 30, 31]. Although these pathological processes basically represent secondary injury, the term "early brain injury" is more appropriate from the perspective of cerebral vasospasm development.

3. THE ACUTE INFLAMMATORY RESPONSE IN STROKE

Inflammation plays a crucial role in the pathophysiology of stroke; on one side the inflammatory response is necessary for tissue repair, on the other side the inflammatory response is an important contributor to secondary brain injury after stroke [32, 33]. The inflammatory response is quite uniform in ischemic and hemorrhagic stroke, including activation of microglia and endothelial cells in the first step, followed by infiltration of affected areas by peripheral leukocytes which subsequently cause tissue damage (Fig. 1) [34-36]. Although the basic concept of inflammatory response after stroke is generally known, there is still a lot of missing information and this concept is constantly updated [37].

Microglia represents resident immune cells in central nervous system responsible for immunological surveillance and phagocytic functions [38]. Microglial activation occurs rapidly after stroke in reaction to ischemia/tissue damage [39-41]. Microglial activation may also be induced by endothelial cells, suggesting the important role of microvasculature after stroke [42]. Activated microglia is morphologically and functionally similar to macrophages [43]. The role of activated microglia is ambiguous. Activated microglia may have neuroprotective role, possibly by its phagocytic functions [39]. But activated microglia is mainly a source of neurotoxic substances, including proteases, excitatory amino acids, nitric oxide and pro-inflammatory cytokines [44, 45].

Endothelial cell activation includes increased expression of selectins and adhesion molecules, which is an essential step for recruitment of leukocytes from peripheral blood [46]. Both E-selectin and P-selectin are involved in leuko-

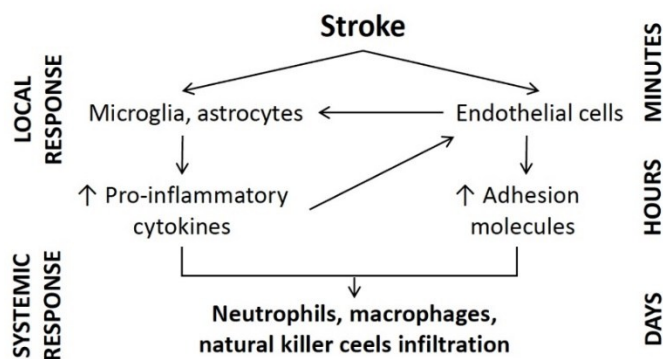


Fig. (1). The inflammatory response after stroke and its spatial and temporal context. Stroke associated tissue ischemia/damage is strong pro-inflammatory signal, which activates resident immune cells (microglia) as well as astrocytes and endothelial cells, resulting in the production of pro-inflammatory cytokines and adhesion molecules. Pro-inflammatory cytokines help to activate endothelial cells locally, but they are mainly released into circulation, where they activate peripheral leukocytes (mainly neutrophils, macrophages and natural killers). Activated leukocytes from the periphery are directed by the adhesion molecules, which allow the leukocytes to infiltrate the affected areas.

cyte rolling and recruitment and are associated with secondary brain injury, while the L-selectin seems not to be related to stroke [47-49]. Adhesion molecules facilitate diapedesis of leukocytes through the vessel wall and while there are strong evidences that ICAM-1 contribute to secondary brain injury, reports about the role of VCAM-1 in the pathophysiology of stroke are controversial [50-55].

Peripheral leukocytes infiltrate the affected area in response to microglial and endothelial activation. The kinetics of leukocytes after ischemic as well as hemorrhagic stroke is as follows: the first infiltrating cells are neutrophils, followed by macrophages and natural killer cells with subsequent infiltration of T lymphocytes [56]. The deleterious effect of escalated inflammatory response is mainly associated with toxic effect of ROS species, arachidonic acid metabolites and pro-inflammatory cytokines which significantly contribute to secondary brain damage [57]. It is still not clear whether the inflammatory response associated with severe stroke may be considered as a deregulated or just exaggerated, but regulated process. Regulatory T lymphocytes play crucial role in maintaining immune homeostasis and controlling inflammation and these cells are recently a subject intensive research [58].

4. INFLAMMATION AND CELL DEATH AFTER STROKE

Inflammation plays crucial role in the pathophysiology of stroke and inflammation is closely associated with cell death [57]. On one side, tissue damage induces an inflammatory response in order to remove debris and repair tissue damage, on the other side, strong/deregulated inflammatory response may aggravate the tissue damage and cell death [57]. As mentioned above, harmful effect of inflammation is associated with a toxic effect of ROS species, proteolytic enzymes and pro-inflammatory cytokines. The immune cells are rich source of these mediators and while the ROS as well as pro-

teolytic enzymes may cause direct tissue damage, the effect of pro-inflammatory cytokines is receptor mediated process (Fig. 2) [57]. Several subtypes of cell death have been described and these are characterized by a different level of regulation, but all subtypes are closely interconnected and associated with inflammation [59]. Apoptosis and necrosis may be considered as two opposite poles of cell death scale. Necrosis is unregulated or minimally regulated, passive process which does not require too much energy, while apoptosis is highly regulated, active process requiring energy [60,61]. Necrosis induces inflammation, while apoptosis does not induce inflammation, on contrary apoptosis may be induced by inflammation [59]. Necrosis occurs under "pathological" conditions, while apoptosis occurs either under "physiological" conditions during development or under "pathological" conditions as a result of intrinsic "self-eliminating" mechanisms or immune surveillance [59]. Necroptosis, pyroptosis and autophagy are the other subtypes of cell death, which will be described below, however, all the subtypes of cell death are closely interconnected, possibly as the backup programs and immune system may coordinate interactions between them [62, 63].

4.1. Necrosis

Although this article is primarily focused on programmed cell death, it is important to consider all forms of cell death and that is why this part was included in this review. Necrosis is a type of cell death, which is minimally regulated or unregulated at all [60, 61]. It usually occurs in the core of infarction or in areas damaged directly by the mass effect of hematoma, so this type of cell death is typically associated with primary injury [7, 9]. Due to extreme conditions, bioenergetic failure may occur with subsequent cytotoxic edema and cellular lysis [7]. Bioenergetic failure is a passive, not regulated, process, which develops quickly and we may assume that no regulation occurs because there is not time and energy for it. In less severe conditions, necrosis may occur as

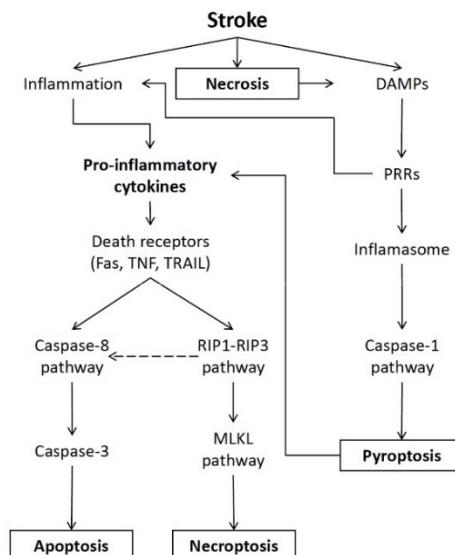


Fig. (2). The inflammatory response and cell death after stroke. DAMPs (damage associated molecular pattern) are released from injured/necrotic cells in response to tissue damage. Inflammation is triggered in response to DAMPs through PRRs (pattern recognition receptors). Pro-inflammatory cytokines act through death receptors (Fas, TNF, TRAIL) triggering either caspase-8 apoptotic pathway (extrinsic), or RIP1-RIP3 (receptor-interacting protein kinases)-MLKL (mixed-lineage kinase domain-like protein) pathway resulting in necroptosis. The RIP1-RIP3 pathway may induce apoptosis when MLKL is not present. PRRs also contribute to inflammation by induction of inflammasome formation and caspase-1 activation, resulting in maturation and releasing of pro-inflammatory cytokines from the pyroptotic cells.

partially regulated process initiated by oxidative stress, high Ca^{2+} cytoplasmic concentrations and mediated by calpain mechanism [64-67]. Necrotic cell death is associated with an activation of immune system [39, 40].

4.2. Apoptosis

Apoptosis is a highly regulated type of cell death, which develops in a delayed manner and which is associated with secondary injury. Apoptosis typically occurs in ischemic penumbra and apoptosis also plays an important role in the pathophysiology of hemorrhagic stroke [7, 68, 69]. Cellular component degradation in apoptosomes and cell shrinkage are characteristics of this type of cell death. Apoptosis is mediated by caspase pathways, which may be triggered by intrinsic or extrinsic factors. The intrinsic pathway is induced by cellular/mitochondrial damage, leading to cytochrome c release from mitochondria, which activates caspase 9 - caspase 3 [70]. The extrinsic pathway is mediated by surface death receptors, from which the most important are Fas, TNF and TRAIL receptors associated with activation of Caspase 8 and Caspase 10 [71]. This may lead to activation of intrinsic pathway or to direct activation of executioner caspase 3 [72]. Caspase 3 is the final step of activation for both intrinsic and extrinsic pathways mediating programmed cell death, mainly by enhancing DNA damage and fragmentation [73]. The immune system plays crucial role in apoptosis regulation and excessive inflammatory response is asso-

ciated with induction of pro-apoptotic and inhibition of anti-apoptotic pathways [74].

4.3. Necroptosis

Necroptosis is a programmed type of cell death combining features of apoptosis (initial phase) and necrosis (executive phase). Necroptosis is mediated by same receptors as apoptosis, different signaling pathways are, however, associated with these two subtypes of cell death. Fas, TNF and TRAIL activation is, in case of necroptosis, caspase independent machinery associated with RIP1-RIP3 (receptor-interacting protein kinases) signaling pathway [75]. RIP1-RIP3 complex activates MLKL (mixed-lineage kinase domain-like protein) which induce necroptotic cell death by permeabilization of plasmatic membrane [76-78]. MLKL is essential for necroptosis execution, because an activation of RIP3 in absence of MLKL induces caspase dependent machinery, which result in apoptosis, suggesting that the selection of "executive program" (necroptosis or apoptosis) possibly depends on the levels of signaling and effector proteins [79]. Necroptosis was suggested as a potential back up plan for the situations when the caspase mediated cell death program fails [80]. Necroptosis was also reported to be associated with extremely pathological extracellular conditions, such as acidosis, which is associated with ASIC1 (acid-sensing ion channel 1) mediated activation of the RIP1-RIP3 system [77]. This may represent a potential shortcut in case

when the severe environmental conditions do not allow realizing apoptotic program.

4.4. Pyroptosis

Pyroptosis is a type of programmed cell death, which is associated with a high level of inflammatory response. The inflammatory response is triggered by PRRs (pattern recognition receptors) which recognize a wide range of microbial structures called PAMPs (pathogen associated molecular pattern), endogenous stress signals called DAMPs (damage associated molecular pattern) or ASC (apoptosis speck-like) proteins and induce formation of inflammasomes [81, 82]. Important compounds of inflammasomes are NLRPs (NOD-like receptors containing pyrin domains), mainly NLRP 1 and NLRP3 which interact with pro-caspase-1 and cleave it into active form [81, 82]. Caspase 1 in turn cleaves precursors of inflammatory cytokines activating mainly IL-1 β , IL-18 and IL-33 which are released from dying cells and induce inflammation [81, 82].

4.5. Autophagy

Autophagy is a basic cellular function enabling degradation of cellular content in autophagosomes [83]. This process is enhanced under stress conditions such as stroke and maybe both beneficial and deleterious [84, 85]. Cellular components degradation and recylation may help to keep energy homeostasis and the physiological level of autophagy was demonstrated to be protective, while insufficient or excessive level of autophagy was shown to be harmful [86]. Three types of autophagy were described: macroautophagy, microautophagy and chaperone-mediated autophagy. Macroautophagy is the predominant pathway involved in recycling and degradation of cytoplasmic content in autophagosomes which fuse with lysosome, forming autolysosome [87]. Microautophagy is non-selective degradation of cytoplasmic substances in the lysosomes [88]. Finally chaperone-mediated autophagy represents selective degradation of cytoplasmic material bound to chaperone [89]. Autophagy plays a crucial role in degradation of cytoplasmic proteins, lipids, as well as whole organelles such as mitochondria, endoplasmic reticulum or ribosomes [90-93]. Autophagy is tightly regulated, the major regulatory pathways are AMPK (AMP-activated protein kinase) pathway which is involved in positive modulation, and mTOR (mammalian target of rapamycin) pathway which represents negative modulator [94].

5. INFLAMMATION AS THERAPEUTIC TARGET AFTER STROKE

Inflammatory response after stroke may be considered as a two step process. The first step is an activation of the immune system and leukocyte trafficking, in the second step infiltrating leukocytes execute their functions with all the consequences. Therapeutic interventions basically target either leukocyte trafficking or crucial mediators of inflammatory response. However, it is necessary to mention that many important details regarding inflammatory response after stroke are still not known, and, for example, the serum levels of TNF α in patients with intracerebral hemorrhage have not been investigated in a robust clinical study [95].

ICAM-1 (intercellular adhesion molecule 1, CD54) is adhesion molecule which is necessary for neutrophil transendothelial migration [96]. In experimental conditions, the anti-ICAM-1 antibody reduced neutrophil infiltration into the brain and this was associated with reduced ischemic brain damage in transient focal cerebral ischemia model [97]. Phase III clinical trial with anti-ICAM-1 antibody enlimomab, however, failed to prove the protective effect of this treatment in acute stroke patients and treatment was even associated with higher infarct volume and mortality [98]. Further examination found that administration of anti-ICAM-1 antibody induced production of host antibodies against the medicament and activation of the immune system including neutrophils, complement and endothelial cells [99].

Blockade of IL-1 receptor using antagonist IL-1RA decreased cerebral ischemia injury in experimental conditions, but in phase II clinical trial IL-1RA did not improve infarct volume, survival or outcome of acute stroke patients [100, 101]. Peripheral administration of medication was suggested as a possible reason for clinical trial failure and intranasal administration was postulated as a potential solution [102].

Minocycline is a broad-spectrum tetracycline antibiotic with neuroprotective, anti-inflammatory and anti-oxidative properties [103, 104]. Minocycline down-regulate microglial activation, TNF α expression and T cell activation [105]. The neuroprotective effect of minocycline was reported in both ischemic and hemorrhagic stroke models [106-108]. Minocycline improved outcome of acute ischemic stroke patients in an open-label, evaluator-blinded study, but the phase IV clinical trial did not confirm the previous results and was terminated for futility [109, 110]. Phase I clinical trial testing the effectiveness of minocycline in patients with intracerebral hemorrhage is currently in progress [111].

Fingolimod is an agonist of sphingosine-1-phosphate receptor, which prevents lymphocytes to egress from lymphoid organs and decreases expression of ICAM-1 [112, 113]. Fingolimod was also reported to decrease activity of cytotoxic T-cells independently of S1P receptor [114]. The beneficial effect of fingolimod was described in both ischemic and hemorrhagic stroke in animal models [113, 115, 116]. In an open-label, evaluator blinded study in acute ischemic stroke patients, orally administered fingolimod decreased circulating lymphocytes, reduced infarct volume, attenuated neurological deficit and promoted recovery [117]. In a multi-center pilot trial, the combination therapy with fingolimod and alteplase reduced reperfusion injury and improved outcome in acute ischemic stroke patients [118]. The neuroprotective effect of fingolimod was also reported in patients suffering from ICH. In a pilot two arms, evaluator blinded study, fingolimod reduced perihematomal edema, attenuated neurological deficit and promoted recovery in patients with a small supratentorial hematoma [119].

PPAR- γ (peroxisome proliferator-activated receptor gamma) agonists have multiple effects including attenuation of inflammatory response. PPAR- γ is a nuclear receptor responsible for transcription of several products participating in lipid and glucose metabolism, as well as in inflammatory response [120, 121]. PPAR- γ agonist rosiglitazone attenuated

experimentally induced ischemic brain injury by reducing pro-inflammatory cytokines and chemokines expression and this was associated with decreased neutrophil infiltration into the brain [122]. Rosiglitazone also decreased neuronal damage and improved functional outcome in experimentally induced intracerebral hematoma and this was associated with the improved phagocytic function of microglia, which promotes hematoma resolution [123]. Phase II clinical trial testing safety of PPAR- γ agonist Pioglitazone for hematoma resolution after intracerebral hemorrhage has been completed [124].

Celecoxib is a selective inhibitor of cyclooxygenase 2 which was reported to reduce inflammation, brain edema and perihematomal cell death in experimentally induced ICH [125]. In a small multi-center study, celecoxib reduced perihematomal edema in patients with ICH [126].

Simvastatin is hypolipidemicum with additional effects including improving endothelial functions and decreasing vascular inflammation [127]. Simvastatin is considered to be an effective prophylaxis against cerebral vasospasm development after SAH and anti-vasospastic and anti-inflammatory properties are recently tested in phase III clinical trial [128, 129].

Etanercept is TNF α inhibitor, which is currently in phase I clinical trial in patients with aneurysmal subarachnoid hemorrhage [130].

CONCLUSION

Inflammation plays an important role in the pathophysiology of stroke and it is closely associated with cell death after stroke. Both the inflammation and cell death are very complex pathophysiological processes. Thanks to both experimental and clinical research, we have a lot of information, but we are still far from understanding these crucial processes in their complexity. And possibly that is why we are not able to effectively control either inflammation or cell death when exaggerated. More research is needed in this field in order to elucidate the interconnections between inflammation and different types of cell death in its complexity. It is very desirable, because it may allow us to develop a more complex therapeutic approach to patients suffering from stroke. Complex therapeutic approach targeting inflammatory response as well as employing new technologies in diagnostics and management may improve the effectiveness of treatment, reducing neuronal cell death after stroke and improving patients outcome after stroke [131, 132].

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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3.2.1.2 IL-6 Levels in the Cerebrospinal Fluid and their Association with Brain Oxygen Partial Pressure and Cerebral Vasospasm Development in Patients with Aneurysmal Subarachnoid Haemorrhage

Duris K, Neuman E, Vybihal V, Juran V, Gottwaldova J, Kyr M, Vasku A, Smrcka M. IL-6 Levels in the Cerebrospinal Fluid and their Association with Brain Oxygen Partial Pressure and Cerebral Vasospasm Development in Patients with Aneurysmal Subarachnoid Haemorrhage. *Cesk. Slov. Neurol. Neurochir.* 2016;79:573–578.

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In this research, we tried to find out whether there is a relation between IL-6, parameters of TCD, and PbtO₂ in patients suffering from SAH. IL-6 is one of the most widely studied markers of inflammation in stroke.

We studied IL-6 levels in CSF on day four and compared them to the parameters of TCD and PbtO₂ on day 5 after the onset of bleeding. This time point represents the period when the highest risk of vasospasm development begins. In total 20 patients with both mild/moderate and severe SAH were enrolled in the study. Although there was a correlation between IL-6 and PbtO₂ in our study group and a clear tendency to higher IL-6 levels in patients with cerebral vasospasm and low PbtO₂ levels, the differences were not statistically significant.

This study demonstrated that IL-6 may be a promising diagnostic marker, however prediction of DCI using this marker alone is problematic.

Vztah likvorových hladin IL-6 ke změnám parciálního tlaku kyslíku v mozku a k rozvoji vazospazmů u pacientů po subarachnoidálním krvácení z ruptury aneuryzmatu mozkové tepny

IL-6 Levels in the Cerebrospinal Fluid and their Association with Brain Oxygen Partial Pressure and Cerebral Vasospasm Development in Patients with Aneurysmal Subarachnoid Haemorrhage

Souhrn

Cíl: Cílem práce bylo zjistit, zda existuje vztah mezi likvorovými hladinami IL-6 a nálezy na transkraniální dopplerometrii či hodnotami parciálního tlaku kyslíku v mozku (PbtO₂) u pacientů po subarachnoidálním krvácení. **Soubor a metodika:** Do souboru byli zařazeni pacienti se subarachnoidálním krvácením z ruptury aneuryzmatu mozkové tepny, kteří byli čtvrtý den po atace v bezvědomí. U pacientů byl hodnocen vztah mezi likvorovými hladinami IL-6 ze čtvrtého dne po atace krvácení a nálezy na transkraniální dopplerometrii či hodnotami parciálního tlaku kyslíku v mozku z následujícího dne. **Výsledky:** Celkem bylo hodnoceno 20 pacientů. U zkoumaného souboru byl patrný trend k vyšším hladinám IL-6 u pacientů s vazospazmy mozkových tepen (med: 3 544; 25 %/75 % = perc: 2 106/6 907 vs. med: 10 080; 25 %/75 % perc: 2 540/13 958; p = 0,0946), avšak nebyla prokázána korelace mezi hladinami IL-6 a rychlostmi toků na magistralních tepnách. Při srovnání hladin IL-6 s hodnotami parciálního tlaku kyslíku v mozku byl patrný trend k vyšším hladinám IL-6 ve skupině s hodnotami PbtO₂ nižšími než 20 mm Hg (med: 2 860; 25 %/75 % perc: 940/8 580 vs. med: 6 937; 25 %/75 % perc: 3 811/14 524; p = 0,0587) a byla prokázána korelace mezi likvorovými hladinami IL-6 a hodnotami PbtO₂ (rs = -0,626; p = 0,002). **Závěr:** Ve zkoumaném souboru pacientů po subarachnoidálním krvácení byla v období reprezentujícím začátek periody největšího rizika vzniku opožděné mozkové ischemie prokázána korelace mezi likvorovými hladinami IL-6 a hodnotami PbtO₂ z následujícího dne. Na základě těchto výsledků lze předpokládat, že IL-6 může představovat slibný marker, který by mohl být použitelný k predikci rizika vzniku opožděné mozkové ischemie.

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Autori deklarují, že v souvislosti s předmětem studie nemají žádné komerční zájmy.

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The Editorial Board declares that the manuscript met the ICMJE "uniform requirements" for biomedical papers.

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Key words

subarachnoid hemorrhage – inflammation – interleukin-6 – brain tissue oxygen monitoring – Licox – transcranial dopplerometry – cerebral vasospasm

VZTAH LIKVIOROVÝCH HLADIN IL-6 KE ZMĚNÁM PARCIÁLNÍHO TLAKU KYSLÍKU V MOZKU A K ROZVOJI VAZOSPASMŮ

Abstract

Aim: The aim of this study was to evaluate whether levels of IL-6 in the cerebrospinal fluid are associated with parameters of transcranial dopplerometry or brain oxygen partial pressure (PbtO₂) in patients with subarachnoid haemorrhage. **Patients and methods:** Patients with aneurysmal subarachnoid haemorrhage, who were unconscious during the fourth day after the onset of bleeding, were enrolled in the study. We explored associations between levels of IL-6 in the cerebrospinal fluid as measured on the fourth day after subarachnoid hemorrhage and transcranial dopplerometry parameters or brain oxygen partial pressure measured one day later. **Results:** A total of 20 patients were enrolled into this study. There was a trend to higher levels of IL-6 in patients with cerebral vasospasm (med: 3,544; 25%/75% = perc: 2,106/6,907 vs. med: 10,080; 25%/75% perc: 2,540/13,958; $p = 0.0946$), however, no correlation between the levels of IL-6 and mean velocities in magistral vessels was observed. There was a tendency to higher levels of IL-6 related to PbtO₂ lower than 20 mm Hg (med: 2,860; 25%/75% perc: 940/8,580 vs. med: 6,937; 25%/75% perc: 3,811/14,524, $p = 0.0587$) and there was a significant correlation between the levels of IL-6 and PbtO₂ in our study group ($r_s = -0.626$; $p = 0.002$). **Conclusion:** In this study group, there was a correlation between levels of IL-6 in the cerebrospinal fluid and subsequent PbtO₂ values at the beginning of the period representing the highest risk of delayed cerebral ischemia development. Based on these results we assume that IL-6 might be considered as a promising marker to predict risk of developing delayed cerebral ischemia.

Úvod

Subarachnoidální krvácení (Subarachnoid Hemorrhage; SAH) je typ cévní mozkové příhody vyskytující se s incidencí asi 10/100 000 obyvatel/rok a je spojeno s až 50% mortalitou [1]. V přibližně 80 % případů je SAH spojeno s rupturou aneuryzmatu mozkové tepny [2]. Ošetření aneuryzmatu představuje zásadní léčebný postup, který má za cíl předejít opakovanému prasknutí aneuryzmatu, což je nejzávažnější komplikace SAH spojená s až 80% mortalitou [2].

Vedle opakovaného krvácení je obávanou komplikací SAH opožděná mozková ischemie (Delayed Cerebral Ischemia; DCI). DCI se obvykle rozvíjí mezi čtvrtým a 14. dnem, postihuje asi 30 % pacientů a je spojena s výrazným nárůstem morbidit a mortality [3]. Vazospazmy mozkových tepen jsou nejčastější příčinou DCI, avšak náhled na DCI se v poslední době přehodnocuje, neboť vazospazmy mozkových tepen nemusí vést vždy k DCI a na druhou stranu DCI může vzniknout nezávisle na přítomnosti vazospasmů [3–5]. Předmětem intenzivního výzkumu posledních let se proto vedle vazospasmů stalo studium časných patologických změn v mozku (Early Brain Injury; EBI). EBI je komplex navzájem provázaných patologií, které časově předcházejí vzniku vazospasmů, mohou být jejich příčinou a zároveň mohou vést k DCI nezávisle na vzniku vazospasmů. K nejvýznamnějším patologiím ze souboru EBI patří přechodný nárůst intrakraniálního tlaku s přechodnou mozkovou ischemií, zánět, apoptóza neuronálních i endotelálních buněk nebo edém mozku. Z molekulárních mechanismů je to porucha produkce a signální dráhy oxidu dusného, dysregulace koagulační kaskády, excitotoxicita a oxidativní stres [6–8].

Zánět hraje klíčovou roli v patofyziologii SAH a je významným činitelem v rozvoji EBI i vazospasmů mozkových tepen [7,9,10]. Nadějným markerem zánětlivé reakce po SAH se jeví interleukin-6 (IL-6), jehož hladiny v likvoru výrazně narůstají časně po atace krvácení, problémem však zůstává fakt, že výše hladin je velmi variabilní. Jsou-li za horní mez normy považovány hodnoty do 15 pg/ml, pohybují se likvorové hladiny po SAH ve stovkách až tisících pg/ml [11,12]. Předchozími pracemi byla popsána možná souvislost mezi likvorovými hladinami IL-6 časně po SAH a pozdějším výskytém vazospasmů i DCI [13,14]. Paralelní srovnání vztahu mezi hladinami IL-6, DCI a rozvojem vazospasmů mozkových tepen časně po SAH však provedeno nebylo, a to bylo cílem této práce.

Materiál a metody

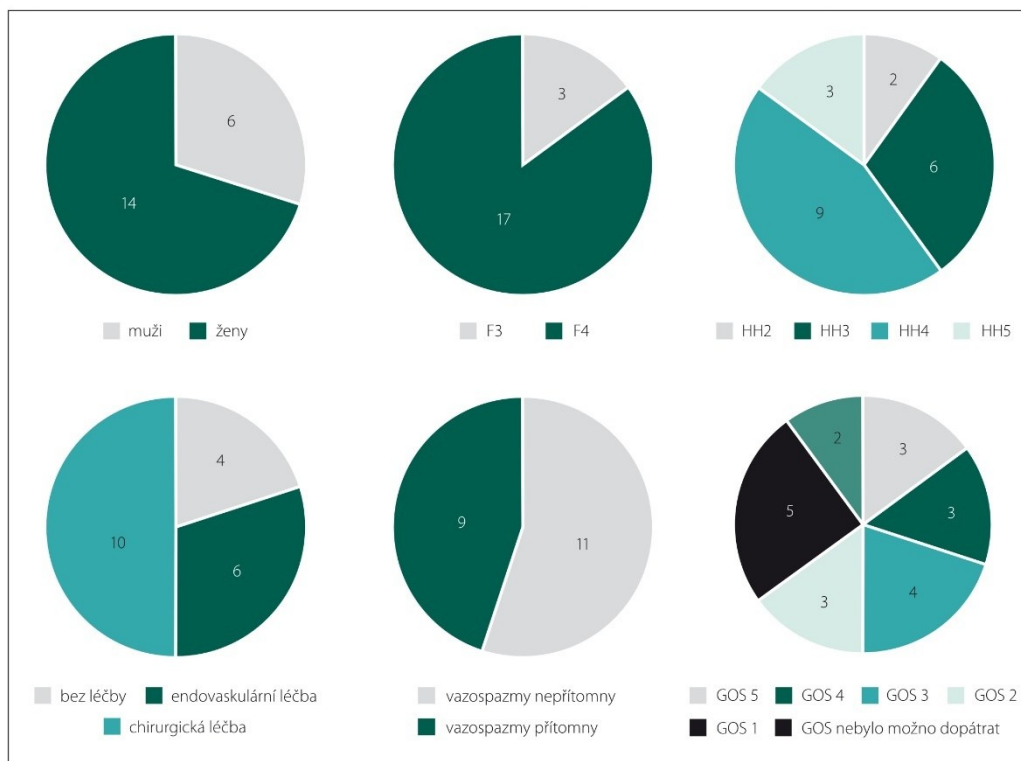
Studie i všechny postupy byly schváleny etickou komisí FN Brno. Účast ve studii byla dobrovolná, což bylo stvrzeno podpisem informovaného souhlasu pacientem nebo příbuznými pacientů v bezvědomí. Pacienti zařazení do této studie tvořili část souboru, který byl hodnocen v rámci grantového projektu probíhajícího na Neurochirurgické klinice FN Brno mezi roky 2013 a 2015. V jeho rámci byli zařazováni pacienti po SAH prokázaným na výpočetní tomografii, které nebylo starší než 24 hod, přičemž klinický stav při přijetí nebyl rozhodující. Z tohoto širšího souboru byli pro potřeby této studie vybráni pacienti, kteří byli čtvrtý den po SAH v bezvědomí (nezávisle na stavu vědomí při přijetí).

Všechny diagnostické a léčebné postupy vč. zavedení zevní lumbální drenáže nebo zevní komorové drenáže či ošetření aneuryzmatu (operace nebo endovaskulární

výkon) probíhala u pacientů zařazených do studie stejným způsobem jako u ostatních pacientů s touto diagnózou. Obecným cílem léčby bylo ošetřit aneuryzma v nejkratší možné době po přijetí a následně udržovat systolický krevní tlak nad horní hranici normy nebo na úrovni hypertenze v případech pacientů s vazospazmy. Léčba byla v jednotlivých případech individualizována s ohledem na věk a celkový stav pacienta s důrazem na kardiiovaskulární onemocnění.

U pacientů zařazených do studie byl hodnocen vztah likvorových hladin IL-6 k následnému rozvoji poruch oxygenace mozkové tkáně a vzniku vazospasmů mozkových tepen. Za tím účelem byly u každého pacienta vyhodnoceny tři vzorky likvoru odpovídající čtvrtému dni po SAH. Vzorky byly odebrány v intervalu 8 hod, byly zpracovány cestou Oddělení klinické biochemie FN Brno za použití standardních laboratorních metod a hladiny IL-6 byly stanoveny pomocí komerčně dostupných ELISA kitů (RD Systems). Za účelem detekce vazospasmů bylo u všech pacientů standardně prováděno vyšetření pomocí transkraniální dopplerometrie (TCD). Ve studii byly použity střední rychlosti toků (V_{mean}), kdy za známky vazospasmů bylo považováno rychlosti vyšší než 120 cm/s na arteria cerebri media (ACM) nebo 100 cm/s na arteria cerebri anterior (ACA), přičemž směrodatné byly hodnoty stranově odpovídající lokalizaci čidla monitoringu oxygenace mozkové tkáně. Jednalo se o jednorázová vyšetření, která byla provedena nejdříve po skončení vzorků likvoru, což odpovídalo pátému dni po SAH. Monitoring oxygenace mozkové tkáně (PbtO₂) byl prováděn pomocí systému Licox, přičemž pro potřeby této studie byla použita reprezentativní hodnota odpovídající 24hod průměru

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Graf 1. Charakteristika souboru.

F – Fisherovo skóre, HH – škála podle Hunt a Hesse, GOS – Glasgow Outcome Scale (škála výsledného stavu).

PbtO₂ odpovídající pátému dni po SAH, což bylo konzistentní s hodnocením TCD. Monitorovaná oblast byla ipsilaterálně k lokalizaci aneuryzmatu. U středochárových aneuryzmat bylo čidlo zavedeno ipsilaterálně k dominantní cévě zásobující aneuryzma. Jestliže nebylo možné určit dominantní cévu zásobující aneuryzma, potom bylo čidlo zavedeno do nedominantní hemisféry.

Likvorové hladiny IL-6 byly hodnoceny v závislosti na přítomnosti vazospasmů (skupina s vazospazmy a skupina bez vazospasmů) a v závislosti na hodnotě PbtO₂ (skupina s PbtO₂ nižší než 20 mm Hg a s PbtO₂ vyšší než 20 mm Hg). Dále byla hodnocena korelace mezi hladinami IL-6 a parametry TCD či PbtO₂. Statistické hodnocení dat bylo provedeno pomocí Mannova-Whitneyova testu a pomocí Spearmanova testu. Graficky jsou data vyjádřena jako medián, 25./75. percentil a minimum/maxi-

mum. Za hladinu významnosti byla při všech analýzách považována hodnota $\alpha = 0,05$.

Výsledky

Charakteristika souboru

Do souboru bylo zařazeno 20 pacientů ve věkovém rozmezí od 26 do 71 let, přičemž průměrný věk souboru činil 48,3 let. Charakteristiky souboru ukazuje graf 1 a lokalizaci aneuryzmat tab. 1. V hodnoceném souboru převažovaly ženy, tíže krvácení odpovídala u většiny pacientů stupni Fisher 4 a většina pacientů byla v těžkém klinickém stavu (HH4 a HH5). Těžkému klinickému stavu zkoumaného souboru odpovídal i výsledný stav, který byl u většiny pacientů neuspokojivý (GOS 1–3). Výsledný stav byl hodnocen s odstupem tří měsíců po atace krvácení a výsledný stav nebyl dopátrán v případě dvou pacientů, kteří nebyli v relevantním časovém intervalu neurologicky vyšetřeni ani

v naší, ani ve spádové neurologické ambulanci. Polovina pacientů ze zkoumaného souboru, tedy 10 pacientů, bylo operováno, u šesti pacientů bylo použito endovaskulární řešení a v případě čtyř pacientů nebylo akutní řešení indikováno. Vazospazmy byly v době hodnocení (pátý den po SAH) přítomny u devíti pacientů a během hospitalizace se objevily ještě u dalších šesti pacientů.

Vztah mezi likvorovými hladinami IL-6 a parametry TCD

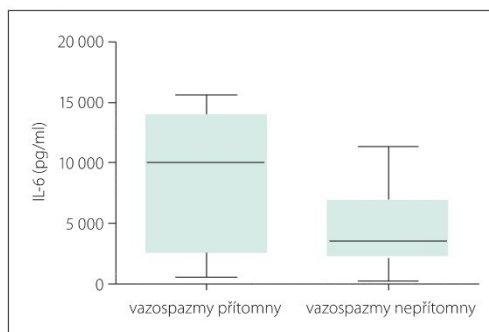
Graf 2 ukazuje porovnání likvorových hladin IL-6 v závislosti na přítomnosti vazospasmů. Rozdíl v likvorových hladinách IL-6 v závislosti na přítomnosti vazospasmů není statisticky významný, avšak je patrný náznak trendu k nižším hladinám IL-6 u pacientů bez vazospasmů (med: 3 544; 25 %/75 % = perc: 2 106/6 907 vs. med: 10 080; 25 %/75 % perc: 2 540/13 958; $p = 0,0946$). Grafy 3 a 4 zná-

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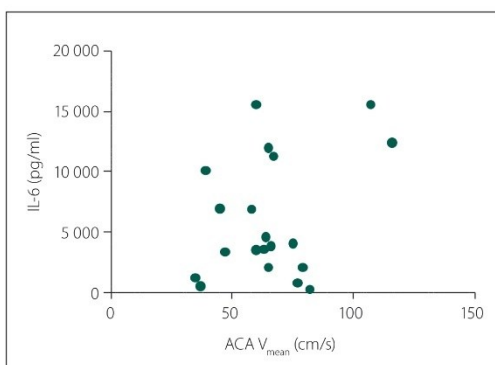
Tab. 1. Lokalizace aneuryzmatu u zkoumaného souboru.

Lokalizace aneuryzmatu	Počet
ACoA	7
ACM (vpravo/vlevo)	6 (3/3)
ACoP (vpravo/vlevo)	2 (2/0)
ACI (vpravo/vlevo)	2 (0/2)
AB	2
AV (vpravo/vlevo)	1 (1/0)

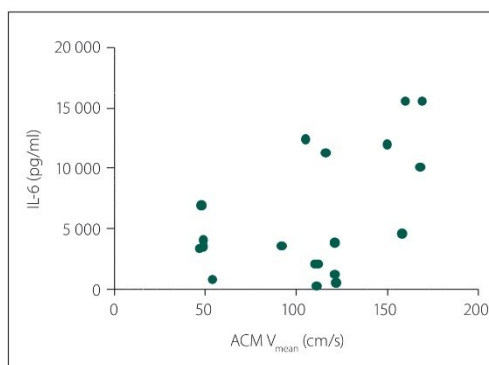
ACoA – arteria communicans anterior, ACM – arteria cerebri media, ACoP – arteria communicans posterior, ACI – arteria carotis interna, AB – arteria basilaris, AV – arteria vertebralis.



Graf 2. Hladiny IL-6 podle přítomnosti vazospasmů.



Graf 3. Korelace likvorových hladin IL-6 s rychlostí toků na arteria cerebri anterior (ACA).



Graf 4. Korelace likvorových hladin IL-6 rychlostí toků na arteria cerebri media (ACM).

zornují korelaci mezi likvorovými hladinami IL-6 a rychlostmi toků na ACA či ACM. U zkoumaného souboru nebyla prokázána korelace mezi hladinami IL-6 v likvoru a rychlostmi toků na ACA ($r_s = 0,13$, $p = 0,2867$, $n = 20$; graf 3) ani ACM ($r_s = 0,327$, $p = 0,082$, $n = 20$; graf 4).

Vztah mezi likvorovými hladinami IL-6 a PbtO₂

Graf 5 ukazuje srovnání likvorových hladin IL-6 u pacientů rozdělených dle hodnot PbtO₂. U pacientů s hodnotami PbtO₂ vyššími než 20 mmHg byl patrný výrazný trend k nižším hladinám IL-6 v likvoru v porovnání s pacienty s hodnotami PbtO₂ nižšími než 20 mmHg (med: 2 860; 25 %/75 % perc.: 940/8 580 vs. med: 6 937; 25 %/75 % perc.: 3 811/14 524; $p = 0,0587$). Graf 6 znázorňuje korelaci mezi likvorovými hladinami IL-6 a PbtO₂. U zkoumaného souboru byla signifikantní korelace mezi hodnotami

PbtO₂ a hladinami IL-6 v likvoru ($r_s = -0,626$, $p = 0,002$, $n = 20$).

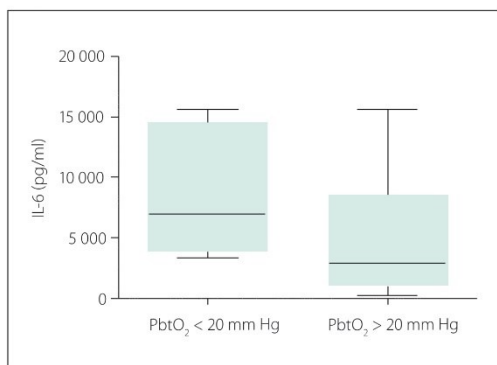
Diskuze

Cílem práce bylo vyhodnotit vztah likvorových hladin IL-6 k rozvoji DCI a vazospasmů mozkových tepen u pacientů po SAH. Zánět hraje významnou roli v rozvoji DCI na podkladě časného poškození mozku i vazospasmů mozkových tepen [7,9,10], a lze tedy předpokládat, že aktivita zánětlivé reakce bude mít vliv na stav mozkové tkáně samotné i cévního řečiště (ať již mikro- nebo makrovasikulárního), čemuž bude odpovídat rozvoj DCI či vazospasmů mozkových tepen s určitým časovým odstupem. Na druhé straně jsou poškození mozkové tkáně i ischemie silnými prozánětlivými podněty, takže zánět, ischemie a tkáňové poškození tvoří bludný kruh.

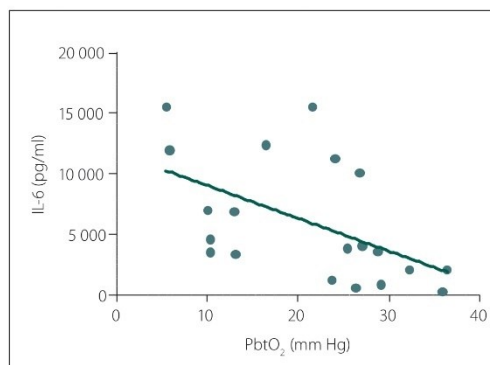
Hodnocení bylo načasováno do počátku období největšího rizika vzniku vazospasmů mozkových tepen s ohledem na dříve po-

psanou dynamiku likvorového IL-6 [15]. Odběry pro stanovení IL-6 byly provedeny čtvrtý den po SAH (tři odběry po 8 hod) a reprezentativní hodnota byla vypočítána jako průměr ze tří odběrů. Likvorové hladiny IL-6 byly vyhodnoceny ve vztahu k parametrum nejblíže TCD vyšetření po skončení odběrů, které korespondovalo s pátým dnem po SAH. Parametry TCD odpovídaly jednorázovému vyšetření, a to může představovat určitý problém, neboť jednorázové vyšetření nemusí vždy poskytnout reprezentativní hodnotu. V klinické praxi se však v rámci pooperačního monitoringu jen zřídka provádí TCD vyšetření častěji než jedenkrát denně a i v rámci klinického výzkumu je běžný monitoring po 24 hod [16]. Kontinuální TCD monitoring není zatím standardně používán a díky potenciálnímu nárůstu teploty tkáně, ke kterému může kontinuální monitoring vést se i zde doporučuje intermitentní použití [17].

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Graf 5. Likvorové hladiny IL-6 podle parciálního tlaku kyslíku v mozku (PbtO₂).



Graf 6. Korelace likvorových hladin IL-6 s hodnotami parciálního tlaku kyslíku v mozku (PbtO₂).

Vztah mezi likvorovými hladinami IL-6 a následnou přítomností vazospasmů byl u zkoumaného souboru podstatně méně patrný než v případě PbtO₂. Z absolutního hlediska byly hodnoceny likvorové hladiny IL-6 ve skupinách s vazospazmy a bez vazospasmů bez ohledu na postižené cévy. Přestože byl ve skupině pacientů s vazospazmy trend k vyšším hladinám IL-6 než u pacientů bez vazospasmů, není tento trend tak jasně vyjádřen jako v případě PbtO₂. Korelační analýza byla provedena pro každou cévu zvlášť a nebyl prokázán vztah mezi likvorovými hladinami IL-6 čtvrtý den po SAH a rychlostmi toků na ACA či ACM následující den, i když v případě ACM může být náznak trendu slabé korelace patrný. Dosud publikované údaje nejsou v otázce likvorových hladin IL-6 jednotné. Některé zdroje souvislost mezi zvýšením likvorových hladin IL-6 časně po SAH popisují, jiné však poukazují na to, že souvislost mezi likvorovými hladinami IL-6 a výskytem vazospasmů je prokazatelná až v pozdějším období [13,18,19].

PbtO₂ monitoring je invazivní metoda sloužící ke kontinuálnímu měření parciálního tlaku kyslíku v tkáni (mozku). Systém Licox, který používáme na naší klinice, funguje na polarografickém principu a v neurointenzivní péči nalézá poměrně široké uplatnění [20,21]. Přestože není definitivně uzavřena otázka fyziologických hodnot PbtO₂, předchozí studie prokázaly, že dlouhodobý pokles PbtO₂ pod 20 mmHg je u pacientů po SAH spojen s horším výsledným stavem a déletrvající pokles PbtO₂ pod 20 mmHg lze považovat za rizikový faktor vzniku DCI [22]. U námi zkoumaného souboru byla jasná tendence k vyšším hladinám likvorového

IL-6 u pacientů, u kterých bylo následně PbtO₂ nižší než 20 mmHg. Za významnější výsledek však považujeme, že byla prokázána korelace mezi IL-6 a hodnotami PbtO₂, což naznačuje souvislost mezi tíží zánětlivé reakce a úrovní oxygenace mozkové tkáně. Vztah mezi likvorovými hladinami a IL-6 a pozdějším rozvojem DCI byl mimo jiné popsán ve studii, ze které také vyplývá, že období mezi čtvrtým a pátým dnem po SAH může být velmi vhodné ke studiu dynamiky likvorového IL-6 v souvislosti s pozdějším rozvojem DCI [15]. Zmiňovaná studie je zajímavá i z toho důvodu, že našla významné rozdíly mezi likvorem a mozkovou extracelulární tekutinou (Extracellular Fluid; ECF) v dynamice i koncentracích IL-6 u pacientů po SAH. Likvorové hladiny IL-6 byly vyšší, avšak hladiny v likvoru i v ECF měly vysokou výpovědní hodnotu stran rozvoje DCI. Rozdíly hladin IL-6 mezi likvorem a mozkovou extracelulární tekutinou autoři připisují dalšímu zdroji, který produkuje IL-6 v likvoru. Jiné studie, které zkoumají vztah IL-6 a DCI po SAH, se většinou zabývají hodnocením hladin IL-6 v mozkové extracelulární tekutině a výsledky jsou stran IL-6 převážně pozitivní, ne však jednoznačné [23–25].

Parametry PbtO₂ monitoringu odpovídaly pátému dni po SAH, což bylo z hlediska načasování konzistentní s hodnocením TCD. Vztah hladin IL-6 k parametřům oxygenace mozkové tkáně i parametřům TCD byl hodnocen výlučně v daném časovém období za účelem zhodnocení možností predikce aktuálního vývoje pomocí parametrů zánětu. Z toho důvodu nebyly hodnoceny ani parametry výsledného stavu pacientů (GOS), ani konečného rozsahu DCI, k čemuž

by bylo vhodnější použít kontrolní CT nebo MR [26].

Přestože časové souvislosti naznačují spojitost zánětu s poruchami oxygenace mozkové tkáně, jsme si plně vědomi, že na základě našich dat se nemůžeme vyjádřit k otázce, zda se jedná o spojitost přímou či nepřímou. Také si uvědomujeme, že jsme zkoumali pouze časovou souvislost zánětporucha oxygenace, ale vzhledem k patofyziologii EBI může zároveň existovat i opačný vliv, neboť – jak již bylo uvedeno výše – z patofyziologického hlediska se jedná o bludný kruh. Uvědomujeme si také, že zkoumaný soubor byl poměrně malý a k učinění přesnějších a reprezentativnějších závěrů by bylo potřeba zařadit více pacientů.

Skutečnost, že likvorové hladiny IL-6 odpovídají spíše hodnotám PbtO₂ než vazospasmům, je kompatibilní s teorií EBI. Oxygenace mozkové tkáně bude zcela jistě ovlivněna vazospazmy mozkových tepen v případě, že jsou hemodynamicky významné. Vedle vazospasmů je však třeba brát v úvahu i další faktory, které mohou zásadně ovlivnit oxygenaci mozku – např. tkáňové poškození na podkladě mikroembolizace nebo právě zánětu. Všechny tyto patologie jsou zahrnuty pod EBI, které je definováno jako komplex navzájem provázaných patologií, jež časově předcházejí vzniku vazospasmů a mohou být jejich příčinou a zároveň mohou vést k DCI nezávisle na vzniku vazospasmů.

Závěr

U pacientů po SAH byla v období reprezentujícím začátek periody největšího rizika vzniku DCI prokázána korelace mezi likvorovými hladinami IL-6 a hodnotami PbtO₂, za-

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tímco parametry TCD takovou výpovědní hodnotu neměly. Na základě těchto výsledků se potvrdilo, že IL-6 může představovat slibný marker, který by mohl být použitelný k predikci rizika vzniku DCI.

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3.2.1.3 Early Dynamics of Interleukin-6 in Cerebrospinal Fluid after Aneurysmal Subarachnoid Hemorrhage

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In this study, we aimed to characterize the early dynamics of inflammatory response after SAH with regard to basic clinical characteristics such as HH or GOS. To characterize inflammatory response, we chose widely used markers TNF α , IL-1 β , and IL-6. Blood and CSF samples were collected every 8 hours for four days.

In total 47 patients with all grades of SAH were enrolled in the study. Compared to blood, CSF was shown to be a better compartment and IL-6 was a better marker than TNF α and IL-1 β for evaluation of early inflammatory response in the brain. Levels of IL-6 were massively increased early after SAH, and the additional increase was visible at 72 hours and later. There was a significant association between IL-6 and GOS in both overall levels (IL-6 was higher in patients with bad outcomes) and dynamics after 72 hours (IL-6 rose more rapidly in patients with bad outcomes after 72 hours).

These findings indicate that a massive inflammatory response is triggered immediately after SAH and that IL-6 is a good candidate as an early marker of inflammation after SAH. However, it was not possible to define clinically relevant threshold levels due to the high variability of IL-6.

Early Dynamics of Interleukin-6 in Cerebrospinal Fluid after Aneurysmal Subarachnoid Hemorrhage

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Abstract

Background Subarachnoid hemorrhage (SAH) is a severe condition associated with high mortality. Early brain injury (EBI) plays an important role in the pathophysiology of SAH, and inflammation is a major contributor to EBI. Inflammation is a widely studied topic in both experimental and clinical conditions; however, just a few clinical studies have focused primarily on the early inflammatory response after SAH, and detailed information about the association between the dynamics of early inflammatory response with main clinical characteristics is lacking. This study analyzes the early dynamics of inflammatory response after SAH and evaluates the possible associations between the markers of early inflammatory response and main clinical characteristics. **Patients and Methods** A total of 47 patients with a diagnosis of aneurysmal SAH within the last 24 hours were enrolled in the study. All treatments, including treatment of aneurysm (surgery/coiling) and implantation of a drainage system (external ventricular drainage/lumbar catheter), were conducted in the same way as in other patients with this diagnosis. Blood and cerebrospinal fluid (CSF) samples were collected three times a day for 4 days. The dynamics of proinflammatory cytokines were assessed, and associations between levels of the proinflammatory cytokines interleukin (IL)-6, IL-1 β , or tumor necrosis factor (TNF) α and main clinical characteristics were evaluated using linear mixed-effect models.

Results The CSF levels of IL-6 were massively increased initially after SAH (up to 72 hours) with an additional increase in later phases (after 72 hours), but there was high variability in IL-6 levels. A significant association was noted between the Glasgow Outcome Scale score and both overall levels of IL-6 ($p = 0.0095$) and their dynamics ($p = 0.0208$); the effect of the Hunt and Hess scale was borderline ($p = 0.0887$). No association was found between IL-6 levels and Fisher grade, modality of treatment (surgery, coiling, no treatment), and later development of cerebral vasospasm.

Keywords

- ▶ subarachnoid hemorrhage
- ▶ early brain injury
- ▶ inflammation
- ▶ IL-6

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Plasmatic levels of IL-6 increased slightly, but no significant association was found. The levels of IL-1 β and TNF α were within the physiologic range in both CSF and plasma.

Conclusions Early dynamics of IL-6 in CSF are associated with a patient's outcome. But it is difficult to use IL-6 alone for outcome prediction due to its high variability. The question is whether the dynamics of IL-6 could be used in combination with other early markers associated with brain injury. More detailed research is required to answer this question.

Introduction

Subarachnoid hemorrhage (SAH), with an annual incidence of ~ 10 per 100,000 and a case fatality rate of $\sim 50\%$, is a severe condition mainly affecting patients of reproductive age.¹ The most serious complication of SAH is a rebleed, associated with high mortality.² Delayed cerebral ischemia (DCI), which usually occurs between day 3 and 14 after the onset of bleeding, is another severe complication affecting $\sim 30\%$ of patients.^{3,4} Cerebral vasospasm was traditionally considered the cause of DCI; however, it is not always associated with DCI, and DCI may occur independently of cerebral vasospasm development.^{5,6} Early brain injury (EBI) represents pathologies that arise before vasospasm development.^{7,8} EBI has been a subject of intensive research in recent years because it may be associated with both DCI and cerebral vasospasm.^{6,9}

Inflammation plays an important role in EBI, and inflammatory response after SAH is a widely studied topic in both experimental and clinical conditions.^{10–12} Interleukin (IL)-6 is a commonly studied marker of the inflammatory response; however, only a few clinical studies have focused on early changes of IL-6 levels following SAH. IL-6 was described as massively increased in cerebrospinal fluid (CSF) early after onset of bleeding, and early CSF levels of IL-6 were associated with clinical status at admission (Hunt and Hess [HH] scale), cerebral vasospasm, as well as with DCI development and the final outcome of patients with SAH.^{13–15} Plasmatic levels of IL-6 were described as physiologic or slightly elevated early after SAH, but the association between early plasmatic levels and DCI or outcome was also described.^{16–18}

Previous studies focused on the association of the early levels of IL-6 with particular clinical characteristics, such as cerebral vasospasm development; however, no detailed study has described the association of the early dynamics of IL-6 with main clinical characteristics in one cohort of patients. Additionally, some important information is still missing, for example, how the dynamics of IL-6 are affected by the surgical treatment of a cerebral aneurysm.

The goal of this study was to analyze the early dynamics of inflammatory response after SAH and evaluate possible associations between the markers of early inflammatory response and main clinical characteristics.

Patients and Methods

All the procedures were approved by the ethics committee at the University Hospital Brno. Patients with a diagnosis of nontrau-

matic SAH hospitalized between 2013 and 2015 in the Department of Neurosurgery at the University Hospital Brno were asked to participate in this study. In cases of an unconscious patient, relatives were asked on behalf of the patient. Participation in the study was voluntary; it was confirmed by the signature of informed consent by the patient or relatives of the unconscious patient. The criterion for inclusion in the study was an aneurysmal SAH within the last 24 hours. SAH was diagnosed using computed tomography (CT), and aneurysm was confirmed by three-dimensional CT angiography. All the treatment, including treatment of the aneurysm (surgery/coiling) and implantation of a drainage system (external ventricular drainage/lumbar catheter), was conducted in the same way as in other patients with this diagnosis.¹⁹ The general goal was the early treatment of cerebral aneurysm (within 48 hours after onset of bleeding). Modality of treatment (surgery, coiling, conservative) was selected individually according to common standards with regard to clinical status and overall condition of the patient, shape and location of the aneurysm, or the presence of an intracerebral hematoma.¹⁹ Postoperative/coiling treatment, including treatment of vasospasm, was conducted according to current guidelines.¹⁹ Conservative no treatment was indicated mainly in patients in a poor overall condition or neurologic condition, as is customary.²⁰

Blood samples and samples of CSF were collected every 8 hours for 4 days after implantation of an external ventricular drainage/lumbar catheter. The drainage system was implanted in particular cases between hours 8 and 16 after the onset of bleeding. Transcranial Doppler (TCD) was performed daily for 2 weeks to detect the development of cerebral vasospasm. Blood flow velocities higher than 120 cm/s in medial cerebral artery or 100 cm/s in anterior cerebral artery were considered a sign of cerebral vasospasm. Samples of blood and CSF were processed at the Department of Clinical Biochemistry at University Hospital Brno using standard laboratory procedures. The levels of tumor necrosis factor (TNF) α , IL-1 β , and IL-6 were analyzed using commercial enzyme-linked immunosorbent assays (R&D Systems, Minneapolis, Minnesota, United States).

Statistical Analysis

The initial analysis of data revealed the massive increase of IL-6 in CSF; all the other markers did not exceed physiologic levels. Further analysis thus focused on the evaluation of IL-6 in CSF.

IL-6 data were transformed logarithmically to achieve an approximately normal distribution that was checked graphically. Linear mixed-effects hierarchical models were used to evaluate IL-6 dynamics in CSF. In these models, repeated

measures were accounted for through random effects modeled for each subject. The mixed-effects hierarchical approach for our models can be described briefly as follows. Two-level linear mixed models were created. At level 1 of the model, linear change of log(IL-6) over time was modeled for each subject. Linear splines with nodes every 24 hours were used to capture possible nonlinearity in the rate of change over the evaluated period of time. However, only a node at 72 hours was found significant and retained in the final model. Further, at level 2, parameters from level 1 were explained using clinical parameters of interest modeled as fixed. Both intercept and slopes were allowed to vary randomly. This is the general composite formula of our models fitted:

$$\begin{aligned} \text{Log(IL6)}_{ij} = & \beta_{00} + \beta_{10}\text{TIME}_{ij} + \beta_{20}(\text{TIME}_{ij} - 72) + \beta_{01}\text{PARAM}_i \\ & + \beta_{11}(\text{TIME}_{ij} * \text{PARAM}_i) + \beta_{21}((\text{TIME}_{ij} - 72) * \text{PARAM}_i) \\ & + [b_{0i} + b_{1i}\text{TIME}_{ij} + \varepsilon_{ij}], \end{aligned}$$

where the last term in brackets represents random components and are assumed to have these distributions: $\varepsilon \sim N(0, \sigma^2 I)$ and $b \sim N(0, \sigma^2 D(\theta))$.

Model Building

At first, all parameters based on the specifications just described were included in the model. However, because parameter $\hat{\alpha}_{11}$ was not significant in either model, it was not kept in any model, and only the effect of a parameter on the rate of change from time 72 (β_{21} parameter) was modeled. Further, a final model was created leaving out nonsignificant interaction terms. If the main (β_{01} parameter) was found insignificant when building a model, it was still retained in the model to demonstrate its negligible effect. To facilitate interpretation, final models were shown graphically. The mean modeled levels of IL-6 with respect to analyzed groups were given in the figures where a modeled mean trend for a specific group is shown on the background of case-profile plots of all data.

The significance of parameter estimates are given contextually with interpretation in the Results section. In further text, when describing an "overall" effect of a parameter, we refer to the β_{01} parameter and effect; when describing "dynamics," we refer to the β_{21} parameter of the model. Statistical analyses were performed using SAS v.9.3 (SAS Institute, Cary, North Carolina, United States).

Sample Size Rationale

Sample size estimation or power analysis for even simple multilevel mixed models is not a common or straightforward task because many statistical and mainly unknown parameters need to be known in advance. In more complex designs such as a power analysis, it is even less feasible. Discussions on a sufficient number of subjects or the accuracy of estimated parameters are mainly based on simulation studies and reviews from experience. Based on these studies and in accordance with the commonly cited rule of thumb, it implies that 20 units for each level are necessary for sufficient accuracy of effect estimates for a simple multilevel mixed model that is similar to what we used. Further, we utilized R software with the

ImmPower procedure (longpower package; www.r-project.org) to estimate sample size using our data. Based on these analyses, we can demonstrate that with 20 to 30 subjects in each group we are able to find ~25% change in rate of change (dynamics) of evaluated parameter under usual statistical error conditions (α 5%, power 80%).

Thus, assuming some imbalances, models with one factor (variable with two to three categories) analyzed at a time (a univariate model) should demonstrate sufficient accuracy with a total of 40 to 50 subjects.

Results

The Study Group and Early Inflammatory Response after SAH

A total of 47 patients were enrolled in this study. The mean age of the study group was 53.9 years. **Fig. 1** lists the main characteristics of the study group. The TCD examination could not be performed in six cases due to the inadequate temporal window. The final outcome (Glasgow Outcome Scale [GOS] score after 3 months) remained unknown in six other cases because of loss to follow-up. The levels of proinflammatory cytokines TNF α , IL-1 β , and IL-6 were evaluated in plasma and CSF. The levels of TNF α and IL-1 β were within physiologic levels, both in plasma and CSF, whereas the levels of IL-6 in plasma were slightly increased above the physiologic level. If physiologic levels of IL-6 reach 12.5 pg/mL, the mean levels (geometric mean) of IL-6 in our study group was 32.7 pg/mL (95% confidence interval [CI], 28.03–37.51). No association between plasmatic levels of IL-6 and main clinical characteristics was found. There was a massive increase in the levels of IL-6 in CSF in our study group, described in the next section.

Levels of IL-6 in Cerebrospinal Fluid Early after SAH

Overall CSF levels of IL-6 in our study group were massively increased, reaching the mean value (geometric mean) of 2,698 pg/mL (95% CI, 2,388–3,048). There was a high variability in CSF levels of IL-6 in our study group (25% percentile: 1,240; median: 2,936; 75%: 7,110). Based on the time model, the dynamics of IL-6 significantly changed at the 72-hour time point (**Fig. 2**). Thus for further analyses two splines with a knot at 72 hours were considered in the model, and various factors were included in the model just described. The association between these characteristics and the levels of IL-6 were found to be insignificant: Fisher grade (**Fig. 3**), aneurysm treatment option (**Fig. 4**), and cerebral vasospasm development that occurred later (**Fig. 5**). The association between HH grade and the dynamic of IL-6 was found to be borderline ($p = 0.0887$; **Fig. 6**). IL-6 levels were similar in all HH grades; however, the increase beyond hour 72 could hardly be discerned in grade HH 1–2. Finally, there was a significant association between GOS and both overall levels of IL-6 ($p = 0.0095$) and their dynamic > 72 hours after SAH ($p = 0.0208$; **Fig. 7**). The lowest IL-6 levels were found in the GOS 5 group with an increase beyond 72 hours. In contrast, higher IL-6 levels were found in the GOS 1 to 2 and 3 to 4 groups at baseline. Moreover, further increase in IL-6 levels could be noticed beyond hour 72 in the GOS 1 to 2 group.

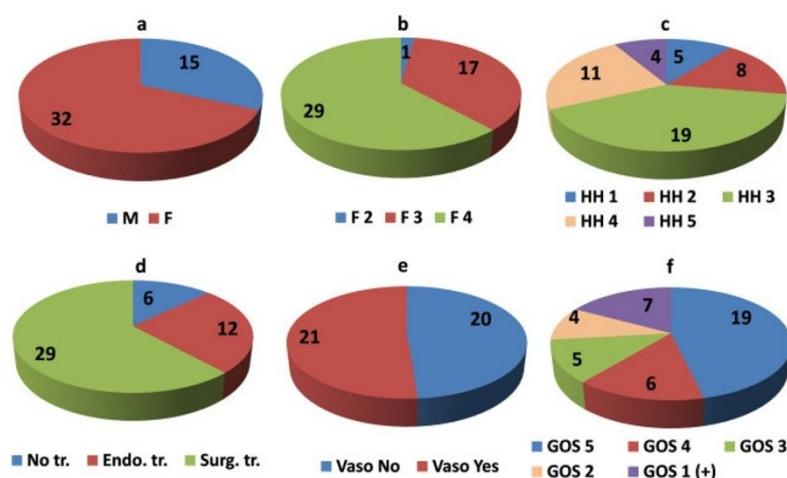


Fig. 1 Characteristics of study group, according to (a) gender, (b) Fisher grade, (c) Hunt and Hess grade, (d) modality of treatment, (e) vasospasm development, and (f) Glasgow Outcome Scale (GOS) score. Endo. Tr, endovascular treatment; F, female; F, Fisher grade; GOS 1 (+), death; HH, Hunt and Hess grade; M, male; No. tr., without treatment; Surg. tr, surgical treatment; Vaso No, without vasospasm; Vaso Yes, with vasospasm.

Discussion

Inflammation plays an important role in the pathophysiology of SAH. Based on the results of experimental research, two phases of inflammatory response following SAH can be distinguished. The first step is activation of the local inflammatory response that occurs early after SAH and is mediated by microglia and endothelial cells.^{21,22} Activation of microglia and endothelial cells is associated with the production of proinflammatory cytokines and adhesion molecules.^{21,22} This leads to the activation of leukocytes in the periphery,

which subsequently infiltrate affected areas and promote the inflammatory response.²³ The immune reaction mediated by infiltrating leukocytes may be considered the second step of the inflammatory response. The production of proinflammatory cytokines in the brain following experimental SAH was also described as biphasic.²²

Most clinical studies have not focused primarily on the early inflammatory response after SAH, and, to our knowledge, no study has described the association of the early dynamics of the

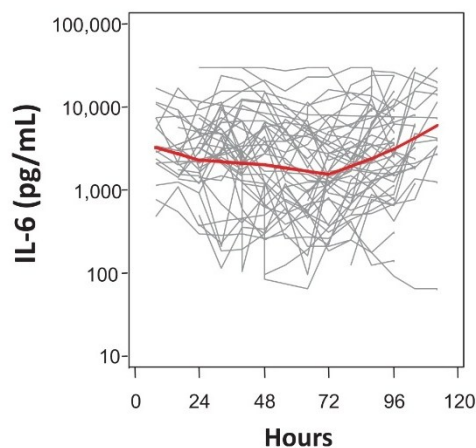


Fig. 2 Overall levels of interleukin (IL)-6 in cerebrospinal fluid ($n = 47$). Individual gray curves in the background represent individual levels of IL-6 second, and mean color curves in the front represent mean model values. Time 0 corresponds to the onset of symptoms.

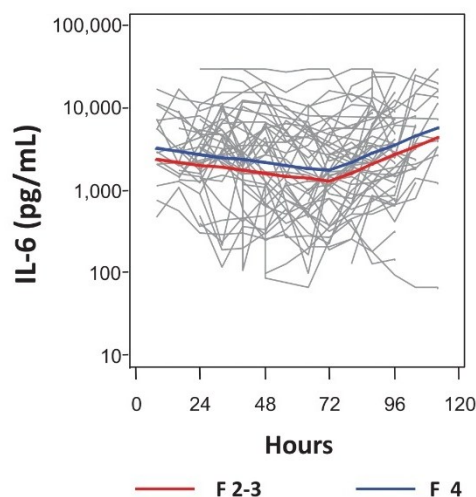


Fig. 3 Association of Fisher (F) grade with cerebrospinal fluid levels of IL-6 ($n = 47$). Individual gray curves in the background represent individual levels of IL-6, and mean color curves in the front represent mean model values for a specific group. Time 0 corresponds to the onset of symptoms.

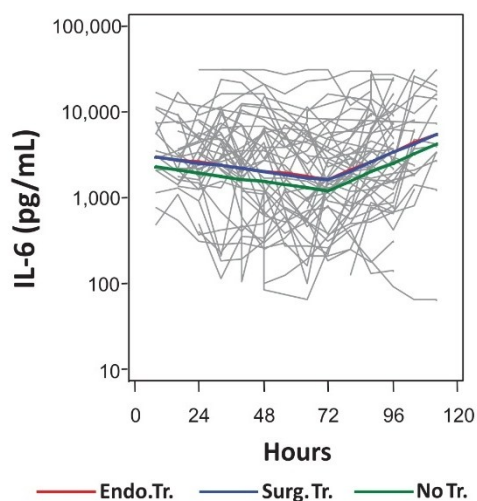


Fig. 4 Association of treatment option with cerebrospinal fluid levels of interleukin (IL)-6 ($n = 47$). Individual gray curves in the background represent individual levels of IL-6, and mean color curves in the front represent mean model values for a specific group. Time 0 corresponds to the onset of symptoms. Endo. tr., endovascular treatment; No tr., without treatment; Surg. tr., surgical treatment.

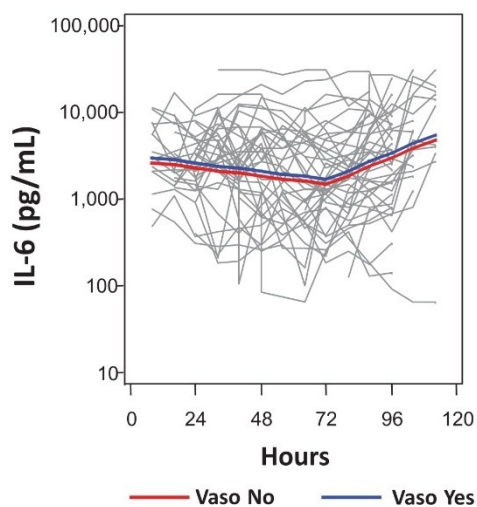


Fig. 5 Association of interleukin (IL)-6 in cerebrospinal fluid with later development of cerebral vasospasm ($n = 41$). Individual gray curves in the background represent individual levels of IL-6, and mean color curves in the front represent mean model values for a specific group. Time 0 corresponds to the onset of symptoms. Vaso No, without vasospasm; Vaso Yes, with vasospasm.

inflammatory response with the main clinical characteristics in one cohort of patients. The goal of this study was to evaluate the dynamics of early inflammatory response after SAH and to

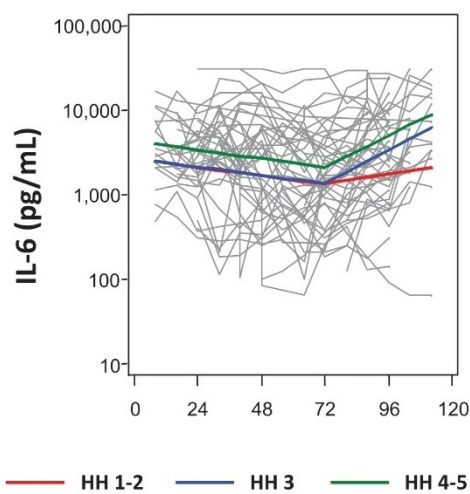


Fig. 6 Association of Hunt and Hess (HH) grade with cerebrospinal fluid levels of interleukin (IL)-6 ($n = 47$). Individual gray curves in the background represent individual levels of IL-6, and mean color curves in the front represent mean model values for a specific group. Time 0 corresponds to the onset of symptoms.

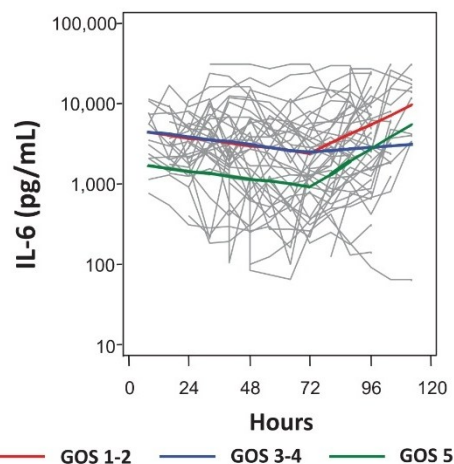


Fig. 7 Association of Glasgow Outcome Scale (GOS) score with cerebrospinal fluid (CSF) levels of interleukin (IL)-6 ($n = 41$). Individual gray curves in the background represent individual levels of IL-6, and mean color curves in the front represent mean model values for a specific group. Time 0 corresponds to the onset of symptoms.

describe the relationships between early inflammatory response after SAH and main clinical characteristics. We chose the most widely used proinflammatory cytokines (IL-1 β , TNF α , and IL-6) as the markers of inflammation. The levels of these cytokines were evaluated in plasma and CSF. The drainage system was implanted in particular cases between hours 8 and 16 after the onset of bleeding, so all the collected samples cover

the interval between 8 hours and 112 hours after SAH where only the interval between 24 and 96 hours represents all the patients.

Plasmatic levels of none of the evaluated markers were substantially increased in our study group. This is consistent with previously published data that mostly describe the plasmatic levels of proinflammatory markers within physiologic range or increased in later stages, but not early after SAH.^{16,24} Levels of TNF α and IL-1 β were within physiologic range in CSF, whereas the levels of IL-6 were massively increased in our study group. Increased CSF levels of proinflammatory markers early after SAH were also described previously as well as the fact that levels of IL-6 early after SAH are higher than levels of TNF α and IL-1 β .^{13,16} All these results suggest that the early activation of the immune system after SAH may be limited to the brain tissue.

The levels of IL-6 in CSF were massively increased initially after SAH (up to 72 hours) with an additional increase in later phases (after 72 hours). These dynamics of IL-6 may be associated with infiltration of leukocytes from the periphery. There was a high variability in the IL-6 levels in our study group, suggesting that early inflammatory response may be considered a highly individual reaction, which in addition to SAH is considerably influenced by other factors such as age or genetic background. The high variability of IL-6 was also noticeable in previously published studies that describe CSF levels varying from hundreds to thousands of picograms per milliliter early after SAH.^{13,25} Mixed models are the ideal tool for finding small systemic changes in dynamics hidden in otherwise very variable data where many unknown and thus unaccountable factors may play a role.

The dynamics of IL-6 were not associated with vasospasm development in our model. Because all the samples were collected within 112 hours after SAH, and the TCD was performed for 14 days, the objective of this analysis was to evaluate whether the early dynamics of IL-6 may predict later development of cerebral vasospasm. Increased levels of IL-6 early after SAH were described as associated with both cerebral vasospasm development and DCI.^{14,26} There are studies, however, that described the peak of association between levels of IL-6 in CSF and cerebral vasospasm in later phases after SAH.^{27,28} The associations of Fisher grade or modality of treatment with the levels of IL-6 were not found to be significant in our model. We consider the latter an interesting finding because surgical stress represents a strong proinflammatory stimulus.²⁹ Based on the fact that the surgery did not cause any additional increase in the production of IL-6, we may speculate that activation of the immune system due to SAH may be very strong, and even a stress such as surgery may not affect it.

The associations of the HH grade or GOS with the dynamics of IL-6 were found to be borderline or significant. While the possible association of IL-6 with HH is visible later than 72 hours after SAH, the association with GOS was noticeable even before this time point. According to the GOS, we divided patients into these groups: GOS 1 + 2 (death and persistent vegetative state), GOS 3 + 4 (severe and moderate disability), and GOS 5 (low/no disability). This is not the traditional way to

evaluate the outcome, but from a pathophysiologic point of view, group GOS 1 + 2 represents patients with "fatal brain damage," group GOS 3 + 4 represents patients with "nonfatal brain damage," and GOS 5 represents patients with "minimal brain damage." The model suggests that the early levels of IL-6 are lower in patients with "minimal brain damage," whereas the levels of IL-6 are higher from the very beginning of the evaluated period in patients with "nonfatal" or "fatal brain injury." Moreover, further increases of IL-6 are noticeable beyond hour 72 in patients with "fatal brain injury," reflecting the most severe condition and inflammation in this group. The different dynamics of IL-6 before and after 72 hours following SAH may refer to a development of early brain injury (EBI).

Increase in CSF levels of IL-6 was previously described both in experimental and clinical conditions.^{11,15,26–28} Most studies focused on the relations between IL-6 and cerebral vasospasm or DCI development. These studies were mainly interested in the time period representing the highest risk of cerebral vasospasm or DCI development, from day 4 onward.^{15,27,28} The details on association between early levels of IL-6 in CSF and GOS is still missing, despite the fact that the levels of IL-6 in CSF early after SAH were previously described.^{15,28} The model presented here of the dynamics of IL-6 demonstrates that the early dynamics of IL-6 are associated with GOS. It is, however, hard to determine threshold levels due to the high variability of data.

This study had several limitations. First, the number of included patients is limited. Although our sample should be sufficient to demonstrate accurate estimates of one factor in a model (univariate models), it does not enable evaluation of more complicated multivariate models. Our conclusions are thus based on univariate analyses, and possible interactions or dependencies on several variables could not be captured. Second, the study had no control group. Standard cut-off values, as used by the Department of Clinical Biochemistry at the University Hospital Brno, were used in this study. Many factors influence the immune system, and thus it is hard to use other patients with CSF drainage as a control group. That is why we used reference values from our Department of Clinical Biochemistry.

Third, the samples were collected via external ventricular drainage or lumbar catheter without differentiation. The external ventricular drainage is indicated in patients with severe SAH as a standard part of neurointensive care, whereas the lumbar catheter is used in patients with mild SAH. We followed this routine because it is not usual to differentiate the CSF samples for the purpose of protein analysis according to route of collection. But we are aware that there may be some differences in protein levels depending on the route of collection. Fourth, IL-6 CSF levels were not adjusted for potential meningitis and intraventricular blood because it is not standard procedure and we were interested in total levels of IL-6, but we are aware that this may influence the results. Fifth, we did not use clinical parameters to evaluate DCI because in case of these parameters, the dynamics should be considered an important factor (e.g., the dynamics of neurologic deterioration), and such an evaluation would be much more complicated and require a more complex model.

Conclusion

Early dynamics of IL-6 in CSF are associated with a patient's outcome. It is, however, hard to use IL-6 solely for outcome prediction due to its high variability. The question is whether the dynamics of IL-6 could be used in combination with other markers associated with EBI. More complex research is required to answer this question.

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3.2.1.4 Early Inflammatory Response in the Brain and Anesthesia Recovery Time Evaluation After Experimental Subarachnoid Hemorrhage

Duris K, Lipkova J, Splichal Z, Madaraszova T, Jurajda M. Early Inflammatory Response in the Brain and Anesthesia Recovery Time Evaluation After Experimental Subarachnoid Hemorrhage. *Transl. Stroke Res.* 2019;10:308–318.

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To better understand the nature of inflammatory response after SAH, we conducted this experimental study in which we studied inflammatory response in different points and various areas of the brain. The main goal of this study was to find out whether the SAH-associated inflammatory response in the brain occurs just in areas affected by the hematoma or in the whole brain.

We used the perforation model of SAH for the purpose of this study. For the final evaluation, we used 84 animals divided into three groups (sham, mild SAH, severe SAH) and four time points (2, 4, 6, and 8 hours). In the above-mentioned time points, we studied the dynamics of mRNA expressions of TNF α , IL-1 β , IL-6, ICAM-1, and VCAM-1 in areas adjacent to hematoma and distant areas. Serum levels of TNF α , IL-1 β , and IL-6 were evaluated at 4 and 8 h timepoints. In the case of severe SAH, the mRNA expressions of pro-inflammatory cytokines were significantly increased in both adjacent and distant areas, while the inflammatory response was less pronounced in mild/moderate SAH. The peak of the initial inflammatory response was at 4 hours after SAH. Expression of mRNA for adhesive molecules ICAM-1 followed the pattern of proinflammatory cytokines mRNA expression, while mRNA expression of VCAM-1 was not significantly different among the groups.

These findings indicate that severe subarachnoid hemorrhage triggers the strong and early inflammatory response not only in areas affected by bleeding (where a certain level of inflammatory response is required for cleaning/reparation), but the inflammatory response is also triggered in areas that are not primarily affected by hematoma. This may suggest either that SAH may cause global damage to the brain (for example by transient global cerebral ischemia) or that severe SAH may lead to deregulation of inflammatory response. Anyway, these results indicate that early inflammatory response after SAH is a more complex phenomenon than just a cleaning reaction.

In this research, we also evaluated the association of SAH severity with anesthesia recovery time. This may be useful for the prediction of severity of SAH immediately after surgery. SAH grade is evaluated after animal sacrificing and removal of the brain, it may be also predicted using neurological testing, which is primarily designed for 24 hours and later time points, however we do not have a tool for earlier prediction of SAH severity. We addressed this issue in the second aim of this research.

Anesthesia recovery time was evaluated for pain reaction recovery time (spinal level),

spontaneous ventilation recovery time (brain stem level), and consciousness recovery time (neocortical level). The anesthesia recovery time of all selected parameters reflected the severity of SAH. The consciousness recovery time test had the best predictive value, but the spontaneous ventilation recovery time test may bring the information in the shortest time.



Early Inflammatory Response in the Brain and Anesthesia Recovery Time Evaluation After Experimental Subarachnoid Hemorrhage

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Abstract

The main objective was to evaluate, whether the subarachnoid hemorrhage (SAH)-associated early inflammatory response has focal or global character, i.e., whether areas distant to hematoma may be affected by an early inflammatory response. The second objective was to evaluate the association of anesthesia recovery time for basic reflexes/neurological functions with severity of SAH. SAH was induced in rats using an endovascular perforation model. Anesthesia recovery time was evaluated for pain reaction recovery time (spinal level), spontaneous ventilation recovery time (brain stem level), and consciousness recovery time (neocortical level). mRNA expressions of TNF α , IL-1 β , IL-6, ICAM-1, and VCAM-1 in areas adjacent and distant to hematoma were evaluated between 2 and 8 h after SAH. Serum levels of TNF α , IL-1 β , and IL-6 were assessed at 4 and 8 h after SAH. Anesthesia recovery time of all selected parameters was associated with severity of SAH. The consciousness recovery time test had the best predictive value, while the spontaneous ventilation recovery time test was able to bring information in the shortest time. The mRNA expressions of pro-inflammatory cytokines were significantly increased in severe SAH groups in both adjacent and distant areas. The inflammatory response in mild/moderate SAH groups was less strong, peaking at 4 h after SAH. Serum levels of pro-inflammatory cytokines were ambiguous. Anesthesia recovery time may be useful for bleeding severity prediction in the SAH model; however, further validation is needed. Severe subarachnoid hemorrhage is associated with the strong early inflammatory response, which has a global character, while mild subarachnoid hemorrhage is accompanied by a weaker inflammation.

Keywords Subarachnoid hemorrhage · Inflammation · Early brain injury · Anesthesia recovery time

Introduction

Early brain injury (EBI) is a complex of pathophysiological processes, which are associated with subarachnoid hemorrhage (SAH), and which may significantly contribute to SAH-related morbidity and mortality [1, 2]. The most important contributors to early brain injury are transient global

cerebral ischemia, oxidative stress, neuronal/endothelial apoptosis, blood-brain barrier disruption, and inflammation [1, 3].

Inflammation plays a major role in EBI; inflammation may be considered as a cross-link among above-mentioned pathologies [4–8]. Activation of the immune system occurs immediately after onset of bleeding and inflammation after SAH may be considered as a two-step process: it is initially a local reaction which is later generalized to systemic response [9, 10]. Local response is represented by the activation of microglia and endothelial cells, which produce pro-inflammatory cytokines and adhesion molecules [11, 12]. Pro-inflammatory cytokines released into blood activate leukocytes in the periphery; adhesive molecules attract them to the affected area, resulting in leukocyte infiltration and subsequent promotion of inflammatory response [11, 13, 14].

Local response, an initial step of inflammatory response after SAH, is a minor topic of current research. Previous studies demonstrated that the local inflammatory response in the brain may be detected within hours after onset of bleeding,

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peaking between 4 and 6 h after SAH [11]. However, it is still not clear whether the local inflammatory response, which reflects activation of the immune system in the brain after SAH, has focal or global character, i.e., whether an early inflammatory response occurs only in areas affected by bleeding or whether it may occur also in distant areas. Information about the spatial context of early inflammatory response SAH may help us to understand whether the early brain injury has global impact on the brain from the very early beginning or whether it develops over time. The main goal of this work was to find out, whether the early inflammatory response after SAH occurs only in areas adjacent to hematoma or whether it may occur also in distant areas of the brain.

Considering experimental SAH, there is currently no test designed for evaluation of initial neurological status. Standard behavioral tests are not suitable for this purpose; these tests are substantially influenced by anesthesia, because the tests are partially based on spontaneous activity (psychological functions) [15, 16]. However, we may assume that basic reflexes/neurological functions, which are crucial for the organism, may be affected by anesthesia in a more consistent way and similar exposures to anesthesia may be associated with similar recovery time unless there is an influence of pathology. Evaluation of recovery time for basic reflexes/neurological functions could be potentially used for evaluation of consistency of the model and prediction of SAH severity. Potential candidates for such evaluation are pain reflex (spinal level), respiratory reflex (brain stem level), and consciousness regaining (cortical level). The second goal of this study was to evaluate whether recovery time for the above-mentioned reflexes/neurological functions may be associated with severity of SAH.

Material and Methods

Animal Experiments

All the experiments were approved by the Masaryk University Institutional Animal Care and by the Ministry of Education, Youth and Sports of the Czech Republic. Adult male Sprague Dawley rats (260–300 g) purchased from the Animal Facility of Masaryk University were used for the purpose of this study. The animals were assigned to sham or SAH group and to one of the following timepoint groups: 2, 4, 6, or 8 h. The animals were anesthetized using isoflurane in 60%/40% medical gas/oxygen mixture. Five percent concentration of isoflurane was used for induction of anesthesia and after the intubation, the anesthesia was maintained using 3% isoflurane concentration. SAH was induced by sharpened 4–0 nylon suture through the left internal carotid artery as described previously [17]. In the case of sham animals, the suture was inserted into the artery, but no perforation was performed. The anesthesia was terminated 5 min after

induction of SAH/suture insertion. At the end of surgery, 1 ml of saline was injected intraperitoneally to prevent dehydration.

After the surgery, animals were kept on mechanical ventilation until spontaneous ventilation was sufficient. If spontaneous ventilation did not recover, the animals were kept on mechanical ventilation during the whole period of monitoring and basic vital signs (heart action, color of eyes) were observed. When basic vital signs were not present in unconscious animals, these were excluded, generating mortality group. In animals which recovered from anesthesia, the following neurological parameters were evaluated: spontaneous ventilation recovery time, pain reaction recovery time, and consciousness recovery time. Respiratory reflex recovery was considered as a criterion for spontaneous ventilation recovery, no matter whether spontaneous ventilation was sufficient. A pain stimulus represented pinch to tail and any escape/defensive reaction was considered as a positive result of the pain reaction recovery test. The criterion for consciousness regaining was a spontaneous awake reaction or awake reaction in response to pain stimulus, no matter whether animal later fell asleep again. These parameters were evaluated every 5 min until full recovery or up to 1 and half hour after surgery. All the evaluations were performed in a blind manner by one person. A heating lamp was used during the whole period of unconsciousness to prevent hypothermia. Detailed protocol of anesthesia and anesthesia recovery time evaluation may be found as a [supplementary file](#).

Animals were sacrificed one of the following timepoints: 2, 4, 6, or 8 h, the picture of the basal part of the brain was taken to evaluate SAH grade, and the samples of the brain and serum were collected. Severity of SAH was evaluated using the SAH grading system [17]. According to the SAH grade, the animals were assigned to mild/moderate SAH group (SAH grade 6–12; below labeled as mild SAH) and severe SAH group (SAH grade 13–18), while animals with SAH grade lower than 6 were excluded for insufficient SAH, as it is usual [17].

Samples

Brain samples were collected from the basal part of the left hemisphere (the area adjacent to hematoma—directly affected by hematoma; below labeled as A samples) and from the right dorsal hemisphere (area distant from hematoma—not affected by hematoma; below labeled as D samples). The samples were homogenized using the glass bead beating technique, frozen in RNA later, and stored at -80°C until processed. Isolation of total RNA was performed with Tripure reagent (Qiagen) according to the manufacturer's instructions. RNA samples were suspended in RNase-free water and quantified spectrophotometrically. Genomic DNA contamination was removed by DNase I treatment (Invitrogen). Single-stranded cDNA was synthesized from 1 μg total RNA in a volume of 50 μl using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland)

(using random hexamers). Gene expression was evaluated by real-time reverse transcription polymerase chain reaction (RT-PCR) analysis using TaqMan chemistry (Applied Biosystems). TaqMan assays were run for TNF α , IL-1 β , IL-6, ICAM-1, VCAM-1, and two housekeeping genes— β -actin and hypoxanthine phosphoribosyltransferase (HPRT1) to determine which housekeeping genes were most suitable for subsequent normalization of data. Amplification reactions were performed on ABI PRISM 7000 Sequence Detection System (Applied Biosystems) with the following thermal cycling conditions: 95 °C for 10 min and then 40 cycles of 95 °C for 15 s and 60 °C for 1 min. An average was calculated for Ct of control after normalization with HPRT1. The calculation of fold change was according to the comparative Ct method ($2^{-\Delta\Delta Ct}$ method). Serum cytokines TNF α , IL-1 β , and IL-6 were measured in samples frozen immediately after sampling and then stored in -80 °C awaiting analysis. The samples were analyzed using commercial ELISA provided by Invitrogen (IL-6, 0–1500 pg/ml; TNF α , 0–750 pg/ml) and USCN Life Science (IL-1 β , 0–800 pg/ml). Intra-assay CV and inter-assay CV for all analysis were < 10%.

Statistical Analyses

The distribution of the data of SAH grade and neurological parameters met the criteria of normal distribution (D'Agostino and Pearson normality test). Ordinary one-way ANOVA followed by Bonferroni's multiple comparison test was used to evaluate these data. ROC analysis was subsequently performed in mild and severe SAH groups. The results of laboratory analyses were log-normally distributed; these data were logarithmically transformed, and the statistical analyses were performed on transformed data using ordinary one-way ANOVA followed by Bonferroni's multiple comparison test. A p value of < 0.05 was considered statistically significant. Statistical analyses were performed using GraphPad Prism version 7.03 for Windows (GraphPad Software, La Jolla, CA, USA, www.graphpad.com). Data are presented as mean and standard deviation (SAH grade and neurological parameters) or geometric mean and geometric SD factor (the rest of the data). The data representing mRNA expressions are presented as a fold change normalized to the absolute control group. In figures, $p < 0.05$ is labeled as *, $p < 0.01$ is labeled as **, and $p < 0.001$ is labeled as ***.

Results

Basic Characteristics of the Study Group

In total, 108 animals were used for purpose of this study, 104 surgeries were performed in this project and another four animals were used as an absolute control (no surgery) for mRNA standardization. From the total amount of operated animals, 16 animals were excluded due to mortality of the model (14.6%) and another 4 animals were excluded because of the insufficient SAH grade. The final laboratory analyses were performed on the group of 84 animals divided into 12 groups according to SAH grade (sham, mild SAH, severe SAH) and timepoint (2, 4, 6, and 8 h), $n = 7$ per group. The evaluation of the initial neurological status was performed in sham, mild, and severe SAH groups with no regard to timepoints used for laboratory analyses.

Three animals did not fully recover from anesthesia and were kept on mechanical ventilation for the whole period of monitoring (2-h timepoint, 1 animal; 4-h timepoint, 1 animal; and 8-h timepoint, 1 animal). Vital signs (color of the eye and heart action) were present in all animals for the whole period of monitoring and reaction to pain recovered in two cases. These animals were not used for evaluation of neurological parameters (infinite recovery time), but all the laboratory parameters were in the range typical for severe SAH groups and these samples were included in the evaluation of inflammatory response.

Basic characteristics of the study group, according to the SAH grade, are summarized in Table 1. In mild SAH groups, SAH grades are comparable in all the timepoints; the mean value of SAH grade is around 9. In severe SAH groups, the SAH grades are also similar in all the timepoints; the mean value of SAH grade is around 16 in these animals.

The Initial Neurological Status

The aim of this assessment was to evaluate, whether recovery time of basic neurological parameters is suitable for evaluation of initial neurological status of experimental animals. We evaluated spontaneous ventilation recovery time, reaction to pain recovery time, and consciousness recovery time. An important prerequisite for such an evaluation was similar exposure to anesthesia in all the groups.

Figure 1 and Table S1 (in supplementary file) demonstrate that the duration of anesthesia was comparable in all the three

Table 1 Basic characteristics of the study group according to SAH grade expressed as a mean (M) and standard deviation (SD); $n = 7$ per group

	Mild 2 h	Mild 4 h	Mild 6 h	Mild 8 h	Severe 2 h	Severe 4 h	Severe 6 h	Severe 8 h
M	9.29	8.57	9.00	8.71	16.43	16.13	15.57	16.14
SD	1.89	1.51	2.10	2.14	1.51	1.89	1.62	1.57

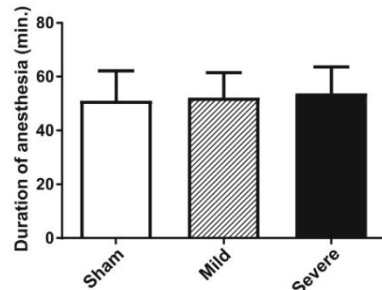


Fig. 1 Duration of anesthesia groups in sham, mild, and severe SAH groups. The data are plotted as a mean and SD; $n = 28$ per group

groups. A total time of anesthesia (including induction of anesthesia) was, in average, around 52 min.

Figure 2 and Table S2 (in supplementary file) show the recovery time of basic vital parameters (A, spontaneous ventilation recovery; B, pain reaction recovery; and C, consciousness recovery). The recovery time was significantly longer in the severe SAH group compared to that in both the sham and mild SAH groups in all three parameters. The highest contrast was observed in the case of consciousness recovery; an average time in the sham group was around 15 min, in the mild SAH group around 20 min while in the severe SAH group, regaining of consciousness took around 45 min on average (Fig. 2c, Tab. S2).

Table 2 summarizes the results of ROC analysis of mild and severe SAH groups for a spontaneous ventilation recovery time, pain reaction recovery time, and consciousness recovery time. In this case, sensitivity represents the ability of assessment to correctly detect absence of particular reflex in the severe SAH group, while specificity represents an ability of assessment to correctly detect presence of the reflex in the mild SAH group. The best predictive value has consciousness recovery time (AUC, 0.95; AUC SE, 0.026); the suitable time for this assessment is around 32nd minute after SAH induction: if the consciousness is recovered, there is a 92% chance that SAH will be mild and if the consciousness is not recovered, there is an 83% chance that SAH will be severe. Finally, if the consciousness will not recover within approximately 38 min after SAH induction, severe SAH may be expected with almost 100% chance and around 67% of severe SAH animals in our study group did not recover within this period. Spontaneous ventilation recovery time has lower predictive value (AUC, 0.87; AUC SE, 0.053) than previous test, but this test may bring us an information earlier. The suitable time for this assessment is around 12th minute after SAH induction: if an animal breathes, there is a 93% chance that SAH will be mild and if an animal does not breathe, there is a 76% chance that SAH is severe. If ventilation will not recover within approx. 18 min, the severe SAH may be expected with

almost 100% chance and around 56% of animals in our study group with severe SAH did not recover ventilation reflex within this period. Pain reaction recovery time does not bring us better information than previous tests, predictive value is lower (AUC, 0.83; AUC SE, 0.060), and the suitable time for this assessment is around an 18th minute after SAH induction.

Inflammatory Response in the Brain Following SAH

The inflammatory response in the brain was evaluated on the level of mRNA expressions of pro-inflammatory cytokines (TNF α , IL-1 β , IL-6) and adhesive molecules (ICAM-1, VCAM-1). Expressions of the above-mentioned parameters

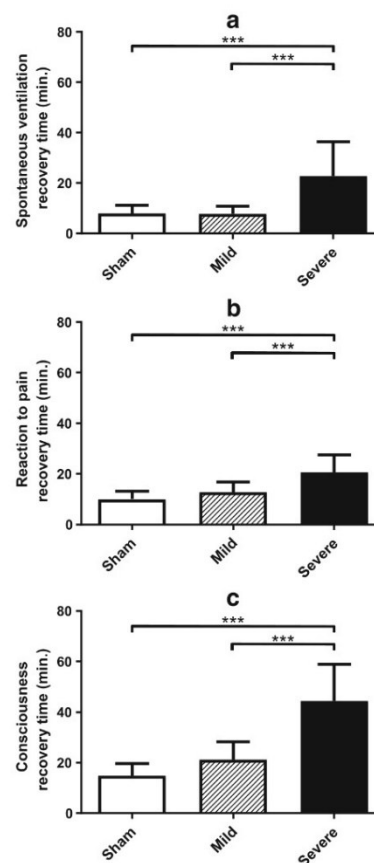


Fig. 2 Spontaneous ventilation recovery time (a), reaction to pain recovery time (b), and consciousness recovery time (c) in sham ($n = 28$), mild ($n = 28$), and severe ($n = 25$) SAH groups. The data are plotted as a mean and SD. $p < 0.001$ is labeled as ***

Table 2 Selected results of ROC analysis expressed in percent and 95% confidence interval range in mild ($n = 28$) and severe ($n = 25$) SAH groups for ventilation recovery time (ventilation), pain recovery time (pain), and consciousness regaining time (consciousness)

	Time (min)	Sensitivity (%)	95% CI	Specificity (%)	95% CI
Ventilation	> 7.5	88	69 to 97%	54	33 to 73%
	> 12.5	76	55 to 91%	93	75 to 99%
	> 17.5	56	35 to 76%	100	87 to 100%
Pain	> 12.5	88	69 to 97%	48	28 to 69%
	> 17.5	72	51 to 88%	88	69 to 97%
	> 22.5	36	18 to 58%	100	86 to 100%
Consciousness	> 22.5	100	86 to 100%	68	47 to 85%
	> 27.5	88	68 to 97%	76	55 to 91%
	> 32.5	83	63 to 95%	92	74 to 99%
	> 37.5	67	45 to 84%	100	86 to 100%

were evaluated 2, 4, 6, and 8 h after induction of SAH in the areas adjacent to hematoma (ipsilateral basal hemisphere) and in the areas distant to hematoma (contralateral dorsal hemisphere).

Figure 3 and Table S3 (in supplementary file) show $\text{TNF}\alpha$ mRNA expression 2, 4, 6, and 8 h after induction of SAH. There was a statistically significant difference between sham and severe SAH groups in all the timepoints both in areas adjacent to hematoma (A) and distant areas (D). The difference between sham and mild SAH groups was statistically significant 4 h after induction of SAH in both areas. The difference between mild and severe SAH groups was statistically significant in 2- and 6-h timepoints in both adjacent and distant areas. Finally, the statistically significant difference in

$\text{TNF}\alpha$ expression between adjacent and distant areas was observed in sham groups 2 h after surgery.

Figure 4 and Table S4 (in supplementary file) show $\text{IL-1}\beta$ mRNA expression 2, 4, 6, and 8 h after induction of SAH. The difference between sham and severe SAH groups was statistically significant in all the timepoints in both areas, while the difference between sham and mild SAH groups was statistically significant at 4 h after surgery in both areas. The difference between mild and severe SAH groups was statistically significant in 2-, 4-, and 6-h timepoints in the adjacent area and in 2- and 6-h timepoints in distant areas. Finally, the statistically significant difference in $\text{IL-1}\beta$ expression between adjacent and distant areas was observed in severe SAH groups at 2 and 6 h after surgery.

Fig. 3 $\text{TNF}\alpha$ expression in areas adjacent (A) and distant (D) to hematoma. The data are normalized to absolute control group and plotted as a geometric mean and geometric SD factor. $p < 0.05$ is labeled as *, $p < 0.01$ is labeled as **, and $p < 0.001$ is labeled as ***; $n = 7$ per group

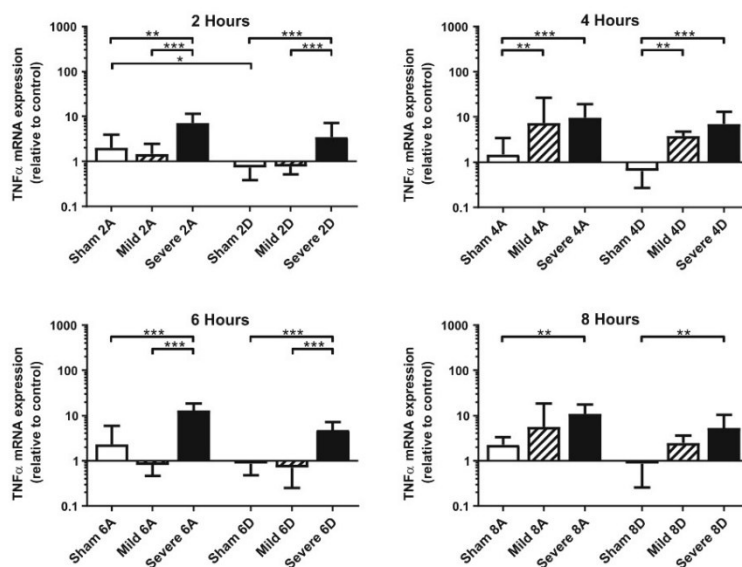


Fig. 4 IL-1 β expression in areas adjacent (A) and distant (D) to hematoma. The data are normalized to absolute control group and plotted as a geometric mean and geometric SD factor. $p < 0.05$ is labeled as *, $p < 0.01$ is labeled as **, and $p < 0.001$ is labeled as ***; $n = 7$ per group

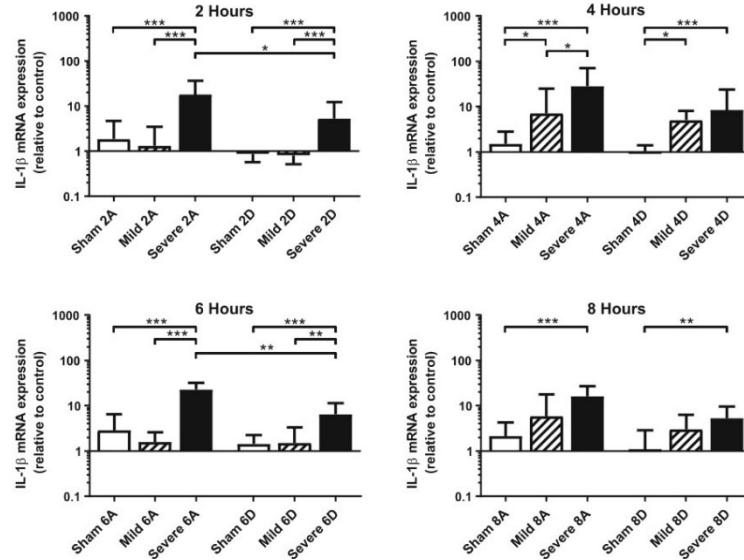


Figure 5 and Table S5 (in supplementary file) show IL-6 mRNA expression 2, 4, 6, and 8 h after induction of SAH. The difference between sham and severe SAH groups was statistically significant in all the timepoints in adjacent areas and in 2-, 4-, and 8-h timepoints in distant areas. The difference between sham and mild SAH groups was statistically significant

in none of timepoints in any areas. The difference between mild and severe SAH groups was statistically significant in all the timepoints in the adjacent area and in 2- and 4-h timepoints in distant areas. Finally, the statistically significant difference between adjacent and distant areas was observed in severe SAH groups at 4, 6, and 8 h after the surgery.

Fig. 5 IL-6 expression in areas adjacent (A) and distant (D) to hematoma. The data are normalized to absolute control group and plotted as a geometric mean and geometric SD factor. $p < 0.05$ is labeled as *, $p < 0.01$ is labeled as **, and $p < 0.001$ is labeled as ***; $n = 7$ per group

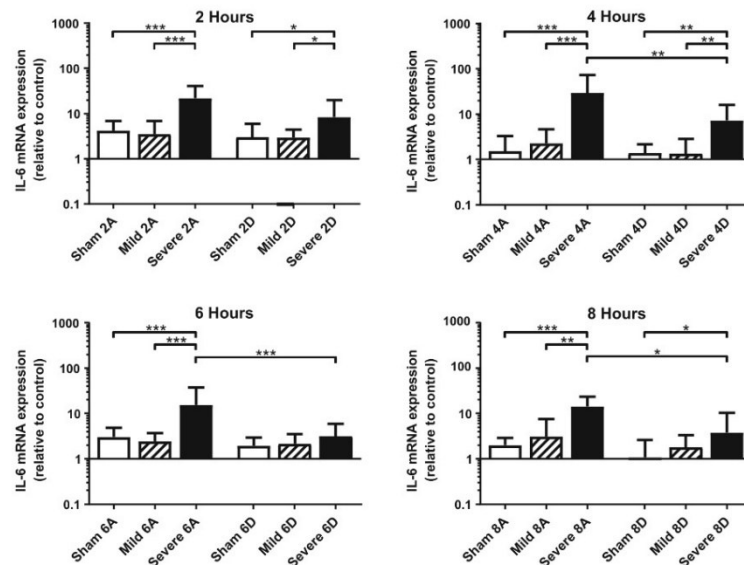


Fig. 6 ICAM-1 expression in areas adjacent (A) and distant (D) to hematoma. The data are normalized to absolute control group and plotted as a geometric mean and geometric SD factor. $p < 0.05$ is labeled as *, $p < 0.01$ is labeled as **, and $p < 0.001$ is labeled as ***; $n = 7$ per group

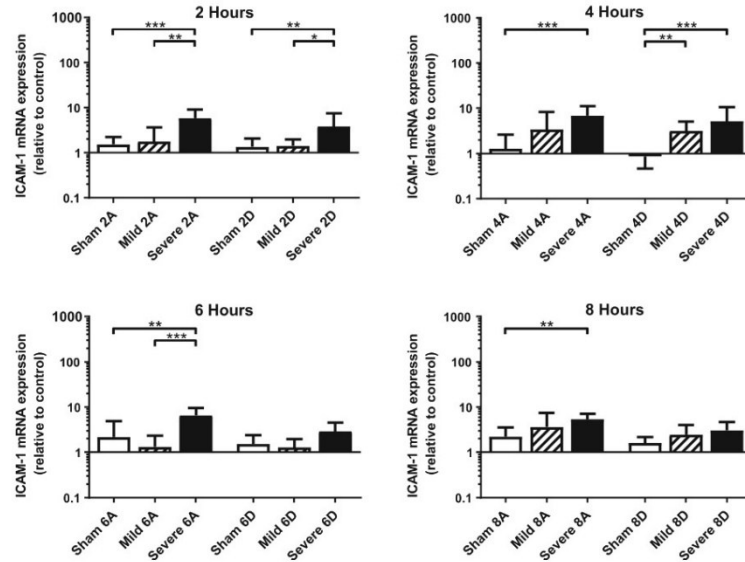
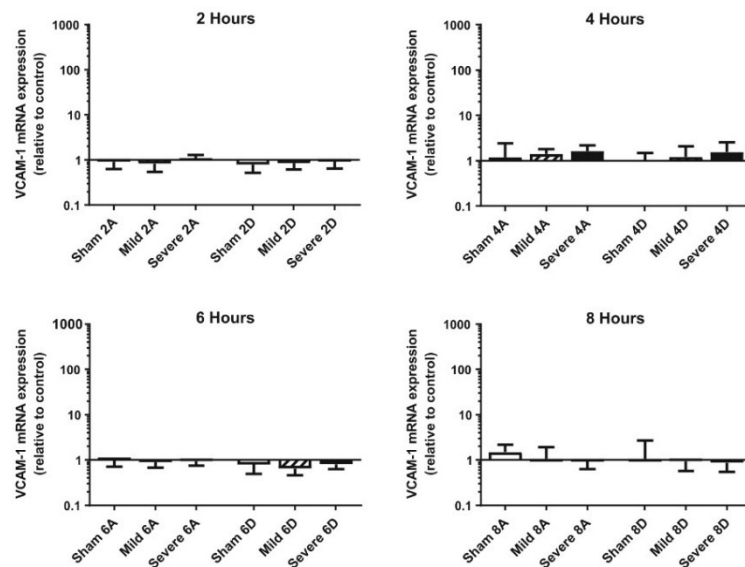


Figure 6 and Table S6 (in supplementary file) show ICAM-1 mRNA expression 2, 4, 6, and 8 h after induction of SAH. There was a statistically significant difference between sham and severe SAH groups in all the timepoints in adjacent areas and at 2 and 4 h in distant areas. Statistically significant difference between mild and severe SAH groups was observed in 2-h

timepoint in both areas and in 6-h timepoint in adjacent areas. Finally, there was statistically significant difference between sham and mild SAH groups in 4-h timepoint in distant areas.

Figure 7 and Table S7 (in supplementary file) show VCAM-1 mRNA expression 2, 4, 6, and 8 h after induction of SAH. In contrast to ICAM-1, no significant difference was

Fig. 7 VCAM-1 expression in areas adjacent (A) and distant (D) to hematoma. The data are normalized to absolute control group and plotted as a geometric mean and geometric SD factor. $p < 0.05$ is labeled as *, $p < 0.01$ is labeled as **, and $p < 0.001$ is labeled as ***; $n = 7$ per group



observed between the groups in any timepoint in case of VCAM-1.

Serum Levels of Pro-inflammatory Cytokines After SAH

Figure 8a, b and Table S8 (in supplementary file) show serum levels of TNF α and IL-1 β in sham and severe SAH groups 4 and 8 h after surgery. No significant differences between sham and severe SAH groups were observed in the case of TNF α and IL-1 β neither 4 nor 8 h after induction of SAH. Overall levels of TNF α were within physiological range, while levels

of IL-1 β were increased above physiological levels in a similar way in all the groups.

Figure 8c and Table S8 (in supplementary file) show serum levels of IL-6 in sham, mild, and severe SAH groups 4 and 8 h after surgery. There was significant difference between sham and severe groups as well as between mild and severe SAH groups 4 h after surgery. No significant difference was observed 8 h after surgery.

Discussion

The main goal of this study was to evaluate whether the early inflammatory response after SAH has in brain focal or global character. In this study, we used timepoints of 2, 4, 6, and 8 h after induction of bleeding and since there is no neurological evaluation designed for such short timepoints, we have also tested whether the anesthesia recovery time of basic reflexes/neurological parameters may be associated with severity of SAH.

The mortality of the model was 14.6% that is lower than typical data published in a perforation model of SAH [18–20]. We attribute it firstly to short timepoints, because we may assume that some of the severe SAH animals, which survived for a short period of time, would not survive for 24 h or longer. Additionally, severe SAH animals with respiration failure may be kept on mechanical ventilation for several hours. It is well known that severe stroke is associated with respiratory failure and the need of mechanical ventilation is often in patients suffering from severe stroke [21]. Keeping animals on mechanical ventilation for limited period allowed us to evaluate severe SAH animals which would die due to respiratory failure. We had three animals which were kept on mechanical ventilation for the whole period of monitoring (2-h timepoint, 1 animal; 4-h timepoint, 1 animal; and 8-h timepoint, 1 animal). The vital signs were monitored in these animals—the heart action and the color of the eyes as a sign of cerebral circulation—because an ophthalmic artery is a branch of the internal carotid artery. These animals were not used for basic reflexes recovery time evaluation, because the recovery time of some of the parameters would be nonsense (infinite). Interestingly, parameters of inflammatory response were within a standard range for severe SAH. Based on these observations (no statistical analysis was performed because of the sample size), we may assume that these animals may be potentially used for evaluation of inflammatory together with all other severe SAH animals.

Basic neurological parameters used for evaluation of anesthesia recovery time were respiratory reflex (spontaneous ventilation recovery), pain reflex, and consciousness recovery time. These parameters reflect activity on the spinal level (pain reflex), brain stem level (respiratory reflex), and neocortical level (consciousness). We used these parameters because standard neurological tests are not designed (and suitable) for

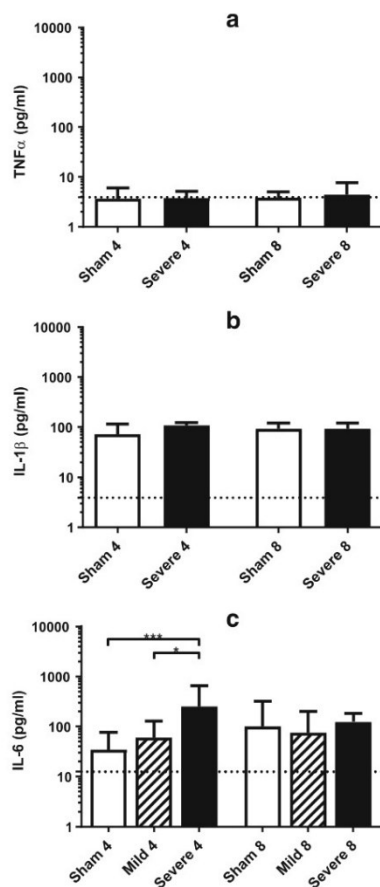


Fig. 8 Serum levels of TNF α (a), IL-1 β (b), and IL-6 (c) at the 4- and 8-h timepoints. The data are plotted as a geometric mean and geometric SD. The dotted line represents the threshold value of physiological levels. $p < 0.05$ is labeled as * and $p < 0.001$ is labeled as ***; $n = 7$ per group

early timepoints [15, 16]. An effect of anesthesia was discussed in the “Introduction.” Additionally, the standard neurological tests are designed as a sort of outcome tests and it has no meaning to perform the tests early after SAH. We used anesthesia recovery time-based tests to evaluate the influence of SAH on the initial neurological status. The prerequisite for this evaluation is similar exposure to anesthesia in all the groups and this criterion was fulfilled in our study groups. We have also paid attention to consistency in the interval between SAH induction and termination of anesthesia; the anesthesia was terminated 5 min after SAH induction/suture insertion. The results of all the evaluated parameters were quite uniform: there was a significant difference in recovery time between severe SAH groups and mild SAH groups while there was no difference between sham and mild SAH groups. The highest contrast was observed in the consciousness recovery time test and this test had the best predictive value. The spontaneous ventilation recovery time was able to bring us relatively accurate information in the shortest time, while the pain reaction recovery test did not bring us better information than previous tests. We think that the most interesting result of ROC analysis is the finding that an absence of respiratory response for more than 18 min after induction of bleeding (or 13 after termination of anesthesia) may indicate severe SAH with almost 100% probability. However, since the above-proposed tests represent different levels of neural activity, we think that it is useful to perform all the tests, even though some of them do not bring valuable information. These tests may be theoretically used in two ways. Firstly, these tests may be used for prediction of SAH severity and for evaluation of consistency of the model. Secondly, these tests may be potentially used for evaluation of the preconditioning treatment effect. Hypothetically, if preconditioning treatment protects against impact of primary injury (for example, by the decrease of oxygen/glucose consumption in neurons), recovery time may be shorter than in cases of untreated animals. And if the recovery time is similar in groups with preconditioning treatment and without treatment, while later evaluated, neurological status is improved in treated groups; this may be indirect evidence that preconditioning targets secondary brain injury. We may thus assume that anesthesia recovery tests may be potentially used in addition to standard neurologic tests and may possibly bring us useful information. However, we are aware that further independent validation is needed before reaching conclusion about the usefulness of anesthesia recovery time tests.

The major goal of this study was to answer the question, whether the early inflammatory response in the brain after SAH has a focal or global character. We evaluated mRNA expressions of widely used pro-inflammatory cytokines (TNF α , IL-1 β , IL-6) and adhesive molecules (ICAM-1, VCAM-1) in areas adjacent to hematoma and the areas distant to the hematoma. In general, the mRNA expressions of selected pro-

inflammatory cytokines were mainly increased in severe SAH groups and this was clear in both adjacent and distant areas in majority of the timepoints. The inflammatory response in mild SAH groups was less strong with a peak at 4 h after SAH. Significant changes were also found in ICAM-1 mRNA expression, while VCAM-1 mRNA expression was not changed. These results indicate that mainly severe SAH is associated with strong activation of the immune system in the brain and the early inflammatory response has a global character. Areas adjacent to hematoma are in contact with blood and possibly damaged. The inflammatory response in these areas may be considered as a “physiological” reaction to the presence of blood and potential tissue damage. The activation of an immune response in adjacent areas has been previously described and our results are in accordance with those of previously published data [11]. Areas distant to hematoma are not primarily affected by hematoma and activation of the immune system in these areas indicates global activation of the immune system in the brain. This suggests that pathophysiological processes associated with early brain injury are complex and may affect all the brain from the very early beginning, especially in the case of severe SAH. A possible reason for activation of the inflammatory response in distant areas may be transient global cerebral ischemia, ischemia caused by acute vasospasms after SAH, or with deregulation of inflammatory response which may occur after stroke [22–24]. ICAM-1 mRNA expression indicates that the inflammatory response may be in severe cases directed to systemic inflammatory response from very early beginning and this reaction may have also global character. Early upregulation of ICAM-1 mRNA expression is known from the ischemic stroke model as well as the fact that VCAM-1 expression may be unaffected [25].

Finally, we have evaluated serum levels of pro-inflammatory cytokines (TNF α , IL-1 β , IL-6) in selected groups and timepoints to find out whether we may, early after SAH, detect an effect of cerebral immune system activation in periphery. Serum levels of pro-inflammatory cytokines were ambiguous. TNF α was physiological in all the groups, while IL-1 β was increased in a similar way in both sham and severe SAH groups in these timepoints. Serum levels of IL-6 were increased in the severe SAH group at 4-h timepoint, while at 8 h after SAH, IL-6 levels were increased in all three groups in a similar way. These results may indicate that the surgical procedure may mask the effect of SAH and make hard to evaluate levels of pro-inflammatory cytokines in periphery early after SAH.

There are several limitations of this study. Firstly, the short timepoints are good to answer the questions about the pathophysiology of early brain injury, but short timepoints cannot give us information about the outcome. We did not use any standard timepoints and this is the limitation. We were, however, focused on evaluation of the initial step of inflammatory response (local reaction in the brain). If we use later timepoints, we would need to take into account inflammation mediated by leukocytes from periphery, while in timepoints

we used, we may assume that inflammatory response represents local reaction [11, 13]. Secondly, we did not evaluate the potential reason for global activation of the inflammatory response. However, in this study, we were focused only on description of the spatial context of the inflammatory response, because the exaggerated inflammatory response itself may cause significant tissue damage [14, 26]. Thirdly, regarding anesthesia recovery time evaluation, we did not evaluate inter-rater reproducibility, because all the evaluations were performed by one person. We assume that inter-rater reproducibility may be potentially consistent, because all the parameters may be considered as relatively objective (presence/absence of reaction). However, final conclusion has to be drawn from the future studies.

Conclusion

Severe subarachnoid hemorrhage is associated with the strong early inflammatory response, which has a global character, while mild subarachnoid hemorrhage is accompanied by a weaker inflammatory response. This indicates that inflammation may affect the whole brain from the very early beginning of SAH pathophysiology, especially in severe cases. The serum levels of pro-inflammatory cytokines do not reflect the early inflammatory response in the brain, possibly because of the effect of surgery on peripheral inflammation. Anesthesia recovery time may be useful for prediction of severity of bleeding in SAH model. Consciousness recovery time has the best predictive value, while spontaneous ventilation recovery time may indicate severe SAH as early as 18 min after induction of SAH. Further validation is needed to evaluate usefulness of anesthesia recovery time tests.

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Compliance with Ethical Standards

All procedures performed in this study involving animals were in accordance with the ethical standards of Masaryk University Institutional Animal Care.

Conflict of Interest The authors declare that they have no conflict of interest.

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26. Blecharz-Lang KG, Wagner J, Fries A, Nieminen-Kelhä M, Rösner J, Schneider UC, et al. Interleukin 6-mediated endothelial barrier disturbances can be attenuated by blockade of the IL6 receptor expressed in brain microvascular endothelial cells. *Transl Stroke Res.* 2018;1–12.

4. SAH-associated inflammation as a problem for diagnostics and treatment

Comprehension of inflammatory response is essential for understanding the pathophysiology of SAH and stroke in general. Stroke-associated inflammatory response is relatively uniform in both ischemic and hemorrhagic stroke and it is also similar in other acute pathologies like traumatic brain injury^{111–113}. A certain level of inflammatory response is essential for cleaning reaction and tissue repair¹¹⁴. However, following severe pathologies, the inflammatory response may be exaggerative and deregulated, possibly resulting in subsequent tissue damage and systemic inflammatory response syndrome with multi-organ dysfunction syndrome¹¹³.

Thus, an inflammatory response associated with severe pathologies may trigger a vicious circle of tissue damage – inflammation – additional tissue damage – more inflammation. An important question related to the inflammatory vicious circle is whether and when the inflammatory response becomes deregulated and whether and eventually how we can prevent it. Every patient in severe condition has some kind of anti-inflammatory treatment that is driven by symptoms/clinical needs, i.e. antiphlogistic to control pain, body temperature, sedation, or general anesthesia in intubated patients. However, we do not have enough consistent markers of inflammation that would help us to conduct anti-inflammatory treatment specifically, because most inflammation-related laboratory markers have high interindividual variability.

A promising option to control inflammation represents vagus nerve stimulation (VNS) which is based on a complex mechanism, cholinergic anti-inflammatory response (CHAIP). CHAIP is a physiological mechanism of immune system regulation by CNS. The main components of CHAIP include the vagus nerve, spleen, and $\alpha 7$ nicotinic acetylcholine receptor ($\alpha 7$ nAChR).

Severe conditions are associated with a disbalance between the activity of the sympathetic (SNS) and parasympathetic nervous system (PNS) because of stress which contributes to activation of SNS. The activity of autonomic nervous system is closely linked to inflammation, physiological activation of the sympathetic nervous system is in general pro-inflammatory, while parasympathetic activity is anti-inflammatory^{115,116}.

VNS decreases inflammation but does not cause immunosuppression¹¹⁶. In experimental conditions, VNS was to decrease inflammation in various severe conditions such as sepsis, septic shock, hypovolemic hemorrhagic shock, or stroke^{117–120}. The effect of VNS is anti-inflammatory, anti-apoptotic, and antioxidative making VNS very complex and promising therapeutic tool^{121,122}.

4.1 The author's publications related to the topic of SAH associated inflammation as a problem for diagnostics and treatment

4.1.1 Evolutionary concept of inflammatory response and stroke

Duris K, Jurajda M. Evolutionary concept of inflammatory response and stroke. *J Neurosci Res.* 2020;98:98–104.

Type of article: Review article

IF (2020): 4,164

Number of citations without self-citations according to WoS (7/2025): 11

In this conceptual review article, we aimed to build a logical construction of arguments that implicate possible reasons for the deregulation of inflammatory response after stroke. We discuss immune system–brain interactions from an evolutionary point of view in order to demonstrate that the immune system cannot be designed to deal with severe pathologies such as stroke.

After a short recapitulation of elementary notes about evolution and epigenetics, we sketch an overview of the functions of the immune system. We are mainly focused on the fact that the immune system has both analytic (immune surveillance) and executive (defense and healing) functions, which make this system very powerful. If we take into account that the immune system has a natural tendency to act aggressively, because dealing with pathogens has to be resolute - the immune system cannot lose (in another case the organism may be lost), it is not a surprise that massive activation of the immune system may cause serious damage to organism.

The next parts are dedicated to a description of the role of the immune system in CNS under physiological conditions and after stroke. We mainly discuss the fact that the immune system cannot be designed to deal with severe pathologies like stroke because such pathologies are typically not compatible with life in wild nature. Even if the organism survives, the evolutionary selection mechanism cannot be applied, because stroke predominantly occurs in the postreproductive period of life.

We may conclude that for above mentioned reasons, evolution has no chance to prepare the immune system for such severe conditions as severe stroke, severe craniotrauma or polytrauma. These conditions may be associated with exaggerated/deregulated inflammatory response, which may cause more damage than initial pathology.

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MINIREVIEW

Evolutionary concept of inflammatory response and stroke

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Abstract

The immune system plays an important role under both physiological and pathological conditions. Immune surveillance as well as defense and healing processes are crucial for the organism, but the immune system has a natural tendency to act aggressively when excessively stimulated. We may assume that the immune system is not designed to deal with severe conditions, such as polytrauma or severe stroke, because these are not compatible with life in the wilderness and evolution has no chance to act in such cases. These conditions are associated with exaggerated/deregulated inflammatory response, which may cause more damage than initial pathology. In this article, we would like to sketch a basic concept of the immune system–brain interactions from the evolutionary point of view and to discuss some implications related to stroke.

KEYWORDS

evolution, inflammation, stroke

1 | INTRODUCTION

Evolution is essential for life in an environment which, in global measure, changes slowly but constantly (Chevin, Lande, & Mace, 2010; Monaghan, 2008). The main challenges for survival in the wilderness are to eat/not to be eaten and to resist/to regenerate. Evolution–development–improvement allows better adaptation in given environment and central nervous system as well as immune system are crucial for survival from this point of view (McEwen & Gianaros, 2010; Medzhitov, 2010). The functions of both central nervous system and immune system are closely linked to the environment and both systems allow us to interact with environment adequately (Blalock, 1984; Morton, Cummings, Baskin, Barsh, & Schwartz, 2006). Better the central nervous system is, better is a chance to find food and avoid danger. Better the immune system is, better resistance and healing functions allowing the organism to keep integrity in more challenging conditions.

The main tool of evolution is selection which is possible due to variability in the population (Chevin et al., 2010; Monaghan, 2008). A certain level of variability is kept in population constantly and the best combination of phenotypes in given conditions has the evolutionary

advantage associated with higher reproducibility rate, resulting in promotion of beneficial features in the population (Chevin et al., 2010; Monaghan, 2008). This relatively simple mechanism is believed to direct development and adaptation of population to global environmental changes, and thus the certain level of variability in population has to be kept constantly (Chevin et al., 2010; Fumagalli et al., 2011; Monaghan, 2008). This mechanism may be considered as a rough tool of development which is fine tuned by an epigenetic mechanism (Nathanielsz & Thornburg, 2003; Tremblay & Hamet, 2008).

Evolution acts on the level of population, while epigenetic modulations act on the level of individual organisms and epigenetics is a dynamic field of science because of its involvement in the pathophysiology of literally any human disease (Nathanielsz & Thornburg, 2003; Portela & Esteller, 2010; Tremblay & Hamet, 2008). The role of epigenetics in inflammation and in stroke pathophysiology has been extensively reviewed too (Lindgren, 2014; McCall, Gazzar, Liu, Vachharajani, & Yoza, 2011; Qureshi & Mehler, 2010a, 2010b, 2011; Shanmugam & Sethi, 2013).

In this article, we would like to sketch a basic concept of the immune system–brain interactions from the evolutionary point of view and to discuss some implications related to stroke.



Significance

The goal of this article is to sketch basic concept of inflammatory response after stroke with a respect to evolution. This approach may possibly help us to understand the limitations of inflammatory response and the consequences for the pathophysiology of stroke.

2 | IMMUNE SYSTEM

The role of immune system for survival is crucial under both physiological and pathological conditions (Medzhitov, 2010; Zhao & Schwartz, 1998). The immune system is the main and universal tool designed to restore a large variety of problems and life-threatening situations (Medzhitov, 2010). The organism is evolutionarily designed to resolve any problems with its own powers and the immune system is a powerful instrument to achieve it (Janeway, 1992; Lee & Mazmanian, 2010).

Considering pathological processes in general, external and internal factors are usually taken into account as determining aspects (Engel, 1960; Tran, Cuesta, Van, Nauta, & Wesdorp, 1993) (Figure 1). External factors represent a threat from outside of the organism and these may be both, one-time thread, such as traumatic event, or longer lasting threads, like invasion and persistence of pathogens in the organism (Engel, 1960; Tran et al., 1993). Internal factors represent features which are more stable, like existing comorbidity, or even permanent, like genetic background (Engel, 1960; Tran et al., 1993).

It seems to be useful to distinguish reaction of the immune system from above-mentioned factors, because immune system represents the dynamic interaction of organism to any pathological condition and immune system is closely connected to both external and internal factors (Strieter, Kunkel, & Bone, 1993; Tran et al., 1993). External factors activate the immune system, while internal factors determine (genetical background) and modulate (comorbidities) function of the immune system. The main functions of the immune system are immune surveillance, defense as well as reparation processes and organism and the level of its activity is proportional to actual needs (Park & Barbul, 2004; Parkin & Cohen, 2001) (Figure 2).

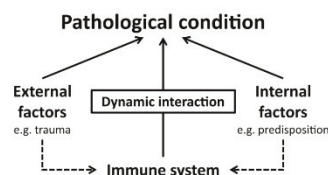


FIGURE 1 The pathophysiological role of immune system
Note. The immune system represents a dynamic interaction, which is influenced by the external factors as well as internal factors and which deals with pathological conditions.

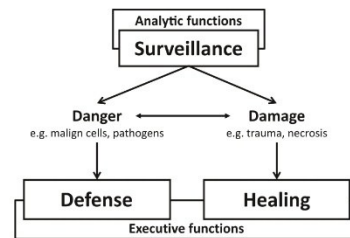


FIGURE 2 The main functions of immune system
Note. The immune system is all in one system combining both analytic and executive functions.

Surveillance may be considered as an analytic function, which is performed continuously, while the defense and healing processes may be considered as executive functions which are performed on demand, when it is needed (Park & Barbul, 2004; Parkin & Cohen, 2001). Surveillance is performed mainly by lymphoid organs and by tissue macrophages, immune cells scattered through the organism, which are devoted to detect danger or damage and either solve the problem by themselves or activate the systemic immune response (Davies, Jenkins, Allen, & Taylor, 2013; Ransohoff & Engelhardt, 2012). Dangerous stimuli, such as pathogen invasion or the presence of potentially malign cell, trigger defense functions to remove dangerous element, while tissue damage initiates immune system-dependent repairing processes (Davies et al., 2013; Ransohoff & Engelhardt, 2012).

Both defense functions and healing processes are closely interconnected, because the potentially dangerous element, like pathogen invasion, may be associated with tissue damage and on contrary structural damage is a risk factor for pathogen invasion. Thus, it seems to be useful that both defense functions and healing processes, which basically represent opposite processes (destruction and reparation), are realized by one system. Moreover, combining analytic and executive functions, immune system represents complex all in one self-regulated system which is very powerful but also potentially dangerous, because the immune system has a natural tendency to act aggressively.

The main functions of immune system reflect a spectrum of problems which immune system is designed to deal with; these are mainly infections and injuries/tissue damage. Infections were the most often cause of death before the era of antibiotics (Adedeji, 2016). Microorganisms have short reproduction time and short reproduction time of pathogens may be possibly associated with aggressivity of the immune system. The immune system has to defeat pathogen quickly and effectively, in the other case the organism is potentially lost. However, it is worth to mention, that although the pathogenic microorganisms are an important issue in medicine, majority of microorganisms are symbiotic (Lee & Mazmanian, 2010). Moreover, interaction with microorganisms and the need to differentiate between symbiotic and pathogen microorganisms is believed to shape the evolution of the adaptive immune system (Lee & Mazmanian, 2010).

The immune system is designed to deal with pathologies which have to be considered as mild from the point of modern medicine. In the wilderness, even the simple fracture has to be considered as a big problem, while severe pathologies, which modern medicine can manage, are not compatible with a life. Therefore, when we take into account that the immune system is on one hand designed to act aggressively and on the other hand is not designed to deal with severe pathologies, the possible consequences are clear. First, severe pathologies may be associated with strong activation/deregulation of the immune system which may cause more damage than initial pathology (Lu et al., 2009). Second, the immune system may also significantly contribute to tissue damage, especially in severe conditions, such as stroke (Lu et al., 2009).

3 | CENTRAL NERVOUS SYSTEM

Similarly to the immune system, the role of nervous system is essential; central nervous system allows us to survive and better adapt in an environment (McEwen & Gianaros, 2010). Higher development of nervous system is associated with higher performance and better adaptation or even control over the environment (Burdet, Osu, Franklin, Milner, & Kawato, 2001). The nervous system is designed for performance, which seems to be evolutionary priority (Wood, 2010).

During evolution central nervous system has developed in a very complex structure, which individually grows step by step during embryonic as well as postnatal period (Crespo, O'Leary, & Maxwell Cowan, 1985; Mrzljak, Uylings, Van Eden, & Judáš, 1991). The weak point of nervous system complexity and successive developmental design is a very limited capability of organism to restore damage and this topic is a major challenge for research (Albensi, 2001; Qin, Zhang, & Yang, 2015).

Practical inability of organism to restore neuronal damage seems to be partially balanced by prevention, which is clearly reflected in anatomy of central nervous system (Melvin & Lighthall, 2002). Skull, meninges, and cerebrospinal (CSF) fluid represent mechanical protection to the outer environment, hematoencephalic barrier represents a homeostatic barrier to inner environment, while glial cells maximally support neuronal functions. Microenvironment in brain is kept in optimal range by these mechanisms, allowing neurons to survive a whole life of an organism (Walz, 1989).

Skull, CSF as well as hematoencephalic barrier are perfect tools to protect the central nervous system against the minor threats of common life, they may, however, represent problem when major pathology occurs, resulting in triggering of the vicious circle of brain damage caused by volume changes, pressure changes, and brain ischemia (Melvin & Lighthall, 2002; Robba, Cardim, Sekhon, Budohoski, & Czosnyka, 2018; Steiner & Andrews, 2006; Thiagarajah, Papadopoulos, & Verkman, 2005).

Intracranial compartmentalization is not only important for stability of microenvironment and for mechanical protection, but it is also significant from immunological point of view. All the three

compartments, i.e. vascular, parenchymal/interstitial fluid and cerebrospinal fluid, are strictly separated by hematoencephalic, hemato-liquor, and meningeal barriers (Laterra, Keep, Betz, & Goldstein, 1999; Saunders, Habgood, Møllgård, & Dziegielewska, 2016; Weller, Sharp, Christodoulides, Carare, & Møllgård, 2018).

The major population of immune cells in the brain is represented by macrophages, which reside both in the parenchyma (microglia) and outside the parenchyma, linked to barriers (perivascular macrophages, meningeal macrophages, macrophages of the circumventricular organs, and macrophages of the choroid plexus) (Herz, Filiano, Smith, Yogeve, & Kipnis, 2017). The immune cells may traffic from CSF to parenchyma and back, but the connection, allowing cellular trafficking between CSF and regional lymph nodes, was established only recently (Engelhardt et al., 2016; Engelhardt, Vajkoczy, & Weller, 2017; Hatterer, Touret, Belin, Honnorat, & Nataf, 2008; Kaminski et al., 2012). This connection is represented by nasal and dural lymphatics which drain CSF to cervical lymph nodes (Engelhardt et al., 2016; Kaminski et al., 2012).

The most numerous myeloid population in the brain is microglia, which has several important functions including synaptic pruning, neurotrophic factors synthesis, phagocytosis, and immune surveillance (Kierdorf & Prinz, 2017; Parkhurst et al., 2013; Schafer et al., 2012; Takahashi, Rochford, & Neumann, 2005). Microglial activation occurs in response to pathological conditions, shifting microglia to pro-inflammatory M1 or anti-inflammatory M2 phenotype. The initial response is typically pro-inflammatory, exaggerating tissue damage, and the second step is usually anti-inflammatory response promoting healing processes (Kanazawa, Ninomiya, Hatakeyama, Takahashi, & Shimohata, 2017).

Non-parenchymal macrophages play an important role in immune surveillance, phagocytosis, barrier-maintaining functions as well as in local intercellular communication (Brendecke & Prinz, 2015; Herz et al., 2017). Non-parenchymal macrophages contribute to antigen processing and closely cooperate with dendritic cells, which are also localized in the meninges and choroid plexus (Chinnery, Ruitenber, & McMennamin, 2010; Henning et al., 2009).

Dendritic cells are not present in brain parenchyma under physiological conditions, but these cells may infiltrate the brain under pathological conditions (Hatterer et al., 2008). After antigen processing, dendritic cells return in the subarachnoid space and reach cervical lymph nodes, where initiate adaptive immune response (Filiano, Gadani, & Kipnis, 2017; Hatterer et al., 2008).

4 | STROKE

Stroke is an acute neurological dysfunction caused by blood supply interruption and tissue damage due to ischemic or hemorrhagic event (Hankey, 2017). The core of infarction or hematoma represent primary injury, which is associated with quick neuronal cell death due to critical ischemia or mass effect of extravasated blood (Brouns & De Deyn, 2009; Xi, Keep, & Hoff, 2006). Adjacent penumbra/perihematomal areas are affected by secondary injury, which may

lead by several mechanisms to delayed neuronal and endothelial cell death with subsequent vasogenic edema formation and possible additional tissue ischemia in severe cases (Hossmann, 1994; Qureshi et al., 2003).

Both primary and secondary injuries contribute to brain ischemia and possibly to immediate or delayed neuronal cell death, according to severity of ischemia and energy depletion (Kitagawa, 2012; Manwani & McCullough, 2013). Tissue damage caused by stroke is immediately detected by the immune system, which triggers an inflammatory response (Iadecola & Anrather, 2011) (Figure 3). The inflammatory response is also modulated by the autonomic nervous system, physiological activation of sympathetic nervous system is in general pro-inflammatory, while parasympathetic activity is anti-inflammatory (Pongratz & Straub, 2014; Tracey, 2009). However, severe conditions, such as stroke or polytrauma may be associated with delayed immunosuppression, possibly due to fact that severe conditions represent very strong stress, which may be associated with overstimulation of sympathetic nervous system and subsequent direct or rebound immunodepression (Jiang, 2008; Yan & Zhang, 2014; Zuo, Shi, & Yan, 2016).

Immune surveillance in the brain is mainly performed by microglia and stroke associated tissue damage is a strong signal for microglia activation (Ahmad & Graham, 2010). Stimulated microglia start to produce pro-inflammatory cytokines, activating endothelial cells, and leukocytes in the periphery (Atangana et al., 2017; Hanisch, 2002). Stimulated microglia also produce chemokines, attracting leukocytes to the affected area (Hanisch, 2002; Pennypacker, 2014). Activated endothelial cells produce adhesive molecules, allowing leukocytes from blood to infiltrate the affected area (Atangana et al., 2017).

Blood-brain barrier represents a problem for leukocyte trafficking, because the blood-brain barrier is not permeable for cell

trafficking (Bechmann, Galea, & Perry, 2007). After extravasation, leukocytes are trapped in perivascular space and to penetrate the glia limitans, the leukocytes release proteolytic enzymes causing blood-brain barrier disruption (Arba et al., 2019; Bechmann et al., 2007). Blood-brain barrier disruption plays an important role in stroke pathophysiology and the inflammation is crucial contributor blood-brain barrier disruption (Blecharz-Lang et al., 2018; Florczak-Rzepka, Grond-Ginsbach, Montaner, & Steiner, 2012).

Infiltrating leukocytes also produce other harmful substances, namely reactive oxygen species and pro-inflammatory cytokines (Wang, Tang, & Yenari, 2007). Reactive oxygen species are massively released after stroke due to several mechanisms, including mitochondrial dysfunction, calcium overload, or inflammation (Lakhan, Kirchgessner, & Hofer, 2009; Wang et al., 2007). Pro-inflammatory cytokines represent potentially harmful substance by several mechanisms, including induction of apoptosis, blood-brain barrier disruption as well as by amplification of the inflammatory response (Behrouz, 2016). This may potentially initiate a vicious circle of inflammation and tissue damage, especially when we consider that the inflammation, oxidative stress, blood-brain barrier disruption, and neuronal apoptosis are closely interconnected and that inflammatory response after stroke is closely related to outcome (Chou et al., 2011; Wang et al., 2018).

Inflammatory response after stroke is quite uniform regardless of stroke subtype (Chen, Yang, Chen, & Zhang, 2015; Jin, Liu, Zhang, Nanda, & Li, 2013). Inflammatory response plays an important role in debris clearing and tissue repair; thus, the certain level of inflammation is required (Patel, Ritzel, McCullough, & Liu, 2013). Immunosuppression may be as harmful as exaggerated inflammation and balanced regulation of inflammatory response, mediated by regulatory B and T cells, is critical (Duffy, Keating, Perera, & Moalem-Taylor, 2018; Han et al., 2016; Liesz & Kleinschmitt, 2016).

5 | CONCLUSION

Neither brain nor immune system is designed to deal with severe pathologies such as severe stroke, craniotraumata, or polytrauma. These pathologies are typically not compatible with life in the wilderness and evolution cannot act in such cases. Considering evolution, stroke typically occurs in postreproductive period, thus even if the organism survives, the evolutionary selection cannot be applied.

An important aspect of stroke pathophysiology is that the immune system, which is designed to act aggressively, interacts with a nervous system which is very sensitive and prone to damage. Exaggerated inflammatory response causing additional damage to initial pathology may be considered as a logical consequence of immune system-brain interaction in severe cases. Additional damage may exaggerate the inflammatory response, which has a tendency for self-propagation, potentially triggering a vicious circle with potentially fatal consequences.

The important challenge for current science is to find out to which point is an inflammatory response exaggerated and when

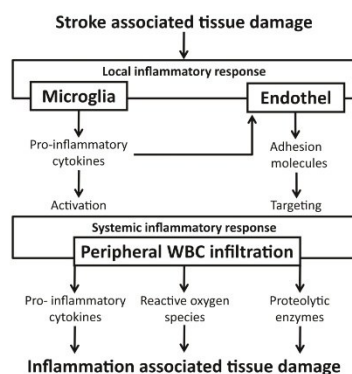


FIGURE 3 Inflammatory response after stroke Note. Inflammatory response after stroke is a two-step process consisting of local and systemic inflammatory response. The local inflammatory response is mediated by microglia and endothelial cells, which activate peripheral white blood cells (WBC) and attract them to the affected area. Systemic inflammatory response is mediated by infiltrating WBC, which may cause additional tissue damage.

deregulation begins. The problem is, how to define deregulation. We may assume deregulation as uncontrolled, chaotic process due to lack of regulatory mechanism. A high level of randomness and unpredictability is characteristic for such a process. But the processes which are not known in detail may be due to lack of information considered as random, chaotic, and deregulated too.

The strong inflammatory response may damage cells in any case, but it is important to distinguish between exaggeration and deregulation, because of potential therapeutic strategies. We may assume that possible therapeutic approach will be different in exaggerated and deregulated inflammatory response. Our knowledge about regulations of inflammatory response in its complexity is still limited, but we hope that "simple" logic of evolutionary concepts should be taken into account in discussion about the pathophysiology of stroke.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

Both authors contributed equally to this manuscript. K.D. and M.J. conceptualized the manuscript; K.D. wrote the original draft; M.J. and K.D. wrote, reviewed, and edited the manuscript; M.J. supervised; and K.D. did the funding acquisition.

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4.1.2 Inflammatory response in sepsis and hemorrhagic stroke

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In this review article, we intended to describe inflammatory responses in sepsis and hemorrhagic stroke side by side to highlight similar features and differences. Sepsis represents an inflammatory response that has a systemic character from the beginning, while hemorrhagic stroke represents pathology, in which inflammation has initially local character and subsequently turns into systemic inflammation. Inflammatory response is often exaggerated/de-regulated in severe cases of both sepsis and hemorrhagic stroke.

The initial part is dedicated to the description of pathophysiological and clinical aspects of sepsis and hemorrhagic stroke including molecular mechanisms and treatment strategies. In the next section, we describe experimental models of both conditions. In the final section, we describe the role of inflammatory response and underlying molecular signaling mechanisms in sepsis and hemorrhagic stroke.



Review article

Inflammatory response in sepsis and hemorrhagic stroke

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ABSTRACT

Exaggerated/deregulated inflammatory response is a major medical problem that is often associated with bad outcomes or even the death of the patient. Sepsis and severe hemorrhagic stroke are two illustrative examples because sepsis represents inflammation that is systemic initially, while stroke is initially a local pathology that in severe cases turns into systemic inflammation. There are clear differences between the two pathological conditions in terms of mechanisms and dynamics of inflammatory response at an early stage, while later the inflammatory response becomes uniform. This article aims to describe side-by-side sepsis and hemorrhagic stroke so that similar features and differences can stand out. We will describe the pathophysiological and clinical aspects of sepsis and hemorrhagic stroke, experimental models, the role of inflammation, and molecular mechanisms of inflammation in both conditions. Comparison of inflammatory response in these two model situations may lead to a better understanding of life-threatening conditions pathophysiology, in which the exaggeration/deregulation of the inflammatory response may occur with potentially severe consequences. This article is kept detailed to provide the reader with in-depth information, which should allow him to find the mechanisms of inflammatory response and to elaborate them by himself in the context of his particular needs.

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1. Introduction

The response of the immune system is an essential pathophysiological mechanism. Immune system reaction in terms of acute or chronic inflammatory response is part of all pathologies. A major medical problem is an exaggerated/deregulated inflammatory response, which often causes bad outcomes or even the death of the patients. Sepsis and severe cases of hemorrhagic stroke are two illustrative examples of how exaggeration/deregulation of the inflammatory response may occur.

Stroke and sepsis are closely interconnected^{1–3}. Patients suffering from both ischemic and hemorrhagic stroke are at risk of sepsis development, most often from lung infection.¹ On the other hand, patients with sepsis are at higher risk of both ischemic and hemorrhagic stroke.^{2–3} The risk of stroke onset is highest in the first two weeks and the risk of stroke remains increased up to 1 year after the sepsis². Although the association of stroke with sepsis is an important topic, the aim of this article is not to describe the rela-

tionship between hemorrhagic stroke and sepsis in terms of stroke-associated etiopathogenesis of sepsis and vice versa.

Instead, this article aims to describe sepsis and hemorrhagic stroke side-by-side so that similar features and differences in inflammatory response can stand out. From this point of view sepsis and hemorrhagic stroke may be illustrative examples of inflammatory response pathophysiology. Sepsis represents inflammation that has a systemic and exaggerated/deregulated character from the beginning, while stroke is initially a local pathology that in severe cases turns into systemic inflammation (as do other severe pathologies). There are clear differences between the two pathological conditions in terms of mechanisms and dynamics of inflammatory response at an early stage, while later the inflammatory response becomes uniform. Understanding both dichotomy and uniformity of inflammatory response in these two model situations may lead to a better understanding of life-threatening conditions pathophysiology, in which the exaggeration/deregulation of the inflammatory response may occur with potentially severe consequences.

In this article, we will describe the pathophysiological and clinical aspects of sepsis and hemorrhagic stroke, experimental models, the role of inflammation, and molecular mechanisms of

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inflammation in both conditions. We keep this article detailed to provide the reader with in-depth information, which should allow him to find the mechanisms of inflammatory response and to elaborate them by himself in the context of his particular needs.

2. Sepsis and hemorrhagic stroke

2.1. Sepsis

Sepsis is a “life-threatening organ dysfunction caused by a dysregulated host response to infection”. Sepsis arises when the organism’s response to an infection injures its own tissues and organs⁴ (Singer et al. 2016). Sepsis is a health issue of physiological, pathological, and biochemical abnormalities induced by infection. It is a major public health concern worldwide, associated with high mortality rates and considerable economic consequences⁵ (Tiru et al. 2015). The incidence of sepsis is increasing, likely due to an aging population with more comorbidities (Chronic obstructive pulmonary disease, cancer, heart failure, etc.)^{4,6–7} (Iwashyna et al. 2010; Singer et al. 2016; Vincent 2009). Although the true incidence is unknown, mainly due to scarce or nonexistent data from low- and middle-income countries, even conservative estimates indicate that sepsis is a leading cause of mortality for patients in intensive care units^{8,9} (Fleischmann et al. 2016; Vincent et al. 2014).

Sepsis has been defined as matching 2 or more symptoms of systemic inflammatory response syndrome (SIRS) plus infection⁴ (Singer et al. 2016). Meaning that sepsis meets at least some SIRS criteria, but not vice versa because SIRS criteria can be met by other etiologies such as trauma, ischemia, or pancreatitis. These pathologies can also increase heart rate, respiratory rate, and white blood cell count, but are not caused by an infection.

The true incidence and mortality of sepsis cases are unknown in any given country. Most data describe only rich countries where it is estimated that the mortality is 2–8 million deaths per year⁸ (Fleischmann et al. 2016). In Europe, the incidence numbers can be estimated to be 100 to 300/100 000 per year, with a mortality of around 30 %⁸ (Fleischmann et al. 2016). The most common infection sites that lead to sepsis are the lungs (64 % of cases), abdomen (20 % of cases), bloodstream (15 % of cases), and renal or genitourinary tracts (14 %)⁷ (Vincent 2009). The most frequently identified organisms responsible for sepsis occurrence are *Staphylococcus aureus* (20.5 %, Gram-positive) and *Escherichia coli* (16 %, Gram-negative)⁷ (Vincent 2009).

The precise mechanisms of sepsis-induced cell injury and organ dysfunction are not fully understood; however, it is commonly accepted that sepsis interferes with the distribution of systemic blood flow via vasodilation and disturbance to the microcirculation.

A well-described immune activation pathway as a response to infection involves microbial pathogen-associated molecular pattern molecules (PAMPs), which are recognized by pattern-recognition receptors (PRRs), such as Toll-like receptors (TLRs) on the innate immune system cells (Fig. 1)¹⁰ (Cecconi et al. 2018). This pathway leads to the activation of nuclear factor κ B (NF κ B) and the release of pro- and anti-inflammatory mediators by neutrophils. Similar signaling occurs with activation by damage-associated molecular pattern molecules (DAMPs)¹¹ (Denning et al. 2019). These inflammatory mediators include cytokines such as interleukin (IL)-1, IL-2, IL-6, IL-8, tumor necrosis factor α (TNF α), and others¹² (Hotchkiss and Karl 2003). They help with neutrophil-endothelial adhesion and activate the clotting and complement pathways, which can lead to the creation of microthrombi^{10,12} (Cecconi et al. 2018; Hotchkiss and Karl 2003) (Fig. 2).

The inflammatory mediators contribute to changes in hemodynamic response. Cytokines, mainly TNF α , are released from mono-

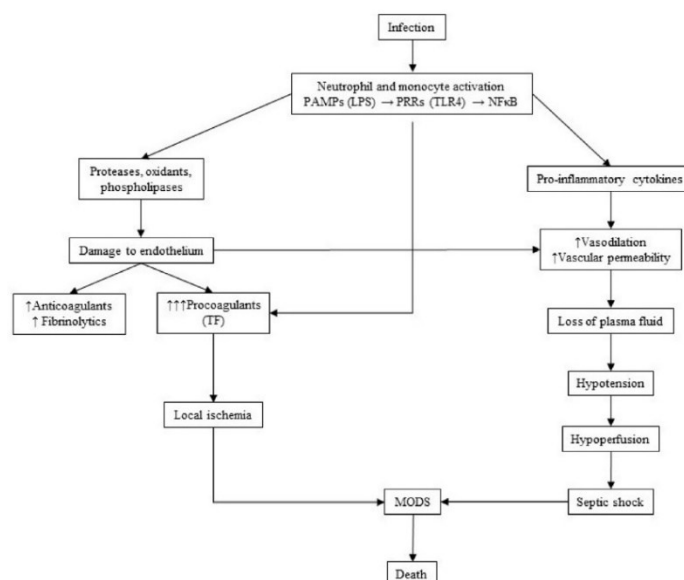


Fig. 1. Pathophysiology of sepsis: the pathogen-associated molecular pattern molecules (PAMPs) are recognized by pattern-recognition receptors (PRRs) on the plasmatic membrane. The recognition through PRRs stimulates the imbalance activation of pro-inflammatory response (leading to cellular damage and tissue impairment) and anti-inflammatory response (leading to immune system impairment). It leads to the secretion of cytokines, proteases, complement, leukocyte, and coagulation activation. The deregulation of inflammatory response together with age, genetics, and comorbidities can contribute to organ dysfunction and ultimately death.

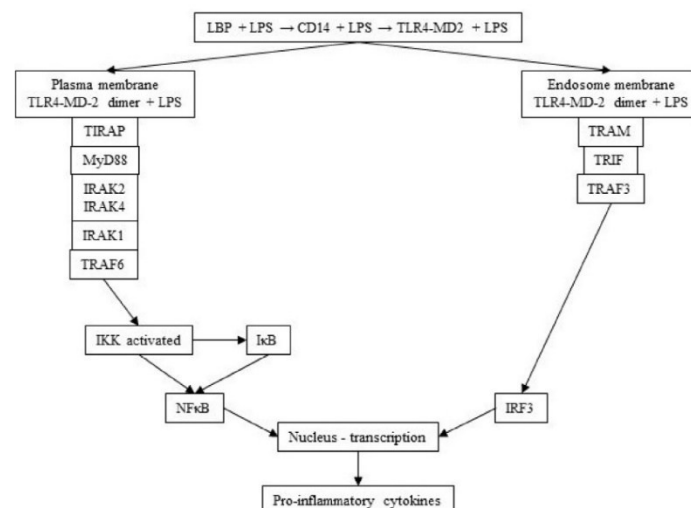


Fig. 2. TLR4 signaling diagram.

cytes/macrophages after the simulation by microbial products, such as lipopolysaccharide of gram-negative bacteria (LPS)¹⁰ (Cecconi et al. 2018). TNF α then binds to receptors on various types of cells and causes vasodilation and increased vascular permeability and consequently loss of plasma fluid¹⁰ (Cecconi et al. 2018). These effects are not clinically apparent at first, because elevated heart rate and cardiac output compensate them to maintain normal blood pressure¹³ (Jacobi 2002). However, as sepsis progresses, this compensation is exceeded which manifests as hypotension¹³ (Jacobi 2002).

Hypotension may be managed by fluid therapy, but in patients, non-responding to fluid therapy hypotension may worsen septic shock¹⁴ (Font, Thyagarajan, and Khanna 2020). Patients with septic shock that do not respond to treatment by inotropic or vasopressor agents are at great risk of developing multiple organ dysfunction syndrome (MODS) from hypoperfusion¹³ (Jacobi 2002). MODS can be associated with a higher death rate¹⁰ (Cecconi et al. 2018) (Fig. 3.).

In the physiological inflammatory response, neutrophils are attracted to the inflammation site, roll along the endothelium, and adhere to it through interaction between endothelial selectins with integrins and chemokine receptors in the surface of leukocytes¹⁵ (Newton and Dixit 2012). Although local adhesion is expected as part of the host defense response, in sepsis systemic activation of leukocytes causes the release of damaging proteases, oxidants, and phospholipases causing damage to the endothelium and increasing microvascular permeability^{13,16} (Duris and Jurajda 2020; Jacobi 2002).

The endothelium has many important functions because it maintains vascular integrity, regulates vascular tone, and contributes to the local balance of coagulation and anticoagulation^{17–18} (Bermejo-Martin et al. 2018, Godo and Shimokawa 2017). The endothelial damage in inflammation causes the release of tissue factors, which is an important trigger of the extrinsic clotting system¹⁷ (Bermejo-Martin et al. 2018). In addition, the tissue factor release is stimulated by cytokines from neutrophils and macrophages¹³ (Jacobi 2002). Other important released molecules are procoagulants (such as plasminogen activator factor and von

Willebrand's factor), anticoagulants (activated protein C, antithrombin), and fibrinolytic factors (tissue plasminogen activator)¹⁷ (Bermejo-Martin et al. 2018). However, in systemic inflammation anticoagulants and fibrinolytic are quickly consumed leading to an overwhelming procoagulant response¹³ (Jacobi 2002). Therefore, microvascular coagulation develops and contributes to further damage because of local ischemia. Inflammatory response and coagulation are closely linked in sepsis and SIRS.

2.2. Hemorrhagic stroke

The cerebrovascular incident, more commonly referred to as a stroke, is a medical condition in which impaired blood flow causes damage or cell death in the brain. Hemorrhagic stroke is caused by bleeding from a ruptured blood vessel into the brain.

Hemorrhagic stroke can be subdivided into intracerebral hemorrhage (ICH), in which the bleeding is to the brain parenchyma, and subarachnoid hemorrhage (SAH) in which the bleeding is to the subarachnoid space.

Hemorrhagic stroke is associated with high morbidity and mortality. 101.5 million people worldwide suffer from stroke in 2019¹⁹ (Virani et al. 2021). Hemorrhagic stroke, ICH and SAH, account for 10 to 30 % of all stroke cases^{19,20} (Chen, Zeng, and Hu 2014; Virani et al. 2021). The socioeconomic burden of stroke is considerable. Cerebrovascular diseases, including ischemic and hemorrhagic stroke, are the second biggest cause of death in the European Union²¹ (Wafa et al. 2020).

The most common risk factors of hemorrhagic stroke are age, race, sex, hypertension, diabetes, and smoking²² (Grysiewicz, Thomas, and Pandey 2008). However, myriad other risk factors contribute to stroke. The risk factor of suffering from stroke doubles every decade, therefore the elderly are the most threatened group of individuals²³ (Ariesen et al. 2003). Generally speaking, Asians, Blacks, and Hispanics are at higher risk of suffering from stroke than Caucasians²⁴ (Labovitz et al. 2005). However, many factors play into reported numbers, such as a higher prevalence of hypertension or poorer control of hypertension in these people. Women are at lower risk of suffering from hemorrhagic stroke than men,

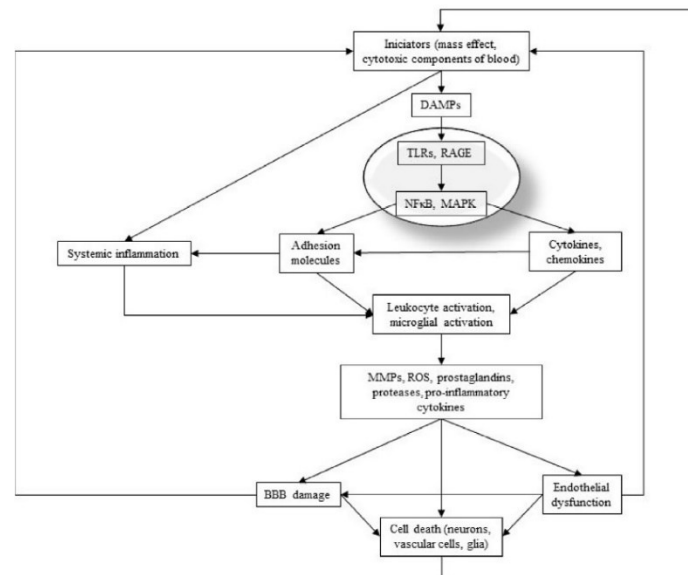


Fig. 3. Inflammatory response in hemorrhagic stroke.

which is the opposite trend from ischemic stroke²² (Grysiewicz, Thomas, and Pandey 2008). Hypertension is associated with a higher risk of developing hemorrhagic stroke^{20,22} (Grysiewicz, Thomas, and Pandey 2008; Chen, Zeng, and Hu 2014). Also, people with a so-called “unhealthy lifestyle”, which includes patients with diabetes, smokers, alcohol users, etc., have been associated with a higher risk of suffering from hemorrhagic stroke^{20,22} (Grysiewicz, Thomas, and Pandey 2008; Chen, Zeng, and Hu 2014).

The common characteristic of ICH and SAH is the formation of hematoma caused by bleeding into the brain. In ICH, the most common sites are basal ganglia and cerebral lobes²⁵ (Muschelli et al. 2015). The most common cause of SAH is a rupture of cerebral aneurysm which is most frequently localized at the circle of Willis at the bifurcation of cerebral arteries²⁶ (Chen et al. 2014). The formation of hematoma disrupts neurons, glial cells, endothelium, and smooth muscle cells, which results in hypoperfusion of the brain, ischemia, neurotransmitter release, mitochondrial dysfunction, cellular swelling, and activation of glial cells (microglia and astrocytes), resulting in inflammation and edema^{26–29} (Chen et al. 2014; Kleinig and Vink 2009; Saand et al. 2019; Testai and Aiyagari 2008).

The primary injury is caused by the mass of hematoma in the cranium, compressing the brain and increasing the intracranial pressure (ICP)^{26–28} (Chen et al. 2014; Kleinig and Vink 2009; Saand et al. 2019). This is referred to as a mass effect. As a consequence of the mass effect, cerebral perfusion pressure and cerebral blood flow are decreased, causing blood flow arrest and subsequently coagulation and ischemia^{27–28} (Kleinig and Vink 2009; Saand et al. 2019).

The secondary injury is caused by inflammation, cytotoxic components of blood (hemoglobin, iron, free radicals) overproduction, excitotoxicity, and upregulation of proteolytic enzymes^{26–30} (Chen et al. 2014; Jelinek, Jurajda, and Duris 2020; Kleinig and Vink 2009; Saand et al. 2019; Testai and Aiyagari 2008). All of this may result in damage to the endothelium, damage to the blood–brain barrier

(BBB), cerebral edema, hydrocephalus, and ultimately neuronal cell death^{26,28,31} (Chen et al. 2014; Saand et al. 2019; Zhang et al. 2020).

3. Management and treatment of sepsis and hemorrhagic stroke

3.1. Sepsis

The management and treatment of sepsis and septic shock is an important part of current research. An effective treatment should focus on an early intervention, which includes the removal of the infection source. Assessment of the source of infection (especially if unidentified) or undrained abscess through appropriate diagnostics is essential for the early management of sepsis^{10,32} (Cecconi et al. 2018; Nishie 2013). Additionally, initial sepsis management should include the initiation of appropriate antimicrobial therapy, and restoration of tissue perfusion through fluid resuscitation³² (Nishie 2013). Prompt intravenous access should be obtained, blood and other appropriate samples should be taken, and assessment for organ dysfunction and tissue hypoperfusion conducted^{10,32} (Cecconi et al. 2018; Nishie 2013).

Fluid resuscitation is important for patients with hemodynamic instability, hypotension, or elevated lactate concentration. This management should be conducted by the administration of crystalloid fluids (30 ml/kg) within the first hour^{10,32} (Cecconi et al. 2018; Nishie 2013). Control of the source of the infection is usually managed by removal of the infected tissue, drainage of an abscess, or removal of an infected device³³ (Marshall et al. 2004). It is done by open surgery or percutaneous drainage³³ (Marshall et al. 2004). Antimicrobial therapy has also been shown to be greatly beneficial to septic patients, especially when conducted promptly³⁴ (Seymour et al. 2017). Vasoactive drugs are an important part of therapy for patients with septic shock to help maintain perfusion pressure¹⁰ (Cecconi et al. 2018). Targeted mean arterial pressure is 65 mm Hg at the initial stage, but a higher targeted mean blood

pressure (80–85 mm Hg) has been suggested to have better survival rates^{10,35} (Asfar et al. 2014; Cecconi et al. 2018). The preferred first-line vasopressor is norepinephrine³⁶ (Daniel et al. 2010).

3.2. Hemorrhagic stroke

There are several different approaches to the treatment of hemorrhagic stroke. The most common ones are blood pressure management, management of raised ICP, hemostatic therapy, antiepileptic therapy, and surgery³⁷ (Lapchak and Araujo 2007).

Blood pressure should be reduced gradually to 150/90 mmHg using various forms of medication (beta-blockers, ACE inhibitors, etc.)^{20,38} (Chen, Zeng, and Hu 2014; Morotti and Goldstein 2020). ICP can be managed by elevating the head to 30 degrees and osmotic agents^{20,38} (Chen, Zeng, and Hu 2014; Morotti and Goldstein 2020). Hemostatic therapy is given to reduce the advancement of the hematoma^{20,39} (Chan et al. 2021; Chen, Zeng, and Hu 2014). Different types of surgical therapy can be used such as the evacuation of hematoma from the craniotomy, endoscopic aspiration, and stereotactic aspiration, while surgical clipping or endovascular coiling can be used in SAH patients^{20,40} (Chen, Zeng, and Hu 2014; van Gijn and Rinkel 2001). The general goal is to reduce the invasiveness of the surgery to a minimum.

4. Experimental models of sepsis and hemorrhagic stroke

4.1. Sepsis

Rodents, such as mice and rats, are the most widely used animal models for studying sepsis. Several main models have been developed so far. They include injection of a toxic agent (LPS, zymosan, CpG, or another PRR ligand), administration of viable pathogens (bacteria or intestinal contents), and impairment of barrier tissue integrity (cecal ligation and puncture model, etc.)^{41–42} (Korneev 2019; Kuzmich et al. 2017). The first two are an example of low-invasive nonsurgical methods, whereas the third is an example of a method where invasive surgery is necessary.

The murine sepsis models described below possess many limitations regarding humans. Age, gender, choice and timing of delivery, anesthetics, analgesics, and antibiotics choices are just a few examples of variation⁴³ (Lewis, Seymour, and Rosengart 2016). There is also a difference in the severity of sepsis caused by each of these methods. Furthermore, rodents are often housed in the pathogen- and microbe-free environments that do not correlate well with the environment of the “real world”⁴³ (Lewis, Seymour, and Rosengart 2016).

4.2. Impairment of barrier tissue integrity models

In the most clinically relevant sepsis models, polymicrobial peritonitis is initiated by disrupting the integrity of the gut, allowing microorganisms to enter the peritoneal cavity⁴² (Kuzmich et al. 2017). These models are widely used because they are considered to mimic human sepsis⁴² (Kuzmich et al. 2017). The endotoxin release is relatively slow and can be modified based on the number and size of punctures⁴⁴ (Hubbard et al. 2005). It is also comparable to human sepsis in regards to the hemodynamic, biochemical, and cytokine changes^{42,44} (Hubbard et al. 2005; Kuzmich et al. 2017). The most used models are the cecal ligation and puncture model, colon ascendens stent peritonitis model, and cecal ligation and incision model.

The cecal ligation and puncture (CLP) model imitates a clinical state of an intraperitoneal abscess and polymicrobial peritonitis because bacteria gradually enter the peritoneal cavity⁴¹ (Korneev 2019). The CLP does not require the administration of toxins or living pathogens. This model offers stimulation of both pro-

inflammatory and anti-inflammatory immune responses⁴⁵ (Rittirsch et al. 2009). It gives a similar dynamic of sepsis in DAMPs production, cytokine response, metabolic alterations, and the subsequent development of SIRS and MODS⁴⁵ (Rittirsch et al. 2009). In some cases, abscess form around the puncture location blocking the release of the cecal content and not allowing the sepsis to progress into septic shock, transforming an acute inflammatory response into a chronic one, which may persist for months⁴⁶ (Nacionales et al. 2016). The severity and dynamics of developing sepsis can to some extent be regulated by the length of the ligation in a certain cecal region, the number of punctures, the needle size, administration of antibiotics or another form of therapy⁴⁷ (Mishra and Choudhury 2018). Because of the varied nature of surgical parameters, such as the aforementioned needle size, ligation length, number of punctures, laparotomy technique, anesthesia, and the reaction of individual rodents, it is difficult to reproduce a specific model⁴³ (Lewis, Seymour, and Rosengart 2016). Therefore, a large sample is required for good reproducibility^{41,43} (Korneev 2019; Lewis, Seymour, and Rosengart 2016).

Colon ascendens stent peritonitis (CASP) mimics the clinical state of polymicrobial acute diffuse peritonitis⁴¹ (Korneev 2019). It better shows the pathophysiological alterations as compared to CLP, because bacteria continuously enter the peritoneal cavity together with cytokine production and MODS development⁴¹ (Korneev 2019). In CASP abscess does not form⁴³ (Lewis, Seymour, and Rosengart 2016). The dynamics of sepsis development may be regulated by varying stent diameter or by removing the stent and suturing the perforation in repeated surgery⁴¹ (Korneev 2019). This model is not as explored as CLP, the hemodynamic and metabolic changes are not completely characterized, and the immune response is not reproduced because the anti-inflammatory cytokines arise almost at the same time as the pro-inflammatory^{41,43} (Korneev 2019; Lewis, Seymour, and Rosengart 2016).

Cecal ligation and incision (CLI) is a relatively new model to mimic the more acute onset of sepsis compared to CLP⁴¹ (Korneev 2019). Unlike CLP, CLI is not used as widely. The metabolic, hemodynamic, and immune response changes have not yet been fully characterized⁴¹ (Korneev 2019).

4.3. Administration of viable pathogens models

Administration of live Gram-positive (e.g. *Staphylococcus* and *Pseudomonas*) or Gram-negative (e.g. *Escherichia coli*) bacteria provides a low-invasive method with high reproducibility to induce sepsis⁴¹ (Korneev 2019). It is a suitable model for studying the mechanisms by which a particular pathogen triggers the immune response, depending on whether clinical isolates or laboratory strains are used⁴¹ (Korneev 2019). A specific polymicrobial injection may be utilized, but more commonly only a single bacteria strain is used⁴² (Kuzmich et al. 2017). Single bacteria strains may be a good model for hospital mono-infections, but it does not mimic polymicrobial sepsis⁴¹ (Korneev 2019).

The magnitude of the host's response depends on whether intravenous or intraperitoneal injection is used, with intraperitoneal showing an attenuated response⁴³ (Lewis, Seymour, and Rosengart 2016). In general, this model poorly reflects the human sepsis state. A single massive administration of the bacteria is used to achieve bacterial infection, whereas in sepsis there is a continuous spread of bacteria from the infection site⁴¹ (Korneev 2019). The single massive administration of bacteria causes many bacteria to die because of complement activation, the resulting development of endotoxemia causes death and limits the full development of sepsis⁴¹ (Korneev 2019). Some researchers tried to resolve this problem by using vascular catheters and infusing bacteria over several hours⁴⁸ (Poli-de-Figueiredo et al. 2008).

The bacteria and fibrin clot implantation model combines standardized amounts of bacteria which serve as a reservoir for bacte-

ria release⁴³ (Lewis, Seymour, and Rosengart 2016). This fibrin clot with bacteria is implanted in the peritoneal cavity which requires surgery and general anesthesia⁴¹ (Korneev 2019). It results in a continuous release of bacteria, better mimicking the gradual spread of bacteria from the infection⁴³ (Lewis, Seymour, and Rosengart 2016). This model prevents early mortality associated with bacterial injection, it is suitable for studying mono-infections and their treatment, it is simpler than CLP or CASP, and sepsis can be regulated by changing the clot density and bacterial concentration^{41,43} (Korneev 2019; Lewis, Seymour, and Rosengart 2016). Some authors conflict about whether this is a reproducible model, there are some difficulties with preparing the infected fibrin clot and surgical technique, however, these parameters are potentially controllable^{41,43} (Korneev 2019; Lewis, Seymour, and Rosengart 2016).

Intraperitoneal injection of a fecal solution or cecal slurry is a simple, low-invasive method that provides polymicrobial sepsis⁴⁹ (Korneev 2019). The dynamics may be regulated by changing the injected amount⁴⁹ (Starr et al. 2014). However, it is difficult to standardize and reproduce metabolic, hemodynamic, and immunological features of this approach, because of the variation in pathogen composition injected⁴⁹ (Starr et al. 2014).

4.4. Induction of administering toxins models

Injection of PRR ligands (LPS, CpG, peptidoglycan, lipoteichoic acids, zymosan, etc.) is an easy-to-perform model for inducing inflammatory response with good reproducibility^{41–42} (Korneev 2019; Kuzmich et al. 2017). There is an early release of high levels of pro-inflammatory cytokines which promote the rapid development of SIRS⁴² (Kuzmich et al. 2017). The disadvantage coming with this is that the pathophysiological aspects of human sepsis are not fully mimicked⁴² (Kuzmich et al. 2017).

The most used model usually consists of either intraperitoneal or intravenous injection of purified LPS into the host animal⁴³ (Lewis, Seymour, and Rosengart 2016). The response to LPS doses is variable, smaller doses cause a hyperdynamic state whereas larger doses produce hypotension and hypothermia mimicking a murine septic shock⁴³ (Lewis, Seymour, and Rosengart 2016). On the molecular level, important pro-inflammatory cytokines such as TNF α , IL-1 β and IL-6 peak earlier and for a shorter duration after intravenous application in endotoxemia, compared to intraperitoneal administration⁴³ (Lewis, Seymour, and Rosengart 2016).

The advantages of this model are low invasiveness, it is easily controllable, standardized, and reproducible⁴¹ (Korneev 2019). There are several alternative methods for LPS administration. There is no need to manipulate pathogens. Although it is easy to do this model, it poses some limitations, LPS is a single component of the many PAMPs associated with Gram-negative bacteria, it ignores the host-pathogen interaction of the polymicrobial sepsis, the model usually results in endotoxemia rather than sepsis. In general, the pathophysiological aspects of human sepsis are not fully mimicked^{41–43} (Korneev 2019; Kuzmich et al. 2017; Lewis, Seymour, and Rosengart 2016).

Besides LPS administration, there is also a method where D-galactosamine (D-GalN) is administered together with LPS for better sensitization to LPS⁴¹ (Korneev 2019). This model possesses all the advantages of the only LPS administration and reduces the amount of LPS needed⁴¹ (Korneev 2019). With this method, septic shock develops rapidly, leading to early death.

4.5. Hemorrhagic stroke

Models of intracerebral hemorrhage

Experimental models of ICH have been around since the 1960 s. The most common models involve the injection of autologous blood or bacterial collagenase into the brain⁵⁰ (Yan, Chopp, and

Chen 2015). Many species have been used in these experiments, including rodents, cats, dogs, and primates⁵⁰ (Yan, Chopp, and Chen 2015).

The autologous blood injection models are straightforward methods that involve introduction of blood to the brain and the subsequent formation of hematoma in a single injection. Blood is taken from the same animal into which it is later injected. Different volumes can be used to establish the hematoma model. The rapid accumulation of blood in the brain parenchyma is applicable to ICH patients⁵¹ (Clark et al. 1998). Cerebral blood flow is reduced around the hematoma and the surrounding brain tissue, but there is no significant alteration in cerebral perfusion pressure⁵² (Krafft et al. 2012).

The collagenase injection models involve the use of collagenases which are proteolytic enzymes that are secreted at sites of inflammation⁵² (Krafft et al. 2012). Brain tissue contains collagen in the basal lamina of its blood vessels⁵² (Krafft et al. 2012). The pathophysiological mechanisms described earlier lead to the release of collagenase from the injured cells⁵³ (Woo and Broderick 2002). Therefore, collagenase injection can be directly used to disrupt the extracellular matrix in the basal lamina⁵² (Krafft et al. 2012). The collagenase injection models are well reproducible for the study of spontaneous intracerebral bleeding that develops over several hours⁵⁴ (MacLellan et al. 2008).

Other models used to induce ICH are balloon inflation models, which involve inducing the ICH using a balloon introduced into the right striatum through a burr hole⁵⁵ (Shi et al. 2010). It was used in piglets⁵⁵ (Shi et al. 2010). The avulsion of cerebral blood vessels model involves stripping the cortical surface of blood vessels, where avulsion of the veins creates cortical hemorrhages⁵⁶ (Xue and Del Bigio 2003). In the avulsion model, there is more necrosis, less hemorrhage, and lesser neutrophil infiltration than in the autologous blood injection model and collagenase injection model⁵⁶ (Xue and Del Bigio 2003).

Models of subarachnoid hemorrhage

SAH is usually caused by the rupture of an arterial aneurysm from which blood flows into the subarachnoid space. The most common models to study the effects of subarachnoid hemorrhage include injecting blood into the cisterna magna (once or twice) and endovascular perforation of an intracranial artery.

The blood injection model uses blood administration to the cisterna magna⁵⁷ (Marbacher, Fandino, and Kitchen 2010). In the single hemorrhage, models an injection is given only once, in the double hemorrhage models a second injection with autologous arterial blood is given 48 h after the first⁵⁰ (Yan, Chopp, and Chen 2015). The model is quite reproducible due to the fixed blood amount injected in the subarachnoid space⁵⁸ (Prunell et al. 2018).

The endovascular perforation model uses the external carotid artery (ECA) as the main access point⁵⁹ (Sehba 2015). The ECA branches are dissected, cauterized, and divided. A suture is inserted in the ECA, advances through the internal carotid artery, and is finally put to the middle cerebral artery where the vessel is punctured⁵⁹ (Sehba 2015). The suture is withdrawn through the same route⁵⁹ (Sehba 2015). This model allows reperfusion and produces a SAH that imitates the clinical situation to the greatest extent possible⁵⁰ (Yan, Chopp, and Chen 2015). The most common animal used in this model are rats, although mice have also been used⁵⁰ (Yan, Chopp, and Chen 2015).

5. Local to systemic inflammatory response

Inflammation is a set of physiological responses of the organism to violation of its integrity⁶⁰ (Medzhitov 2010). Inflammatory response likely evolved as a response to restore homeostasis⁶¹

(Kotas and Medzhitov 2015). Inflammation can be triggered by noxious stimuli and conditions, such as infection, chemical and physical tissue damage, and ischemia⁶² (Medzhitov 2008). It leads to adaptive reactions that result in the localization of the damage, its elimination, and healing.

The classical signs of local inflammation are redness (rubor), swelling (tumor), pain (dolor), heat (calor), and loss of function⁶⁰ (Medzhitov 2010). It is generally thought that a controlled inflammatory response is beneficial (for example when fighting an infection), but it can become harmful when deregulated (for example causing septic shock)¹⁶ (Duris and Jurajda 2020).

Immune system reaction occurs almost instantly after an injury and is considered to be a two-step process, local and systemic⁶³ (Lucke-Wold et al. 2016). Acute inflammation can be triggered by infection or injury and happens in a horizon of minutes and hours⁶⁴ (Kumar et al. 2004). This inflammatory response can be best characterized by microbial infection (particularly bacterial). In this setting, the inflammatory response is triggered by receptors of the innate immune system, such as PAMPs and DAMPs^{12,62} (Barton 2008; Newton and Dixit 2012). After recognition, mastocytes and tissue-resident macrophages mediated the initial inflammatory response⁶⁵ (Barton 2008). They give the first signals for the development of an inflammatory response by the production of a variety of inflammatory mediators (cytokines, chemokines, eicosanoids, etc.)⁶⁶ (Wright 1997). Endothelial cells upregulate adhesion molecules⁶⁷ (Duris et al. 2019).

The main effect of these inflammatory mediators is to elicit plasma proteins and leukocytes to access the site of infection, thus initiating the systemic inflammatory response¹⁵ (Newton and Dixit 2012). Leukocytes and plasma proteins are normally restricted to the blood vessels, but now the activated endothelium allows selective extravasation¹⁵ (Newton and Dixit 2012). This selectivity is mediated by the interaction of endothelial selectins with integrins and chemokine receptors on the surface of leukocyte-monocytes/macrophages and neutrophils¹⁵ (Newton and Dixit 2012). Leukocytes then follow gradients of cytokines and other inflammatory mediators to the exact site of infection⁶⁸ (Kratofil, Kubes, and Deniset 2017). In the affected tissue site, neutrophils become activated, either by cytokines from the tissue-resident cells or by direct contact with the pathogen⁶⁸ (Kratofil, Kubes, and Deniset 2017). Neutrophils try to kill the invading agents by phagocytosis or degranulation of various toxic components including reactive oxygen species (ROS), reactive nitrogen species, proteinase 3, cathepsin G, and elastase⁶⁹ (Nathan 2006). These effective components do not differentiate between microbial and host targets, so collateral damage to the host tissue is unavoidable^{16,70} (Duris and Jurajda 2020; Nathan 2002).

A successful inflammatory response results in the elimination of the infectious agents and the promotion of repair phase. If the acute inflammatory response fails to eliminate the infectious agents, the inflammatory process persists and acquires new attributes.

The systemic inflammatory response can occur even without local inflammation if there is a massive infiltration of microorganisms into the bloodstream, such as in septic shock⁷¹ (Cai, Deitch, and Ulloa 2009). Alternatively, by massive degranulation of basophils and complement activation by non-infectious intravascular antigen stimuli, such as in anaphylactic shock⁷² (Reber, Hernandez, and Galli 2017). Both states are life-threatening events, because of the extensive release of inflammatory mediators, which cause strong vasodilatation, leading to hypotension, circulatory failure, multiple organ dysfunction syndrome (MODS), and ultimately death^{10,72} (Cecconi et al. 2018; Reber, Hernandez, and Galli 2017).

When the inflammatory response is exaggerated it can evolve into systemic SIRS. SIRS is an inflammatory state that affects the whole body. Symptoms of SIRS are fever ($>38^{\circ}\text{C}$) or hypothermia

($<36^{\circ}\text{C}$), tachycardia (heart rate $> 90/\text{min}$), tachypnea (respiratory rate $> 20/\text{min}$ or $\text{PaCO}_2 < 32 \text{ mm Hg}$) and leukocytosis ($>12\,000/\text{mm}^3$) or leukopenia ($<4\,000/\text{mm}^3$)⁴ (Singer et al. 2016). Two or more symptoms must be met to diagnose SIRS⁴ (Singer et al. 2016). Fever is induced by stimulation of the anterior hypothalamic nucleus and the adjacent preoptic area of the hypothalamus by prostaglandin E2 (PGE2)⁷³ (Garami, Steiner, and Romanovsky 2018). PGE2 synthesis is induced by proinflammatory cytokines that stimulate its creation from arachidonic acid by cyclooxygenase⁷³ (Garami, Steiner, and Romanovsky 2018). This hypothalamic stimulation leads to the activation of hypothalamus – pituitary gland – adrenal glands axis, ultimately increasing tissue metabolism⁷³ (Garami, Steiner, and Romanovsky 2018). Temperature increase in itself is an important activator of metabolic processes in immunocompetent cells⁷⁴ (Evans, Repasky, and Fisher 2016).

6. Inflammatory response in sepsis and stroke

Inflammatory response plays a pivotal role in the pathophysiology of both sepsis and hemorrhagic stroke. It is associated with overexpression of adhesion molecules on monocytes, neutrophils and endothelial cells lead to enhanced presence of blood phagocytes in organs. Complement activation also contributes to organ injury and lethality in sepsis⁷⁵ (Ward 2010). Also, in both of these conditions SIRS may develop⁷¹ (Cai, Deitch, and Ulloa 2009).

6.1. Sepsis

Inflammation is the core of sepsis. In sepsis, there is evidence of overactivation of phagocytes and dendritic cells, but later there is the functional deterioration of these cells⁷⁶ (Delano et al. 2011). It results in reduced innate immunity functions⁷⁶ (Delano et al. 2011). The onset of sepsis is accompanied by inadequate innate and adaptive immune responsiveness which can contribute to immunosuppressant effect early on in sepsis⁷⁷ (Ward 2011). An immunosuppressive environment impairs the efficient removal of infectious organisms (especially those that triggered the septic response)⁷⁸ (Bosmann and Ward 2013). Immunosuppression in sepsis is caused by extensive apoptotic depletion of T and B cells from their reservoirs⁷⁵ (Ward 2011). It is caused by activation of both intrinsic (mitochondrial) and extrinsic (Fas and Fas ligand, TNF α and its receptors) pathway⁷⁹ (Takahashi et al. 2012). Macrophages and dendritic cells depletion also occur, resulting in defective antigen presentation to T cells and compromising adaptive immunity⁷⁵ (Ward 2011).

During inflammatory response in sepsis, neutrophils and macrophages produce high amounts of reactive oxygen species (ROS) and in some cases nitric oxide (NO), which appears to intensify SIRS⁸⁰ (Andrades et al. 2011). This oxidative state quickly lowers antioxidants by depleting them³⁰ (Jelinek, Jurajda, and Duris 2020).

Septic patients have confirmed or suspected infection by definition. It can be accompanied by endotoxemia if endotoxin can be detected in the bloodstream⁸¹ (Dickson and Lehmann 2019). Endotoxin is released by microorganisms during growth and when they are destroyed⁸¹ (Dickson and Lehmann 2019). The immune system induces a strong response to endotoxins. Lipopolysaccharide (LPS) is a part of the cell membrane of Gram-negative bacteria (such as *Escherichia coli*) and it is a classic endotoxin with well-established signaling and a role in inflammatory pathways. Other bacterial components (for example peptidoglycan or lipoteichoic acid) from both Gram-negative and Gram-positive bacteria can also induce systemic inflammatory response⁸¹ (Dickson and Lehmann 2019). As LPS is the most common endotoxin used in sepsis

and endotoxemia models we will describe the molecular signaling in the model of LPS.

From the structural point of view, LPS consists of three parts, the lipid A endotoxin, core oligosaccharide, and O-antigen⁸² (von Kügelgen et al. 2020). The lipid A endotoxin is responsible for LPS toxicity in sepsis, the O-antigen part varies between bacterial species and gives a species-specific serotype⁸² (von Kügelgen et al. 2020).

6.2. TLR signaling in sepsis

Molecular signaling in response to LPS involves an interaction between LPS and a specific membrane receptor of the immune cell. The TLR is a family of receptors that triggers innate immune response after recognizing bacterial PAMPs. LPS activates TLR4 and myeloid-differentiation factor 2 (MD-2), which is an associated protein needed for correct LPS signaling⁸¹ (Dickson and Lehmann 2019). Two more molecules are needed for the signaling, serum lipopolysaccharide-binding protein (LBP) and membrane-associated CD14 protein⁸³ (Park et al. 2009). They allow LPS to bind to TLR4. LPS binds to the hydrophobic pocket in MD-2 through interaction with lipid A⁸³ (Park et al. 2009). This signaling results in the formation of a dimer consisting of two copies of the TLR4-MD-2 complex and an LPS bridge^{42,83} (Kuzmich et al. 2017; Park et al. 2009). The signaling cascade continues intracellularly by recruiting an intracellular domain of the complex which can activate MyD88-dependent and MyD88-independent pathways. The intracellular signal can follow the TLR4/MyD88/NFκB or TLR4/TRIF/IRF3 pathways.

The TLR4/MyD88/NFκB pathway starts from the LPS/TLR4/MD-2 dimer located on the plasma membrane⁴² (Kuzmich et al. 2017). CD14 plays an important role in promoting the internalization of this complex into endosomes⁸⁴ (Zanoni et al. 2011). In this MyD88-dependent pathway, TIRAP adapters bind to the intracellular TIR-domains of the dimerized TLR4 and together they form a platform for the arrival and binding of MyD88⁴² (Kuzmich et al. 2017). The MyD88 molecules then bind serine/threonine kinases called interleukin-1 receptor-associated kinases (IRAK) 2 and 4⁴² (Kuzmich et al. 2017). Together they form the so-called myddosome, which promotes IRAK4 autophosphorylation⁸⁵ (Gay, Gangloff, and O'Neill 2011). IRAK1 can as well bind to the MyD88-IRAK4 complex and be phosphorylated by its kinase domain⁴² (Kuzmich et al. 2017). Then, TNF receptor-associated factor 6 (TRAF6) is recruited to bind to IRAK1, forming a trimer⁸⁶ (Guvén-Maiorov et al. 2015). E3 ubiquitin ligase poly-ubiquitinates chains of TRAF6 which are recognized by TAB2/TAB3 adaptor proteins and IKKγ subunit of IKK-complex⁴² (Kuzmich et al. 2017). It allows the recruitment and activation of TAK1 and phosphorylation of the IκB complex⁴² (Kuzmich et al. 2017). IκB complex phosphorylation promotes degradation and the release of NFκB⁴² (Kuzmich et al. 2017). TAK1 along with NFκB induces the production and release of pro-inflammatory cytokines TNFα, IL-1β, and IL-6⁴² (Kuzmich et al. 2017).

The TLR4/TRIF/IRF3 transduction begins after complex internalization into endosomes⁴² (Kuzmich et al. 2017). Then, the TRIF-related adaptor molecule (TRAM) binds to the TLR4's TIR-domains⁴² (Kuzmich et al. 2017). TRAM has an analogous bridging function to the TIRAP adapter of the MyD88-dependent signaling pathway. TRIF binds to this TLR4-TRAM complex⁸⁷ (Gay et al. 2014). Phosphorylation of TRIF is required to activate and recruit IRF3 by TRAF3⁸⁷ (Gay et al. 2014). Phosphorylated IRF3 dimer is translocated to the nucleus to initiate the transcription of the IFN-β genes⁸⁷ (Gay et al. 2014). In some situations, TRAF6 can bind to TRIF and activate NFκB⁸⁷ (Gay et al. 2014).

Toll-like receptor 4 (TLR4) signaling does not have to be activated only by pathogen-associated molecular patterns molecules (PAMPs) (e.g., LPS). Damage-associated molecular pattern mole-

cules (DAMPs) which come from damaged and necrotic tissues can also activate TLR4 response⁴² (Kuzmich et al. 2017). Other endogenous stimuli such as high-mobility group box 1 (HMGB1) have also produced TLR4 activation⁸⁸ (Mu et al. 2018). LPS possesses a relatively conserved lipid A structure that ensures the optimal interaction with CD14 and MD-2 molecules⁴² (Kuzmich et al. 2017). On the other hand, DAMPs are a structurally diverse group of molecules and the role of CD14 and MD-2 in TLR4 activation is not yet fully understood⁴² (Kuzmich et al. 2017). DAMPs are implicated to cause TLR4 activation in many pathologies, such as atherosclerosis, rheumatoid arthritis, neuroinflammation, trauma, and hemorrhage^{42,88} (Kuzmich et al. 2017; Mu et al. 2018).

Sepsis is the most serious PAMPs/TLR4 disease. Septic shock with increased LPS levels in the bloodstream leading to violation of local and general hemodynamics and endothelial dysfunction has been shown to signal via the TLRs signaling pathway⁸⁹ (Keynan et al. 2011). Among others, sepsis is a possible complication after severe influenza⁸⁹ (Keynan et al. 2011).

TLR4 signaling and the subsequent release of NFκB from the IKK-complex plays a role in the expression of inflammasome NLRP3⁹⁰ (Danielski et al. 2020). NLRP3 is another important component of the complex pathophysiology of sepsis and can be associated with pyroptosis. It is a macromolecular protein complex that regulates the activation of caspase-1 and secretion of pro-inflammatory cytokines (IL-1β and IL-18)⁹⁰ (Danielski et al. 2020). Inhibiting the assembly and activation of NLRP3 inflammasome may help to ameliorate the inflammatory response in sepsis⁹⁰ (Danielski et al. 2020). Similar signaling involving the NLRP3 role has been also described in hemorrhagic stroke⁹¹ (Luo, Reis, and Chen 2019).

6.3. Hemorrhagic stroke

Sepsis and hemorrhagic stroke have many common features that were discussed above. However, unlikely in sepsis, a strong inflammatory response in hemorrhagic stroke is not caused by pathogen infection, it is an aseptic inflammation.

The clinical manifestation of systemic inflammation in hemorrhagic stroke is very similar to the clinical signs in sepsis. It is no surprise because these criteria were originally selected for acute diseases or injuries such as sepsis⁴ (Singer et al. 2016). Several studies have also reported a positive correlation between the development of SIRS and higher risk for other SAH and ICH complications and worse outcome^{92–98} (Boehme et al. 2018; Dhar and Diringer 2008; Elmer et al. 2017; Festic et al. 2014; Rass et al. 2018; Tam et al. 2010; Yoshimoto, Tanaka, and Hoya 2001). Meeting SIRS criteria were also associated with larger hematoma volume, suggesting that SIRS may be a response or associative marker for SAH and ICH severity. SIRS seems to be an important clinical feature in hemorrhagic stroke and may contribute to general outcome and associated complications.

The interaction between local cerebral inflammation and systemic inflammation is of great importance in the pathophysiology of hemorrhagic stroke. As mentioned above, peripheral immune activation influences stroke outcome. For example, an experimental study where rats underwent splenectomy found that it had a beneficial effect on the reduction of infarct size and inflammatory infiltrate⁹⁹ (Ajmo et al. 2008; Lee et al. 2008). Thus, having an anti-inflammatory effect^{99,100} (Ajmo et al. 2008; Lee et al. 2008). Another study, focusing on RANTES (also known as CCL5) in ICH, found that RANTES-deficient mice had significantly blunted effects of ICH in terms of leukocyte and platelet adhesion, BBB permeability and tissue infarction¹⁰¹ (Terao et al. 2008). RANTES is a potent chemotactic factor¹⁰¹ (Terao et al. 2008).

Immune system reaction is initiated very almost instantly after the onset of bleeding. It is considered to be a two-step process with

local and systemic components⁶³ (Lucke-Wold et al. 2016). The local response happens over a horizon of minutes and hours and involves the activation of glial cells (microglia and astrocytes) and endothelial cells⁶⁷ (Duris et al. 2019). After activation glial cells upregulate the production of pro-inflammatory cytokines, and endothelial cells upregulate adhesion molecules⁶⁷ (Duris et al. 2019). Both processes contribute to the infiltration of neutrophils and macrophages, which is a part of the systemic inflammatory response and happens in the horizon of days.

The inflammatory responses of ICH and SAH are somewhat similar, but there are some important differences. In ICH, blood components are introduced directly to the brain parenchyma. Thrombin in particular seems to play a pivotal role in the initiation of inflammatory mechanisms following ICH. When heparinized blood is experimentally introduced to the brain it causes minimal edema, where thrombin infusion causes massive leukocyte infiltration and edema²⁷ (Kleining and Vink 2009).

Several studies have focused on the connection between the elevation of circulating leukocytes and complications associated with worsened clinical outcome,^{28,92–103} (Boehme et al. 2018; Chou et al. 2011; Saand et al. 2019; Tapia-Pérez et al. 2016). In SAH, leukocytosis has been associated with an increased incidence of vasospasms¹⁰² (Chou et al. 2011). It is thought that SAH patients with a higher risk for complications and poor outcomes, have a different inflammatory response to the initial insult over time²⁸ (Saand et al. 2019). This difference in immune response can distinguish between patients at risk for vasospasm and/or poor outcome²⁸ (Saand et al. 2019). Studies focusing on leukocyte elevation associated with ICH outcome have provided conflicting results^{103–105} (Morotti et al. 2016; Tapia-Pérez et al. 2016; Yu et al. 2016).

Fever, although a physiological response with beneficial effects in infection, is generally associated with worse outcomes for many acute diseases, specifically patients with ICH and SAH^{106–107} (Bush 2018; Kramer et al. 2017). SAH patients with fewer fever hours had better clinical outcomes¹⁰⁸ (Pegoli et al. 2015). Moreover, the fever seems to be an independent risk factor for delayed cerebral ischemia (DCI) following SAH¹⁰⁹ (Park 2018). Fever is very prevalent in ICH patients and a higher fever burden is associated with neurological deterioration and worsened outcome,^{94,106–110} (Bush 2018; Elmer et al. 2017; Gillow et al. 2017). Therefore, fever management and targeted temperature management are recommended¹¹¹ (Madden et al. 2017). The general evaluation of whether fever control can improve outcome and reduce complications associated with hemorrhagic stroke remains to be determined.

6.4. Inflammatory response after ICH

For the inflammatory response following ICH is typical rapid activation of resident microglia and infiltration of circulating inflammatory cells¹¹² (Zhou et al. 2014). Excessive microglial activation promotes inflammatory injury by releasing pro-inflammatory mediators, which promote infiltration of peripheral inflammatory cells¹¹³ (Zhang et al. 2017). Recruitment of peripheral leukocytes occurs early after experimentally induced ICH⁵⁰ (Yan, Chopp, and Chen 2015). The increased neutrophil count is associated with deterioration in ICH patients¹¹² (Zhou et al. 2014). Therefore, inhibition of both microglial and leukocyte activation attenuates BBB damage, brain edema, and subsequent cell death and poses a promising target for treatment in ICH patients.

T cells, both CD8+ and CD4+, are increased after ICH and contribute to inflammatory injury. Decreased total counts of T cells in the perihematomal area improve neurological function and reduces brain edema after ICH in mouse models^{114,115} (Rolland et al. 2013; Xie, Qin, and Yin 2020). The number of reactive astrocytes also increases in the perihematomal area and contributes to secondary injury through induction of matrix metalloproteinase

(MMP) 9 activity,^{50,116–117} (Munakata et al. 2013; Yan, Chopp, and Chen 2015; Pan, Liu, and Liu 2021).

Taken together, microglial activation, neutrophil infiltration from the periphery, increase in T-cells, and reactive astrocytes have unfavorable effects after ICH.

6.5. Inflammatory response after SAH

In SAH inflammatory response glial activation, of microglia and astrocytes, is caused by transient ischemia¹¹⁸ (D'Souza 2015). Activated glial cells release pro-inflammatory cytokines, which promote leukocyte infiltration and activation of the endothelium. Activation of endothelium results in upregulation of adhesion molecules, which further reinforces leukocyte infiltration. Leukocyte infiltration is considered to be a systemic inflammatory response.

Infiltrate leukocytes cause collateral damage by the production of MMPs, ROS, and continuous release of pro-inflammatory cytokines making it a positive feedback loop⁶³ (Lucke-Wold et al. 2016). These molecules can cause BBB damage, endothelial apoptosis, vasospasms, and neuronal cell death⁶³ (Lucke-Wold et al. 2016).

6.6. Molecular signaling in hemorrhagic stroke

Similarly, to sepsis, the initiators of the inflammatory response to hemorrhagic stroke are TLRs. It has been demonstrated that TLRs have many endogenous ligands and function as “danger sensors” by recognizing damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs). DAMPs are released early after the tissue injury. High mobility group box 1 (HMGB1) is potent DAMPs that are expressed after brain injury, such as hemorrhagic stroke¹¹⁹ (Paudel et al. 2020).

HMGB1 can interact with TLR2, TLR4, or TLR9 to ultimately trigger NF- κ B and interferon regulatory factors (IRF) activation⁸⁸ (Mu et al. 2018). Similarly, to the signaling described in the molecular signaling in sepsis, TLRs can signal through MyD88-dependent and MyD88-independent pathways¹²⁰ (Wang et al. 2013). In the MyD88-dependent pathway, TLRs activity upregulates MyD88, which interacts with interleukin-1 receptor-associated kinases (IRAKs) to activate p38 mitogen-activated protein kinase (MAPK) or TNF-receptor associated factor (TRAF) 3 or TRAF6 to induce activation of downstream factors like NF- κ B and IRF¹²⁰ (Wang, Ge, and Zhu 2013). In the early stages of SAH, the HMGB1 mediated TLR4/MyD88/NF- κ B signaling pathway induces neuronal apoptosis, nerve injury, and brain edema¹²¹ (Zhang et al. 2016). The MyD88-independent pathway signals through TIR-domain-containing-adaptor-inducing interferon (INF)- β (TRIF) which interacts with TRIF-related adaptor molecule (TRAM), activating TRAF3 or TRAF6 and finally phosphorylating I κ B ϵ to activate IRF3, IRF7, and IFN- β ¹²² (Wang, Ge, and Zhu 2013).

TLR signaling induces the synthesis and secretion of pro-inflammatory cytokines like IL-1 β , IL-6, and TNF- α ¹²⁰ (Wang, Ge, and Zhu 2013). They promote both the local and systemic inflammatory response and upregulate the expression of HMGB1 and receptors for advanced glycation end-products (RAGE), forming a positive feedback loop^{88,120} (Mu et al. 2018; Wang, Ge, and Zhu 2013).

HMGB1 can interact with RAGE. RAGE activates MAPK, extracellular signal-regulated kinase (ERK) 1, and ERK2, which in turn causes phosphorylation and degradation of the inhibitor of nuclear factor κ B (I κ B) to activate NF- κ B^{88,123} (Mu et al. 2018; Murakami et al. 2011; Yang et al. 2010). RAGE is more expressed around the hematoma, which promotes the activation of NF- κ B in the early stages^{124,125} (Li et al. 2014; Zhao et al. 2016).

Another important signaling cascade involves Janus kinase (JAK) and signaling transducer and activator of transcription (STAT). The most prominent in hemorrhagic stroke are JAK2 and STAT1 and STAT3¹²⁶ (Nicolas et al. 2013). The signaling occurs

when the ligand bind to its corresponding receptor, which phosphorylates JAK2; JAK2 then phosphorylates STAT1 or STAT3¹²⁷ (Raible, Frey, and Brooks-Kayal 2014). They dimerize and are translocated to the nucleus, where they bind to specific promoter region and activates transcription¹²⁷ (Raible, Frey, and Brooks-Kayal 2014). This signaling can produce both pro- and anti-inflammatory mediators, depending on the specific molecules and ligands involved¹²⁶ (Nicolas et al. 2013).

PI3K/Akt/mTOR pathway has also been discussed in recent studies^{128,129} (Okada 2019; Sun 2019). The most important molecules in this signaling pathway are receptor tyrosine kinase (RTK), phosphatidylinositol 3-kinase (PI3K), Akt also known as protein kinase B (PKB), and mammalian target of rapamycin (mTOR). To put it simply, the RTK is activated by its corresponding ligand (cytokine, growth factor, hormone, etc.) and autophosphorylation occurs. RTK activates PI3K which in turn phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-trisphosphate (PIP3)¹³⁰ (Manning and Cantley 2007). PIP3 binds to Akt which by activation of other downstream molecules can activate mTOR¹³⁰ (Manning and Cantley 2007). PI3K/Akt/mTOR is an important signaling pathway that is involved in cell metabolism, proliferation, cancer, apoptosis, autophagy, and many other processes. This pathway is also important in hemorrhagic stroke. It is a key regulator of autophagy in the early stages where it has neuroprotective effects¹²⁴ (Li et al. 2020). Inhibiting the activity of mTOR decreases neuronal damage in hemorrhagic stroke^{131–132} (Kardideh et al. 2019; You et al. 2016). Modulation of the PI3K/Akt/mTOR pathway by certain molecules (such as OX40) can lead to the elevation of anti-apoptotic markers¹³³ (Wu et al. 2020).

7. Conclusion

Exaggeration/deregulation of inflammatory response is a medical problem, because it may be associated with additional morbidity and mortality. Sepsis represents a model pathology of inflammatory response exaggeration/deregulation. Understanding similar features of inflammatory response in sepsis and hemorrhagic stroke may help us better understand the mechanisms of inflammatory response exaggeration/deregulation. This is important for the development of new treatment strategies to control the inflammatory response in stroke.

CRedit authorship contribution statement

Matyas Jelinek: Visualization, Writing – original draft, Writing – review & editing. **Kamil Duris:** Conceptualization, Writing – review & editing.

Ethical statement

This review article does not contain any studies with human participants or animals performed by any of the authors.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. All the authors have consented to the publication of this manuscript.

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4.1.3 Cholinergic Anti-inflammatory Pathway and Stroke

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CHAIP represents a physiological mechanism by which CNS regulates an activity of an immune system and controls inflammation. In this review article, we discuss the role of CHAIP in stroke and VNS as a possible tool to control inflammation.

After brief introduction notes on stroke and inflammatory response, we describe the main components of CHAIP which include the vagus nerve, spleen, and $\alpha 7$ nicotinic acetylcholine receptor ($\alpha 7$ nAChR). We cover anatomy, physiology, and molecular signaling pathways in CHAIP. In the next part, we overview CHAIP as a potential target for therapy after stroke. We discuss pharmacological stimulation of $\alpha 7$ nAChR as well as both direct and indirect vagus nerve stimulation.



REVIEW ARTICLE

Cholinergic Anti-inflammatory Pathway and Stroke

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Abstract: Background: Stroke is devastating cerebrovascular event which is responsible for 6.7 million deaths each year worldwide. Inflammation plays an important role in the pathophysiology of stroke. Targeting inflammation after stroke is highly actual topic for both experimental and clinical research.

Methods: Research articles related to cholinergic anti-inflammatory pathway (CHAIP) and stroke were reviewed. The first part of review describes the basic characteristics of inflammatory response after stroke, main components and function of CHAIP. The second part reviews studies focused on CHAIP as a therapeutic target for ischemic and hemorrhagic stroke. Both pharmacological stimulation of $\alpha 7$ nAChR and vagus nerve stimulation after stroke are reviewed.

Results: Cholinergic anti-inflammatory pathway (CHAIP) is a physiological mechanism by which central nervous system regulates immune response and controls inflammation. Vagus nerve, spleen and $\alpha 7$ nicotinic acetylcholine receptor ($\alpha 7$ nAChR) are the main components of CHAIP.

Conclusion: Targeting cholinergic anti-inflammatory pathway is a promising way of immunomodulation which attenuates inflammation in a complex manner without causing immunosuppression.

Keywords: Cerebral ischemia, cholinergic anti-inflammatory pathway, inflammation, intracerebral hemorrhage, stroke, subarachnoid hemorrhage, Vagus nerve stimulation, $\alpha 7$ nicotinic acetylcholine receptor ($\alpha 7$ nAChR).

1. INTRODUCTION

Stroke is devastating cerebrovascular event which is a major cause of long term disability and premature death [1, 2]. Stroke is responsible for 6.7 million deaths each year (11.9% of all deaths) and it represents the number two cause of the death worldwide [3]. The majority of stroke (around 80%) is of ischemic etiology due to vessel obstruction mainly by arterial thrombosis or by embolization from the heart [4, 5]. The remaining 20% of strokes are of hemorrhagic etiology due to vessel or aneurysm rupture, resulting in intracerebral (ICH) or subarachnoid hemorrhage (SAH) [4].

Inflammation plays an important role in the pathophysiology of stroke. Despite the differences in the pathophysiology of ischemic and hemorrhagic stroke, which may be traced on the level of gene expression, inflammatory response is quite uniform regardless of stroke type and below described steps of inflammatory response were previously reviewed elsewhere [6-9]. Ischemia, extravasation of blood from vessels as well as direct tissue damage are strong

pro-inflammatory signals inducing activation of microglia, astrocytes and endothelial cells, which start to produce pro-inflammatory cytokines and adhesion molecules. Pro-inflammatory cytokines activate leukocytes in the blood and spleen and these are attracted to affected areas by adhesive molecules. Infiltrating leukocytes produce large amount of cytotoxic substances, including matrix metalloproteinases, pro-inflammatory cytokines or reactive oxygen species resulting in subsequent damage of extracellular matrix as well as neurons and endothelial cells [6-9].

The inflammatory response is an essential reaction participating in healing processes and certain degree of inflammation is necessary for tissue reparation after the stroke [10]. Severe pathologies, such as shock, trauma or extensive ischemia may be, however, associated with deregulation of inflammatory response which may cause more tissue damage than initial pathology [11, 12]. Targeting inflammation after stroke is thus highly acute topic for both experimental and clinical research [13-15]. Inflammatory response after stroke has been recently targeted in several clinical trials with mixed results [16-21] indicating that selective manipulations with the immune system may be problematic and even counterproductive [19].

In this review, we aim to describe the basic characteristics of cholinergic anti-inflammatory pathway (CHAIP) and

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to review studies focused on CHAIP as a therapeutic target for ischemic and hemorrhagic stroke. Both pharmacological stimulation of $\alpha 7$ nAChR and vagus nerve stimulation after stroke are reviewed. According to our best knowledge, the similar review is still missing.

2. CHOLINERGIC ANTI-INFLAMMATORY PATHWAY

Big advances in molecular biology and biotechnologies have been made during the past decade. Potential new therapeutic targets and strategies evolve constantly [22-24], new ways of drug delivery using micro/nano technologies are tested intensively [25, 26], the possibilities of gene expression silencing are investigated extensively and the top of current science is a stem cell research [27, 28]. Immunomodulation represents one of the progressive therapeutic approaches which is intensively studied in a variety of pathologies [29-31].

Cholinergic anti-inflammatory pathway (CHAIP) is a physiological mechanism by which central nervous system regulates immune response and control inflammation [32]. Targeting cholinergic anti-inflammatory pathway is a promising way of immunomodulation which attenuates inflammation in a complex manner without causing immunosuppression [33]. Stimulation of CHAIP attenuates inflammation in experimentally induced sepsis [34], pancreatitis [35], post-operative ileus [36], renal ischemia/reperfusion injury [37], ischemic [38] or hemorrhagic shock [39]. Vagus nerve, spleen and $\alpha 7$ nicotinic acetylcholine receptor ($\alpha 7$ nAChR) are the main components of CHAIP [32].

2.1. Vagus Nerve

Vagus nerve is the longest cranial nerve innervating thoracic and most of the abdominal cavity (down to flexura coli sinistra). Vagus nerve monitors visceral environment and majority of fibers (around 80%) are afferent [40]. Previous studies demonstrated that the vagus nerve may be activated by both inflammation inducers (LPS) [41] and inflammatory cytokines [42]. Noninfectious production of pro-inflammatory cytokines is associated mainly with tissue damage [11]. Interestingly, the similar information is carried by nociceptive pathways which may be also activated by pro-inflammatory cytokines [43]. Based on central processing of information about inflammation, the activity of the sympathetic (SNS) and parasympathetic (PNS) nervous system is modulated; increased sympathetic activity is pro-inflammatory while increased parasympathetic activity is anti-inflammatory [33, 44]. The overall response of the immune system depends on the ratio between activation of the SNS and PNS [33].

Severe conditions such as polytrauma are predominantly associated with massive activation of the SNS and deregulation of inflammation [45]. Enhancing the activity of PNS by vagus nerve stimulation or direct pharmacological activation of $\alpha 7$ nAChR seems to be promising therapeutic intervention which may attenuate inflammation [46].

2.2. Spleen

The spleen plays major role in the inflammatory response [47]. Spleen represents a reservoir of leukocytes which may be released in response to tissue damage and which infiltrate

the affected area [48, 49]. The spleen is crucial for CHAIP and it was demonstrated that vagal nerve stimulation as well as analogical pharmacological stimulation of $\alpha 7$ nAChR failed to attenuate inflammation in splenectomized animals [50]. Interestingly, there is no direct parasympathetic innervation of spleen and communication between vagus nerve and spleen is mediated by SNS (splenic nerve); vagus nerve regulates activity of the splenic nerve through ganglion coeliacum [51]. Details about the involvement of particular receptor systems was for a long time obscure and last details have been described only recently [52]. CHAIP is associated with $\alpha 7$ nAChR and PNS, while SNS acts through adrenoceptors. $\beta 2$ adrenoceptors were described to be critical for the anti-inflammatory effect of vagus nerve stimulation and vagus nerve stimulation failed to attenuate inflammation in $\beta 2$ knock-out mice [53]. It was also demonstrated that $\beta 2$ signaling is mediated by splenic regulatory lymphocytes [53]. The final piece of the puzzle was described in 2011, when splenic T-lymphocytes were shown to produce acetylcholine [52]. Communication between vagus nerve and splenic leukocytes is most probably as follows (Fig. 1): vagus nerve (PNS - acetylcholine) regulates the activity of splenic nerve (SNS-norepinephrine) through ganglion coeliacum. Splenic nerve regulates activity of splenic T-lymphocytes through $\beta 2$ signaling and these produce acetylcholine. Acetylcholine has anti-inflammatory effect on splenic leukocytes *via* $\alpha 7$ signaling. More recently, an alternative concept has evolved postulating that the effect of vagus nerve stimulation is mediated by T-lymphocytes from an outside of the spleen and splenic nerve is activated by these T-lymphocytes [54, 55]. This interesting concept basically extends the effect of vagus nerve stimulation to all the immune system which sounds very reasonable, but this concept has to be proved.

2.3. $\alpha 7$ nAChR

$\alpha 7$ nAChR is a subtype of nicotine receptors, which are ligand gated channels consisting of five transmembrane subunits [56]. $\alpha 7$ nAChR is homopentameric receptor composed of five $\alpha 7$ subunits [56] and it is an ion channel selectively permeable to calcium [57]. Acetylcholine, choline or nicotine are natural ligands activating $\alpha 7$ nAChR in a non-selective manner, α -bungarotoxin is a selective antagonist of $\alpha 7$ nAChR [58].

$\alpha 7$ nAChR is expressed in a variety of cells involved in immune response, including leukocytes, lymphocytes and endothelial cells [59]. $\alpha 7$ nAChR is also widely expressed in the CNS where it can be found in astrocytes [60], microglia [61], endothelial cells [50] and neurons [59]. Localization of $\alpha 7$ nAChR in neurons may be postsynaptic, generating excitatory responses, or presynaptic/perisynaptic, modulating signal transduction [62]. In hippocampal neurons $\alpha 7$ nAChR plays a major role in synaptic plasticity [63] and synaptic formation during development [64]. Stimulation of $\alpha 7$ nAChR has anti-inflammatory [65], antioxidative [66] and anti-apoptotic [67] properties (Fig. 2).

Anti-inflammatory effect of $\alpha 7$ nAChR signaling is associated with JAK2/STAT3/NFkB pathway [68]. $\alpha 7$ stimulation was demonstrated to decrease production of pro-inflammatory cytokines in liver [39], macrophages [69]/

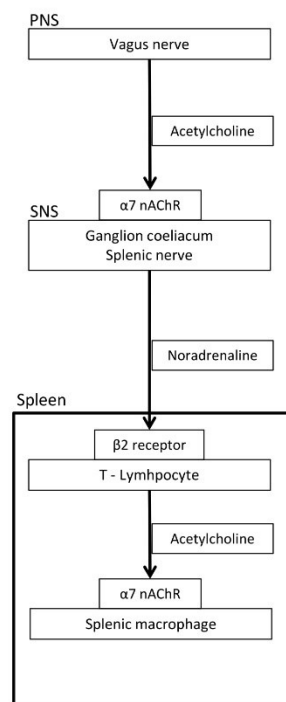


Fig. (1). Current model of vagus nerve associated neurohumoral regulations. Neural components include both parasympathetic (PNS) and sympathetic (SNS) nervous system, while the production of acetylcholine by T-lymphocytes should be considered as a humoral component of splenic macrophage regulations.

monocytes [70]. $\alpha 7$ stimulation was also demonstrated to decrease production of adhesive molecules in endothelial cells and subsequent leukocyte trafficking [65]. Anti-apoptotic effect of $\alpha 7$ signaling is linked to JAK2/PI3/Akt pathway [71] and ERK1/2-PKA [72]. $\alpha 7$ stimulation was demonstrated to decrease expression of proapoptotic proteins like cleaved caspase 3 or GSK-3 β [71] and increase expression of anti-apoptotic proteins like BCL-2 in neuronal cells [67]. The antioxidative effect of $\alpha 7$ signaling was demonstrated to be connected to both JAK2/STAT3/NF κ B [73] and JAK2/PI3/Akt [74] pathways. $\alpha 7$ signaling was referred to be associated with both increased expression of antioxidant enzymes like superoxide dismutase or glutathione peroxidase and decreased expression of prooxidative enzymes like NADPH oxidase [66].

3. CHAIP AS A THERAPEUTIC TARGET AFTER STROKE

Targeting of CHAIP is a promising therapeutic approach of immunomodulation which attenuates inflammation without causing immunosuppression [33]. There are two options

of CHAIP activation: pharmacological stimulation of $\alpha 7$ nAChR and electrical stimulation of the vagus nerve (VNS) [32].

The effect of both VNS and pharmacological targeting of $\alpha 7$ nAChR is similar outside of the CNS and this effect is antioxidative and anti-inflammatory [32], since $\alpha 7$ nAChR is expressed in a variety of cells involved in immunity including leukocytes or endothelial cells [59]. The effect of VNS and pharmacological stimulation of $\alpha 7$ nAChR may, however differ in the brain. In the brain, $\alpha 7$ nAChR is expressed not only in cells related to immunity (astrocytes [60], microglia [61] and endothelial cells [50]) but $\alpha 7$ nAChR is widely expressed also in neuronal cells [59]. Pharmacological stimulation of $\alpha 7$ nAChR has thus antioxidative and anti-inflammatory effect related to immune cells [75, 76] and antioxidative and anti-apoptotic effect in neuronal cells [67]. The effect of VNS on the brain is not directly related to CHAIP because central projections of vagus nerve are not associated with acetylcholine and $\alpha 7$ signaling, but these very complex projections are associated predominantly with norepinephrine signaling *via* locus coeruleus [77, 78]. Norepinephrine signaling was reported to be neuroprotective in a very complex way, including attenuation of neuroinflammation or reduction of oxidative stress and this was reviewed elsewhere [79]. However VNS was also described to be associated with up-regulation of $\alpha 7$ nAChRs [80], so in the brain, VNS may influence CHAIP indirectly.

3.1. Pharmacological Stimulation of $\alpha 7$ nAChR

3.1.1. Ischemic Stroke

The first study focused on CHAIP in ischemic stroke model was conducted by Norman in 2011 [81]. $\alpha 7$ agonist GTS-21 reduced microglial activation (MAC-1 expression), mRNA expression of TNF- α , IL-1 β and IL-6 as well as neuronal cell death in global cerebral ischemia model in mice [81].

Kalappa described that $\alpha 7$ positive allosteric modulator PNU-120596 reduced neuronal injury in hippocampal cell cultures subjected to oxygen glucose deprivation (OGD) and significantly reduced infarct size in transient middle cerebral artery occlusion (MCAO) model in rat [82]. The neuroprotective effect of PNU-120596 was described to be present even when the drug was administered 6 hours after onset of transient MCAO in rat [83].

In a series of experiments Han studied effect of $\alpha 7$ agonist PHA568487 in permanent MCAO model in mice [66]. Treatment improved neurological status, reduced infarct size and neuronal apoptosis. Treatment was associated with a decrease in M1 (pro-inflammatory) and an increase in M2 (anti-inflammatory) microglia/macrophage populations. Treatment was also associated with increased expression of anti-oxidants (Superoxide dismutase - SOD1 and Glutathione peroxidase - GPX1) and decreased expression of pro-oxidative NADPH oxidase. Neuronal injury, neuroinflammation and oxidative stress were aggravated by additional stress represented by tibia fracture and this was also reversed by the treatment. Based on these results the authors concluded that the neuroprotective effect of PHA568487 is associated with reduction of neuroinflammation and oxidative stress [66].

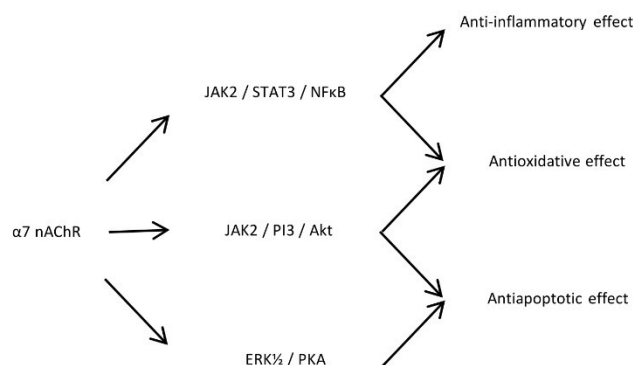


Fig. (2). The effects and the main signaling pathways associated with a stimulation of $\alpha 7$ nAChR.

In a study conducted by Parada, $\alpha 7$ agonist PNU-282987 reduced production of reactive oxygen species, TNF- α and reduced cell death in hippocampal cultures subjected to OGD [84]. The neuroprotective effect was not present in microglia depleted cultures or in cultures from Nrf2 deficient mice, suggesting that the effect of the drug was associated with heme oxygenase -1 (HO-1) pathway in microglia. In the same study, the authors performed *in vivo* experiment in which PNU-282987 reduced infarct size and improved neurological status in photothrombotic stroke model in mice. The beneficial effect of the drug was eliminated by inhibition of HO-1 expression. Based on these results the authors concluded that activation of $\alpha 7$ nAChR associated pathway in microglia regulates neuroinflammation and oxidative stress via a Nrf2/HO-1 pathway and this mechanism is responsible for neuroprotection in ischemic brain [84]. Neuroprotective effect of $\alpha 7$ agonist PNU-282987 against oxidative stress was studied by the same scientific team using neuronal cell cultures [74]. The authors used *in vitro* model of mitochondrial ROS production using rotenone/oligomycin. Neuronal cultures were exposed to rotenone/oligo for 8 hours and left for another 16 hours in medium without rotenone/oligo. Such experimental conditions induced apoptosis in 30% of cells. Treatment with PNU-282987 after rotenone/oligo incubation prevented cell death in a concentration dependent manner. In following experiments authors used PNU-282987 with specific inhibitors and found that activation of $\alpha 7$ nAChR was linked to JAK2/PI3/Akt pathway which induced antioxidant enzyme HO-1 [74].

In hippocampal slices subjected to OGD $\alpha 7$ agonist galantamine protected neural cells and this was reversed by JAK2 antagonist AG490 [73]. The authors describe two mechanisms of action associated with galantamine linked $\alpha 7$ -JAK2 signaling. The first, galantamine inhibited NADPH oxidase (NOX) and production of reactive oxygen species. The second, galantamine reduced NF κ B/iNOS induction and subsequent production of NO which was promoted by OGD [73].

The effect of oral administration of $\alpha 7$ agonist choline after permanent MCAO in rats was studied by Jin [85]. Treatment for 10 days improved neurological deficit, re-

duced infarct volume and neuronal cell death. Higher expression of $\alpha 7$ nAChR, HIF-1 α and VEGF was found in the ischemic cortex in the treated group and higher levels of VEGF were also found in serum. Effect of treatment was reversed by selective $\alpha 7$ antagonist methylacconitine as well as by HIF-1 α siRNA. Choline induced angiogenesis and this was also confirmed by *in vitro* study. The authors concluded that choline attenuates brain ischemic injury in a permanent MCAO model in rats, possibly by facilitating pial arteriogenesis and cerebral-cortical capillary angiogenesis by up-regulating $\alpha 7$ nAChR and inducing the expression of HIF-1 α and VEGF [85].

3.1.2. Hemorrhagic Stroke

The first study focused on CHAIP in experimentally induced intracerebral hemorrhage (ICH) was published by Lee in 2010 [86]. The authors were testing whether they could mimic vagus nerve stimulation by central activation of parasympathetic nervous system and whether this effect could be neuroprotective. Intraventricular administration of muscarine was associated with improved neurological deficit, reduced brain water content and decreased expression of pro-inflammatory cytokines (TNF- α , IL-1 β) and pro-apoptotic Fas ligand. Treatment with muscarine was ineffective in reducing brain water content after splenectomy, suggesting that the effect of muscarine was associated with CHAIP [86].

Mechanism of neuroprotection associated with $\alpha 7$ signaling was systematically studied by Krafft in mice ICH model [71, 87]. Administration of $\alpha 7$ agonist PHA-543613 improved neurological outcome, reduced brain edema formation and reduced neuronal cell death in perihematomal area. Treatment was associated with decreased expression of pro-apoptotic GSK-3 β and cleaved caspase 3 in neurons through PI3-Akt pathway [71]. Involvement of GSK-3 β in blood-brain barrier (BBB) disruption was studied by the same authors in other work using an identical model [87]. In this study, the authors described that inhibition of GSK-3 β by PHA-543613 was associated with stabilization of β -catenin which is involved in regulation of gene expression of proteins essential for BBB [87].

The first study focused on the role of CHAIP in the pathophysiology of subarachnoid hemorrhage (SAH) was conducted in 2006, when Bowman studied the effect of $\alpha 7$ signaling in posthemorrhagic vasospasm development in rat femoral artery model [88]. Pretreatment with $\alpha 7$ agonist CNI-1493 was associated with dose-dependent attenuation of posthemorrhagic vasospasm and with a reduction in levels of pro-inflammatory cytokine IL-6 [88].

The neuroprotective effect of $\alpha 7$ signaling was described also in the rat SAH model [89]. Administration of PNU-282987 was associated with improved neurological outcome, reduced brain edema formation and reduced neuronal cell apoptosis. PNU-282987 significantly increased phosphorylated Akt levels and significantly decreased cleaved caspase 3 levels in neuronal cells in ipsilateral hemispheres and the effect of the drug was reversed using specific inhibitors [89].

3.2. Vagus Nerve Stimulation

The first report about the neuroprotective effect of VNS after ischemic stroke was reported in 1993 when Masada and colleagues described reduced neuronal cell death in hippocampus following stimulation of left cervical vagus nerve in forebrain ischemia model in gerbil [90]. In the same model Miyamoto *et al.* studied neuroprotective effect of VNS stimulation in hippocampus using microdialysis and Doppler flowmetry. They found that VNS stimulation was associated with decreased accumulation of glutamate extracellularly and decreased cerebral blood flow, suggesting that the neuroprotective effect of VNS contribute to suppression of glutamate excitotoxicity as well as to the suppression of reperfusion injury [91].

In 2009, Ay reported that stimulation of cervical vagus nerve ipsilaterally to the vessel occlusion reduced infarct size and improved neurological status in rat transient MCAO model [92]. In further study the same authors described that stimulation of vagus nerve contralaterally to vessel occlusion has similar effect as the stimulation of ipsilateral nerve [93]. The main goal of this study was, however, to describe the effect of VNS on cerebral blood flow. The author described a transient decrease in arterial blood pressure and regional cerebral blood flow during stimulation (30s) which immediately returned to the original levels at the end of each train of stimulation. Stimulation was performed every 5 minutes for 1 hour and overall cerebral blood flow during entire stimulation period was not significantly affected [93]. Similar results were reported by Hiraki, who also reported reduced infarct size, improved neurological outcome as well as a hemodynamically insignificant transient decrease in cerebral blood flow during train of stimulation in transient focal ischemia model [94]. These results suggest that the neuroprotective effect of VNS after ischemic stroke is not attributed to alteration in CBF [93].

Sun *et al.* compared effect VNS in rat models of both transient and permanent focal cerebral ischemia. VNS reduced infarct size and improved neurological score after transient focal cerebral ischemia and reduced infarct size, but not improved neurological score in permanent ischemia model [95].

Ekici *et al.* reported antioxidant properties of VNS in transient focal cerebral ischemia model. The authors referred that VNS was associated with increased levels of antioxidant glutathione and decreased levels of lipid peroxidation marker MDA, suggesting that VNS may be effective against free radical generation and lipid peroxidation [96].

In a series of studies, Jiang *et al.* studied anti-inflammatory and anti-apoptotic properties of VNS in transient focal cerebral ischemia. VNS was associated with decreased production of pro-inflammatory cytokines TNF- α , IL-1 β and IL-6 in the ischemic penumbra. VNS stimulation was also associated with p-Akt up-regulation/ CC3 down-regulation and decreased level of neuronal apoptosis in the ischemic penumbra. Interesting finding of this study was that ischemia reperfusion injury is associated with down-regulation of $\alpha 7$ nAChR and this was reversed by VNS [80]. Additionally the neuroprotective, anti-inflammatory properties of VNS were also associated with up-regulated expression of PPAR γ in the ischemic penumbra [97].

The same authors studied the effect of VNS on the level of microRNA (miR) and they reported neuroprotective effect of miR-210 expression after VNS stimulation. Increased miR-210 was associated with increased p-Akt expression, decreased levels of cleaved caspase3 and decreased neuronal apoptosis. Increased miR-210 was also associated with reduction in oxidative stress, particularly decreased the lipid peroxidation (MDA levels), and increased antioxidative capacity (SOD and GSH). Neuroprotective effects were reversed after miR-210 blockade using miR-210 antagomir [98].

3.3. Transcutaneous Vagus Nerve Stimulation

Vagus nerve stimulation is a promising way of neuromodulation, but it is an invasive procedure. The most recent way of research is therefore focused on indirect vagus nerve stimulation. Indirect stimulation is based on electrostimulation of skin area which is innervated by vagus nerve or which is above vagus nerve. External ear (concha and the external auditory meatus) is the only skin area which is innervated by vagus nerve [99, 100]. Transcutaneous auricular vagus nerve stimulation (TA-VNS) is the most often use way of indirect stimulation and TA-VNS was shown to suppress inflammatory response [101]. So far three studies were conducted to test the effect of indirect vagus nerve stimulation after ischemic stroke.

In 2015, Ay *et al.* referred that TA-VNS reduced infarct size and improved neurological outcome in transient MCAO model in rat [102]. Using immunohistochemistry the authors demonstrated that stimulation of left cavum conchae resulted in bilateral activation of nucleus tractus solitarius and locus coeruleus [102].

The neuroprotective effect of TA-VNS in the transient MCAO model was also reported by Jiang *et al.* Beside reduced infarct size and improved neurological outcome the authors described that TA-VNS was also associated with increased angiogenesis and expression of angiogenic factors (brain-derived neurotrophic factor, vascular endothelial growth factor and endothelial nitric oxide synthase) in ischemic penumbra [103].

Finally Ay *et al.* reported effectiveness of transcutaneous stimulation using surface electrodes applied on the skin above the cervical vagus nerve. The authors used transient MCAO model and neuroprotective effect was detectable when stimulation was initiated up to 4 hours after stroke [104].

4. FUTURE DIRECTIONS

Despite the fact that we have enough evidence about the beneficial effects of CHAIP stimulation on inflammation, more information is needed for better understanding of CHAIP in its complexity.

The interesting part of CHAIP problematic is an involvement of SNS (splenic nerve, β_2 signaling), suggesting that at least in some situations the SNS and PNS act complementarily in a highly organized manner (cooperation between SNS and PNS is also well known in control of erection/ejaculation). More information about cooperation/anticipation between SNS and PNS could bring us better knowledge about autonomic nervous system with many potential clinical implications.

Another interesting point which should be clarified by future studies is the effect of CHAIP stimulation on lymph nodes. The potential role of T-lymphocytes from an outside of the spleen has been already suggested [54, 55] and this was mentioned above.

Finally, the most important step in research of CHAIP as a potential treatment of stroke will be clinical testing.

CONCLUSION

Inflammation plays an important role in the pathophysiology of stroke. CHAIP represents promising therapeutic target, because CHAIP stimulation attenuates inflammation in a complex manner without the side effect of immunosuppression. Stimulation of CHAIP also reduces inflammation associated pathologies, such as oxidative stress and apoptosis. Neuroprotective effects of both vagus nerve stimulation and pharmacological stimulation of α_7 nAChR have been proven in various stroke models and new approaches such as non-invasive indirect vagus nerve stimulation may help to introduce this therapeutic option into the clinical practice. However, this is an initial phase of research in this field and more experimental studies are needed to understand the possibilities and limits of CHAIP stimulation in stroke treatment.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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PATIENT CONSENT

Declared none.

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4.1.4 Vagus nerve stimulation as immunomodulatory therapy for stroke: A comprehensive review

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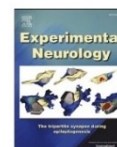
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In this review article, which may be considered as an update of previous article, we aimed to summarize current knowledge about the immunomodulatory effect of VNS. We extensively review signaling pathways associated with VNS and bring a comprehensive overview of experimental studies focused on targeting CHAIP after stroke.



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Research paper

Vagus nerve stimulation as immunomodulatory therapy for stroke: A comprehensive review

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ABSTRACT

Stroke is a devastating cerebrovascular pathology with high morbidity and mortality. Inflammation plays a central role in the pathophysiology of stroke. Vagus nerve stimulation (VNS) is a promising immunomodulatory method that has shown positive effects in stroke treatment, including neuroprotection, anti-apoptosis, anti-inflammation, antioxidation, reduced infarct volume, improved neurological scores, and promotion of M2 microglial polarization. In this review, we summarize the current knowledge about the vagus nerve's immunomodulatory effects through the cholinergic anti-inflammatory pathway (CAP) and provide a comprehensive assessment of the available experimental literature focusing on the use of VNS in stroke treatment.

1. Introduction

Stroke is a serious cerebrovascular event in which poor blood flow to the brain causes cell death. The global prevalence is around 101.5 million; 76% of these causes are of ischemic etiology due to vessel obstruction mainly by arterial thrombosis or embolization from the heart (Foulkes et al., 1988; Virani et al., 2021). The remaining stroke cases (24%) are of hemorrhagic etiology mainly due to vessel or cerebral aneurysm rupture resulting in intracerebral (ICH) or subarachnoid hemorrhage (SAH) (Foulkes et al., 1988; Virani et al., 2021). It is also a major cause of premature death as it represents the second highest cause of death worldwide with 6.6 million deaths every year (Virani et al., 2021; Kim et al., 2020). 3.3 million individuals died of ischemic stroke; 2.9 million of ICH; and 0.4 million of SAH (Virani et al., 2021).

Inflammation plays a crucial role in the pathophysiology of stroke. Despite the differences in the pathophysiology of ischemic and hemorrhagic stroke, the inflammatory response is quite similar regardless of the stroke type (Aronowski and Zhao, 2011; Chen et al., 2015). Ischemia, blood extravasation, and direct tissue damage pose strong pro-inflammatory signals. They induce endothelial cells, microglia, and astrocytes to start producing pro-inflammatory mediators and adhesion molecules. Pro-inflammatory cytokines activate leukocytes in the blood circulation and the spleen, chemokines attract them to the site of the injury, and adhesion molecules mediate their rolling, activation, stable

arrest, and transmigration (Ren et al., 2011). To manage the injury, infiltrating leukocytes produce large amounts of pro-inflammatory mediators, cytotoxic molecules, reactive oxygen, and nitrogen species which results in collateral damage of surrounding cells (neurons, glia, and endothelial cells) as well as extracellular matrix (Aronowski and Zhao, 2011; Chen et al., 2015; Jelinek et al., 2021).

The inflammatory response is a physiological process that is necessary for the elimination of harmful stimuli and the starting of the healing processes, therefore the inflammation is to a certain extent essential for tissue repair after stroke (Xiong and Yang, 2015). However, pathologies like major ischemia, trauma, or shock may be associated with the deregulation of inflammatory response causing further damage to the already serious initial pathology (Duris and Jurajda, 2020; Lu, 2009). Targeting inflammation after stroke is thus a highly sought-after topic in both clinical and experimental research (Dziedzic, 2015).

The vagus nerve (VN, cranial nerve X, Latin: *nervus vagus*, wandering nerve), a mixed nerve with efferent fibers that are primarily cholinergic, participates in the pathophysiology of stroke through its anti-inflammatory, neuroprotective, and immunomodulatory effects. The VN is involved in regulating the autonomic, cardiovascular, respiratory, gastrointestinal, immune, and endocrine systems (Berthoud and Neuher, 2000). Thus consequently, the VN regulates heart rate, blood pressure, vascular resistance, airway diameter, respiration, digestion, peristalsis, and release of enteroendocrine mediators (Berthoud and

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Neuhuber, 2000). After stroke, the VN can be activated, leading to a decrease in inflammation and improved neurological function.

Vagus nerve stimulation (VNS) is a therapeutic technique that involves electrically stimulating the vagus nerve. VNS works by stimulating the VN to release neurotransmitters, such as acetylcholine and norepinephrine, which have a variety of effects, including anti-inflammatory properties, modulating the immune system, inhibiting pain signals, regulating mood and anxiety, and improving cognitive functions.

The goal of this review is to summarize the basic characteristics of the cholinergic anti-inflammatory pathway (CAP), describe vagus nerve stimulation (VNS) as a promising immunomodulatory method that could be used to regulate inflammation and give a systematic list of studies where VNS was used to treat stroke.

2. The vagus nerve role in the immune system

The vagus nerve modulates the immune response in two main ways: (1) the afferent vagus nerve fibers interacting with the anti-inflammatory hypothalamic-pituitary-adrenal (HPA) axis, and (2) the efferent vagus nerve fibers activating the cholinergic anti-inflammatory pathway (CAP) (Fig. 1).

2.1. The afferent vagus nerve and HPA axis pathway

The afferent vagus nerve is a major component of the anti-inflammatory hypothalamic-pituitary-adrenal (HPA) axis pathway. The vagus nerve is an integral part of the neuro-endocrine-immune axis, providing neural, behavioral, endocrine, and immune responses as a part of first-line innate defense against infection/inflammation (Johnston and Webster, 2009). The VN is particularly sensitive to pro-inflammatory cytokine levels, such as interleukin (IL)-1 β , IL-6, and tumor necrosis factor (TNF) α (Hosoi et al., 2005). These cytokines are released by macrophages and other immune cells in various

inflammatory conditions such as sepsis (Hosoi et al., 2005).

The afferent VN fibers are outfitted with IL-1 β receptors at the paranglia level. They convey the information to the nucleus of the solitary tract. There, neurons based in the A2 noradrenergic group are activated and project information to the parvocellular zone of the paraventricular nucleus of the hypothalamus (*nucleus paraventricularis hypothalami*) around corticotropin-releasing hormone (CRH)-containing neurons. The CRH neurons stimulate hypophysis to release the adrenocorticotrophic hormone (ACTH) which stimulates the release of glucocorticoids by the adrenal glands to decrease peripheral inflammation (Bonaz et al., 2016). Thus, making up the hypothalamic-pituitary-adrenal axis and giving the VN anti-inflammatory properties.

Furthermore, the HPA axis can also be activated by circulating pro-inflammatory cytokines on the circumventricular organs (Buller, 2001). These structures are located outside of the blood-brain barrier (BBB) and stimulate nearby neurons to activate the CRF neurons of the paraventricular nucleus, hence also activating the HPA axis (Buller, 2001).

2.2. The efferent vagus nerve and the CAP

Another anti-inflammatory pathway that the VN is involved in is the cholinergic anti-inflammatory pathway. Essentially, it is a vago-vagal reflex, where afferent VN fibers activate efferent VN fibers. The anti-inflammatory effect is due to the release of acetylcholine (ACh) at the distal end of the VN. ACh inhibits the release of pro-inflammatory cytokines such as IL-1 β , IL-6, and TNF α by macrophages and other immune cells. It is mediated through the connection of ACh with α -7-nicotinic acetylcholine receptors (α 7nAChR) which can be found on macrophages. This connection is called the cholinergic anti-inflammatory pathway (CAP).

CAP is a physiological mechanism by which the central nervous system controls immune response and regulates inflammation (Rosas-Ballina and Tracey, 2009). Targeting CAP is a promising way of

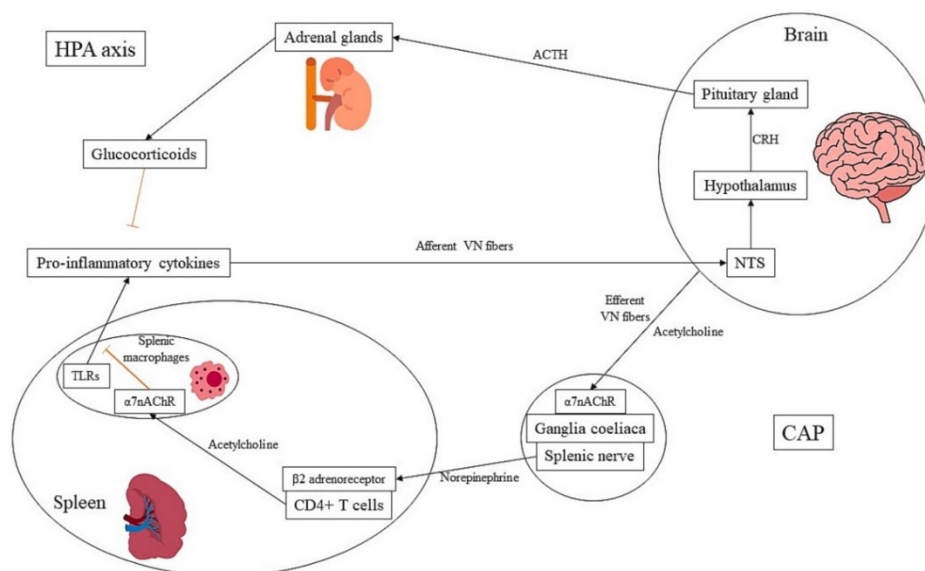


Fig. 1. Diagram of HPA axis and cholinergic anti-inflammatory pathway. α 7nAChR - α -7-nicotinic acetylcholine receptor; ACTH - Adrenocorticotrophic hormone; CAP - Cholinergic anti-inflammatory pathway; CRH - Corticotropin-releasing hormone; HPA axis - Hypothalamic-pituitary-adrenal axis; NTS - nucleus of the solitary tract; TLRs - Toll-like receptors; VN - Vagus nerve.

immunomodulation that attenuates inflammation without causing immunosuppression (Tracey, 2009).

3. Cholinergic anti-inflammatory pathway (CAP)

Three main components play a role in the complex mechanism by which the CAP functions, the vagus nerve, spleen, and the $\alpha 7$ nAChR.

3.1. Vagus nerve in CAP

The vagus nerve can be activated by inflammatory inducers (e.g. LPS) and inflammatory cytokines (Hosoi et al., 2005; Goehler et al., 1997). Based on the central processing of information about inflammation, the activity of the sympathetic and parasympathetic nervous system is modulated (Tracey, 2009; Pongratz and Straub, 2014). Both sympathetic and parasympathetic nervous cooperates and acts as one functional unit in which the overall response depends on the ratio between sympathetic and parasympathetic activity. Increasing the tone of the sympathetic nervous system promotes a pro-inflammatory response, whereas increasing the tone of the parasympathetic nervous system has anti-inflammatory properties. In severe conditions (such as stroke or sepsis), there is a massive activation of sympathetic activity, which promotes deregulation of the inflammatory response (Tracey, 2009).

3.2. Spleen

Spleen is a major part of the immune system thus it plays an important role in the inflammatory response (Offner et al., 2006). The spleen acts as a leukocyte reservoir, so they can be released in response into the bloodstream and transported to the affected area (Pennypacker, 2014; Swirski et al., 2009). The spleen is essential for the CAP, which was demonstrated in animals with splenectomy, where vagus nerve stimulation or pharmacological stimulation of the $\alpha 7$ nAChR failed to attenuate inflammation (Huston et al., 2006). Interestingly, there is no direct link between the VN and the spleen. The parasympathetic nervous system regulates spleen activity by regulating the sympathetic nervous system activity via the celiac ganglia (*ganglia coeliaca*) and the splenic nerve, which are part of the sympathetic nervous system (Rosas-Ballina et al., 2008). The VN and CAP are associated with $\alpha 7$ nAChR and the parasympathetic nervous system, whereas sympathetic activity is associated with adrenoceptors. $\beta 2$ adrenergic receptors are essential for anti-inflammatory response to VNS, which was proven using $\beta 2$ adrenoceptor knock-out mice (Vida et al., 2011). It was also shown that $\beta 2$ signaling is moderated by splenic CD4⁺ T-cells and a particular subset of these splenic CD4⁺ T-cells can produce acetylcholine (Vida et al., 2011; Rosas-Ballina et al., 2011). Acetylcholine binds to $\alpha 7$ nAChR on splenic macrophages and results in the inhibition of the production of TNF α and other cytokines (Murray and Reardon, 2018).

In summary, it seems that the signaling between the VN and splenic leukocytes starts with the VN (parasympathetic, acetylcholine), which through the celiac ganglia regulates the activity of the splenic nerve (sympathetic, norepinephrine). The splenic nerve then modulates the activity of splenic T-lymphocytes through the $\beta 2$ adrenoceptor and these lymphocytes produce acetylcholine which has an anti-inflammatory effect on splenic macrophages via their $\alpha 7$ nAChR, inhibiting the release of TNF α and other pro-inflammatory cytokines.

3.3. $\alpha 7$ nAChR signaling

$\alpha 7$ nAChR ($\alpha 7$ receptor) is a type of nicotinic acetylcholine receptor (Millar, 2003). It is a ligand-gated calcium ion channel which consists of five transmembrane subunits (Millar, 2003). It is a homopentamer composed of five $\alpha 7$ subunits (Millar, 2003). Natural agonists are for example acetylcholine (ACh), choline, and nicotine, a positive allosteric modulator is, for instance, ivermectin, and antagonists include compounds such as ethanol or α -bungarotoxin (Clarke, 1992; Krause et al.,

1998).

$\alpha 7$ nAChR is expressed on several immune cells, including macrophages, lymphocytes and endothelial cells (Clementi et al., 2000). In the CNS, $\alpha 7$ nAChR can be found on astrocytes, microglia, endothelial cells and neurons (Huston et al., 2006; Clementi et al., 2000; Sharma and Vijayaraghavan, 2001; Shytle et al., 2004). In neurons, $\alpha 7$ nAChR appears to have a critical role in memory, learning and attention (Berg and Conroy, 2002; Yakel, 2014). Its localization can be presynaptic/perisynaptic, in which case $\alpha 7$ nAChR modulates signal transduction; or postsynaptic, where it generates excitatory responses (Berg and Conroy, 2002).

On a molecular level, $\alpha 7$ nAChR interacts with three primary signaling pathways, which regulate a wide range of cellular activities and physiological processes. These signaling pathways are: 1) JAK2/STAT3/NF κ B; 2) JAK2/PI3K/Akt; and 3) ERK/PKA. Through the network of complex and overlapping signal transductions, $\alpha 7$ nAChR is involved in various cascades that have anti-inflammatory, anti-oxidative, and anti-apoptotic effects (Fig. 2).

Typically, stimulation of $\alpha 7$ nAChR increases intracellular levels of calcium ion (Ca^{2+}), because as mentioned the $\alpha 7$ nAChR serves as an ion channel (Fucile, 2004). Ca^{2+} can then activate phosphatidylinositol-3-kinase (PI3K) and phospholipase C (PLC) (Blanchet et al., 2006). These two proteins have profound effects on inflammatory pathways.

NF κ B is an important protein complex involved in the transcription control of many inflammatory mediators, like IL-1 β , IL-6, and TNF α . It can be activated in response to several cellular processes such as stress, cytokines, reactive species, oxidation, and viral or bacterial antigens (Liu et al., 2017). The activation of NF κ B involve ubiquitination of I κ B, which is one of the most important inhibitors of NF κ B (Rioux and Castonguay, 2000). NF- κ B predominantly exists as a heterodimer of p50 and p65 subunits, that are usually sequestered in the cytoplasm by a family of inhibitory proteins. Once the inhibitor is disintegrated, p50 and p65 subunits get released and translocated to the nucleus where they regulate the transcription of NF κ B-related genes (Rioux and Castonguay, 2000). The interaction between NF κ B and $\alpha 7$ nAChR is based on $\alpha 7$ nAChR ability to prevent I κ B breakdown, which in turn prevents the nuclear translocation of p65 and/or p50 (Saeed et al., 2005).

Another important nuclear factor modulated by $\alpha 7$ nAChR signaling is nuclear factor erythroid 2-related factor 2 (Nrf2), which is a transcription factor involved predominantly in response to oxidative stress. Kelch-like ECH-associated protein 1 (Keap1) is the main negative regulator/repressor of Nrf2. Cullin 3 ubiquitinates Nrf2, while Keap1 was reported to be a substrate adaptor protein that facilitates proteasomal degradation (Cuadrado et al., 2019). Keap1 contains highly reactive cysteines that upon modification prevent Keap1 from targeting Nrf2 (Cuadrado et al., 2019). Nrf2 is then quickly translocated to the nucleus. It binds to antioxidant response elements (ARE), thus regulating the transcription of ARE genes (Cuadrado et al., 2019). $\alpha 7$ nAChR activation induces Nrf2-dependent expression of heme oxygenase-1 (HO-1), which is an antioxidant enzyme involved in heme group degradation (Funes et al., 2020; Navarro et al., 2015). $\alpha 7$ nAChR agonists can be used to upregulate Nrf2 and associated antioxidant genes (Patel et al., 2017). Also, astrocytes lacking Nrf2 have reduced anti-inflammatory response to $\alpha 7$ nAChR stimulation (Patel et al., 2017).

The strongest anti-inflammatory effects show signalization via JAK/STAT pathway. This cascade involves three main components: Janus kinase (JAK), signal transducer and activator of transcription proteins (STAT), and receptor. This pathway participates in inflammation and immune response. However, STAT3 signaling typically promotes anti-oxidative and anti-apoptotic pathways. It has been shown that JAK2 can be recruited to the $\alpha 7$ subunit of the nicotinic acetylcholine receptor, which, in turn, phosphorylates STAT3 (de Jonge et al., 2005). Simultaneously, STAT3 phosphorylation can also be dependent on activation of many other kinases, such as PI3K or mitogen-activated protein kinase (MAPK) (Tanaka et al., 1999). Most of them function side-by-side with JAK2 and are sensitive to intracellular levels of Ca^{2+} . As mentioned,

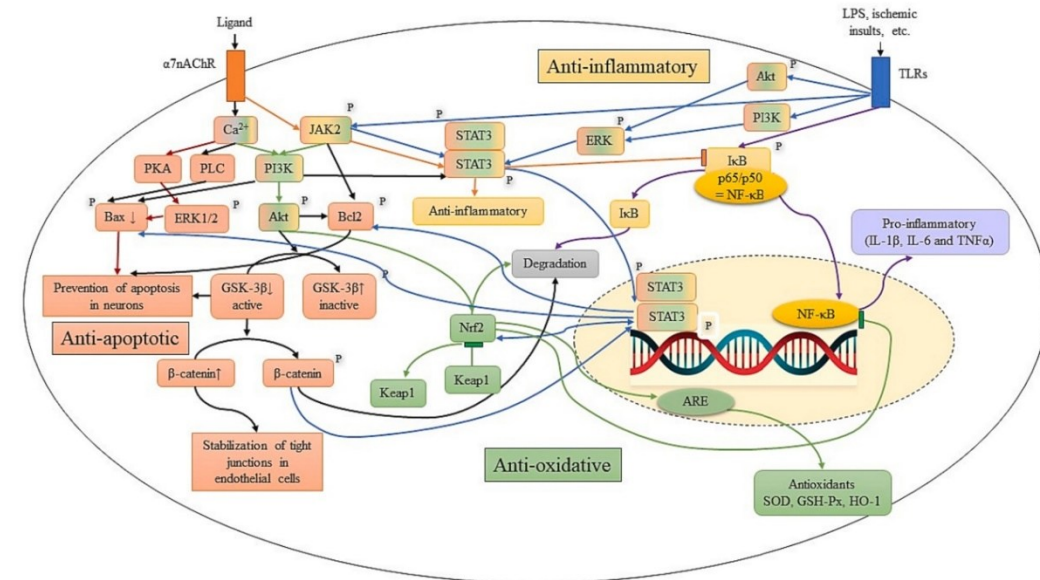


Fig. 2. Main signaling pathways associated with stimulation of $\alpha 7$ nAChR, along with examples of their effects.

$\alpha 7$ nAChR - α -7-nicotinic acetylcholine receptor; Akt - protein kinase B; ARE - antioxidant response elements; Bax - bcl-2-like protein 4 (pro-apoptotic protein); Bcl-2 - B-cell lymphoma 2 (anti-apoptotic protein); Ca^{2+} - calcium ions; ERK - extracellular signal-regulated kinases; ERK1/2 - extracellular signal-regulated kinases 1/2; GSH-Px - glutathione peroxidase; GSK-3 β - glycogen synthase kinase 3 beta; HO-1 - heme oxygenase-1; I κ B - inhibitor of NF κ B; IL - interleukin; JAK2 - janus kinase 2; Keap1 - kelch-like ECH-associated protein 1 (repressor of Nrf2); NF κ B - nuclear factor κ -light-chain enhancer of activated B cells; Nrf2 - nuclear factor erythroid 2-related factor 2; p65/p50 - NF κ B p50/p65 heterodimer; PI3K - phosphoinositide 3-kinase; PKA - protein kinase A; PLC - phospholipase C; SOD - superoxide dismutase; STAT3 - signal transducer and activator of transcription 3; TLR - Toll-like receptor; TNF α - tumor necrosis factor alpha.

$\alpha 7$ nAChR is an ion channel and activator of JAK2. Thus, $\alpha 7$ nAChR can manipulate with JAK2/STAT3 signaling through multiple mechanisms (Maldifassi et al., 2014; Park et al., 2008). Moreover, it has been shown that STAT3 signaling may have a dual effect in inflammation potentially activating pro-inflammatory as well as anti-inflammatory pathways (Park et al., 2008).

$\alpha 7$ nAChR signaling associated with JAK2/STAT3/NF κ B was shown to have anti-inflammatory effects by decreasing the production of pro-inflammatory cytokines and adhesive molecules (Saeed et al., 2005; de Jonge et al., 2005; Wang et al., 2003).

$\alpha 7$ nAChR signaling associated with JAK2/PI3K/Akt and ERK1/2/PKA was shown to have anti-apoptotic effects by decreasing expression of pro-apoptotic proteins like cleaved caspase 3, Bax or GSK-3 β and increasing expression of anti-apoptotic proteins Bcl-2. Additionally, GSK-3 β regulates the expression of its downstream protein β -catenin, an important regulator of intercellular junction structure, which affects blood-brain barrier integrity and permeability (Dajas-Bailador et al., 2002; Krafft et al., 2012; Egea et al., 2012). Anti-oxidative effects can be also linked to JAK2/PI3K/Akt/Nrf2/HO-1 and JAK2/STAT3/NF κ B pathway via increased expression of antioxidants such as HO-1, superoxide dismutase, and glutathione peroxidase, and decreased expression of superoxide producing NADPH oxidase (Egea et al., 2012; Han et al., 2014; Parada et al., 2010).

4. Vagus nerve stimulation (VNS)

Vagus nerve stimulation similarly to pharmacological stimulation of $\alpha 7$ nAChR stimulation may be a promising method of controlling inflammation.

4.1. Direct and indirect VNS

Vagus nerve stimulation (VNS) is a promising therapeutic approach that stimulates the $\alpha 7$ nAChR and activates the cholinergic anti-inflammatory pathway. It has immunomodulatory properties by which it attenuates inflammation without causing immunosuppression (Tracey, 2009). As mentioned above, it prevents the systemic release of pro-inflammatory cytokines, thus it has an anti-inflammatory effect. Furthermore, it has also been shown to have anti-apoptotic, anti-oxidative properties and prevent hypotension (Kanashiro et al., 2017).

It has been shown that the VN plays a crucial role in anti-inflammatory signaling. Blockage of VN signaling in vagotomized animals or $\alpha 7$ nAChR-deficient animals results in aggravated levels of pro-inflammatory cytokines and accelerated shock development, compared to the control (Wang et al., 2003; Borovikova et al., 2000).

Vagus nerve stimulation can be performed by direct stimulation of the VN. This is a method that requires an invasive surgery. A more recent approach focuses on indirect stimulation of the skin area which is innervated by VN. The external ear (concha and external auditory meatus) is the only area of the skin that is innervated by the VN (He et al., 2013; Frangos et al., 2015; Huston et al., 2007). Therefore, it is often called transcutaneous auricular vagus nerve stimulation (taVNS). Indirect stimulation and taVNS were shown to ameliorate inflammatory response (Zhao et al., 2012). There is also so-called transcutaneous VNS (tcVNS) where the cervical branch of the VN is stimulated indirectly through the skin (Yap et al., 2020). tcVNS seems to be a safe and feasible method in the setting of acute ischemic stroke (Arsava et al., 2022).

The clinical applicability of VNS on humans is limited by the invasiveness of the surgical procedure that is required for a direct VNS.

5. Vagus nerve stimulation in stroke treatment - a systematic review

This systematic review section was written using published articles that examined the use of VNS on stroke. A search of electronic databases SpringerLink, ScienceDirect, and PubMed/Medline was conducted. The research terms used were 1) “vagus nerve stimulation” OR VNS AND stroke; 2) “vagus nerve stimulation” OR VNS AND “cerebral ischemia”; 3) “vagus nerve stimulation” OR VNS AND “subarachnoid hemorrhage”; 4) “vagus nerve stimulation” OR VNS AND “intracerebral hemorrhage”. We have found 215 articles with these search terms. Articles were excluded if they were irrelevant to this review’s topic (e.g. not focusing on the use of the immunomodulatory properties of the VN such as the recovery of upper limb motor functions); were reviews, meta-analyses, comments, editorials; were in a different language than English. After this exclusion, we were left with 32 articles. The results of these studies are summarized in Table 1.

As mentioned above, we have not found any clinical trial that would attempt to use VNS anti-inflammatory properties in stroke treatment. Within the experimental articles, 32 studies focused on the use of VNS in ischemic stroke (both transient and permanent) and one study focused on subarachnoid hemorrhage. For ischemic stroke, the middle cerebral artery occlusion model was used in all included studies. For subarachnoid hemorrhage, it was elastase injection. We have not found any studies where VNS was used in the treatment of intracerebral hemorrhage.

Cervical (direct) VNS, trans-cutaneous (indirect) VNS, and trans-auricular (indirect) VNS were used in experimental stroke treatment. Cervical VNS was used in 24 studies, transcutaneous VNS in 6, and trans-auricular VNS in 5 studies. The reason that the sum of VNS methods used exceeds the number of studies is the fact that [Lindemann et al. \(2020\)](#) used four different experimental settings as indicated in [Table 1](#).

The effects of VNS on stroke in these experimental studies showed a variety of effects; among others neuroprotection (Ay et al., 2011; Chen et al., 2018; Jiang et al., 2016; Li et al., 2020a); anti-apoptotic (Jiang et al., 2014; Jiang et al., 2015a; Xu et al., 2018; Zhang et al., 2017; Zhang et al., 2021b; Zhao et al., 2019), anti-inflammatory (Jiang et al., 2014; Ay et al., 2011; Ay et al., 2015; Ay et al., 2016; Karson et al., 2013; Chen et al., 2018; Jiang et al., 2015a; Jiang et al., 2015b; Jiang et al., 2016; Li et al., 2020a; Li et al., 2020b; Lindemann et al., 2020; Lu et al., 2017; Ma et al., 2016; Suzuki et al., 2019; Tang et al., 2022; Xiang et al., 2015; Xu et al., 2018; Yang et al., 2018; Zhang et al., 2017; Zhang et al., 2021b; Zhang et al., 2022; Zhao et al., 2019; Zhao et al., 2022) and anti-oxidative effects (Meyers et al., 2018; Karson et al., 2013; Chen et al., 2018; Jiang et al., 2015a; Li et al., 2020b; Liu et al., 2023; Suzuki et al., 2019); reduced infarct volume (Ay et al., 2009; Hiraki et al., 2012; Jiang et al., 2014; Ay et al., 2011; Ay et al., 2015; Ay et al., 2016; Karson et al., 2013; Chen et al., 2018; Jiang et al., 2015a; Jiang et al., 2015b; Jiang et al., 2016); improved neurological deficit scores (Ay et al., 2009; Hiraki et al., 2012; Jiang et al., 2014; Ay et al., 2011; Ay et al., 2015; Ay et al., 2016; Karson et al., 2013; Chen et al., 2018; Jiang et al., 2015a; Jiang et al., 2015b; Jiang et al., 2016; Li et al., 2020b; Liu et al., 2023; Lindemann et al., 2020; Liu et al., 2016; Lu et al., 2017; Ma et al., 2016; Sun et al., 2012; Suzuki et al., 2019; Tang et al., 2022; Xiang et al., 2015; Zhang et al., 2017; Zhang et al., 2021a; Zhang et al., 2021b; Zhang et al., 2022; Zhao et al., 2019); and promotion of M2 microglial polarization (Zhang et al., 2022; Zhao et al., 2019; Zhao et al., 2022).

The most commonly used parameters of VNS are initiation of stimulation 30 min after ischemia, with 30 s of stimulation every 5 min for 1 h and electrical parameters of 20 Hz and current of 0.5 mA.

The most commonly used animals were unsurprisingly rats and mice, but gerbils were also used.

There are several things that future vagus nerve stimulation research

Table 1

A summary of articles from our systematic research. cVNS – cervical vagus nerve stimulation; MCAO – middle cerebral artery occlusion; SAH – subarachnoid hemorrhage; taVNS – trans-auricular vagus nerve stimulation; tcVNS – transcutaneous vagus nerve stimulation.

Article	Animal model	VNS type	Stroke type	Stroke model	Stimulation parameters (time; frequency; el. current)	Stimulation description	Timepoint	Markers	Effect
Ay et al., 2009	Rats	cVNS	Ischemic stroke - transient	MCAO	30 s; 20 Hz; 0,5 mA	VNS initiated 30 min after ischemia; group 1: 30 s stimulation at every 30 min for 3 h; group 2: 30 s stimulation every 5 min for 1 h	24 h	Infarct volume; Neurological score	Smaller ischemic lesion; better functional score Neuroprotection after ischemic brain injury is not mediated by changes in CBF; stimulation of both the right and left vagus nerve have comparable effects, VNS is effective after ipsilateral and contralateral focal ischemia
Ay et al., 2011	Rats	cVNS	Ischemic stroke - transient	MCAO	30 s; 20 Hz; 0,5 mA	VNS initiated 30 min after ischemia; 30 s stimulation every 5 min for 1 h VNS initiated 30 min after ischemia; 30 s stimulation every 5 min for 1 h	24 h; 3 days	Infarct volume; CBF; Neurological score	Reduced infarct volume; better neurological score nVNS inhibits ischemia-induced immune activation and reduces the extent of tissue injury and functional deficit in rats without causing cardiac or hemodynamic adverse effects when initiated up to 4 h after MCAO
Ay et al., 2015	Rats	taVNS	Ischemic stroke - transient	MCAO	30 s; 20 Hz; 0,5 mA	VNS initiated 30 min after ischemia; 30 s stimulation every 5 min for 1 h	24 h	Infarct volume; Neurological score	Antioxidative properties of VNS; smaller infarct area
Ay et al., 2016	Rats	tcVNS	Ischemic stroke - transient	MCAO	2 min; 25 Hz; 7,2 V	VNS initiated 30 min after ischemia; 2 min stimulation every 10 min for 1 h VNS initiated 10 min before ischemia and continued for 30 min of reperfusion VNS initiated 30 min after ischemia; 30 s stimulation every 5 min for 1 h	7 days	Infarct volume; Neurological score	Smaller ischemic lesion; better functional score; CBF in MCA area did not differ between groups The BBB permeability, infarct volume, activation of microglia and astrocytes and oxidative stress decreased in VNS treated mice and this neuroprotection was associated with the suppression of BBB permeability, VNS alleviated CMI and this neuroprotection was associated with suppression of BBB permeability, neuroinflammation and oxidative stress Better neurological deficit scores, reduced cerebral infarct volume, and decreased number of TUNEL positive cells; the levels of pro-inflammatory cytokines and cleaved caspase 3 protein were significantly decreased,
Karson et al., 2013	Rats	cVNS	Ischemic stroke - transient	MCAO	30 s; 20 Hz; 1 mA	VNS initiated 30 min after ischemia; 30 s stimulation every 5 min for 1 h	30 min; 24 h	Infarct volume; neurological score; MDA, SOD, GSH	
Hiraki et al., 2012	Rats	cVNS	Ischemic stroke - transient	MCAO	30 s; 20 Hz; 0,5 mA	VNS initiated 30 min after ischemia; 30 s stimulation every 5 min for 1 h	21 days	Infarct volume; neurological score; CBF	
Chen et al., 2018	Mice	cVNS	Ischemic stroke - transient	MCAO	30 s; 5 Hz; 0,5 mA	VNS initiated 30 min, 1 d, 2 d, 3 d after CMI; VNS for 30 s every 5 min for 1 h	36 days	BBB permeability; histology	
Jiang et al., 2014	Rats	cVNS	Ischemic stroke - transient	MCAO	30 s; 20 Hz; 0,5 mA	VNS initiated 30 min after ischemia; 30 s stimulation every 5 min for 1 h	24 h	Neurological score, infarct volume, neuronal apoptosis (cleaved caspase-3, TUNEL); pro-inflammatory cytokines; a7nAChR, p-Akt	

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Table 1 (continued)

Article	Animal model	VNS type	Stroke type	Stroke model	Stimulation parameters (time; frequency; el. current)	Stimulation description	Timepoint	Markers	Effect
									and the levels of $\alpha 7nAChR$ and phosphorylated Akt were significantly increased
Jiang et al., 2015a	Rats	cVNS	Ischemic stroke - transient	MCAO	30 s; 20 Hz; 0,5 mA	VNS initiated 30 min after ischemia; 30 s stimulation every 5 min for 1 h	24 h	Infarct volume; neurological score; miR-210; MDA, SOD, GSH; TUNEL	Increased miR-210 expression; decreased apoptosis; increased antioxidant stress response PPAR γ diminished the extent of ischemic infarct, alleviated neuronal injury, and suppressed pro-inflammatory cytokine expression and immune cell activation
Jiang et al., 2015b	Rats	cVNS	Ischemic stroke - transient	MCAO	30 s; 20 Hz; 0,5 mA	VNS initiated 30 min after ischemia; 30 s stimulation every 5 min for 1 h	24 h	PPAR γ ; neurological score; infarct volume; pro-inflammatory cytokines (TNF α , IL1 β , IL6, HMGB1)	aVNS had significant neuroprotective effects and enhanced angiogenesis; improvements in the behavioral scores and brain histopathology, including increased levels of microvessel density and endothelial cell proliferation surrounding the infarct area; BDNF, eNOS and VEGF were expressed at higher levels
Jiang et al., 2016	Rats	taVNS	Ischemic stroke - transient	MCAO	30 s; 20 Hz; 0,5 mA	VNS initiated 30 min after ischemia; 30 s stimulation every 5 min for 1 h twice daily until the time of sacrifice	24 h; 21 days	Infarct volume; neurological score; BDNF, VEGF, eNOS	Neuroprotection in the chronic phase of stroke; enhanced axonal plasticity; activation of the BDNF/cAMP/PKA/p-CREB, axonal plasticity
Li et al., 2020a	Rats	taVNS	Ischemic stroke - transient	MCAO	30 s; 20 Hz; 0,5 mA	VNS initiated 30 min after ischemia; 30 s stimulation every 5 min for 1 h twice daily until the time of sacrifice	28 days	mRNA ($\alpha 7nAChR$, GAP43) cAMP, PKA, p-CREB, axonal plasticity	Upregulated PPAR- γ expression; better neurobehavioral recovery, alleviated neuronal injury, and increased angiogenesis
Li et al., 2020b	Rats	taVNS	Ischemic stroke - transient	MCAO	30 s; 20 Hz; 0,5 mA	VNS initiated 30 min after ischemia; 30 s stimulation every 5 min for 1 h twice daily until the time of sacrifice	28 days	PPAR γ ; neurological score; infarct volume; BDNF, VEGF, P-eNOS	VNS decreased the levels of pro-inflammatory phenotype markers iNOS and TNF α and increased the expression of regulatory phenotype markers Arg1 and TGF β through activating $\alpha 7nAChR$; phenotypic microglial conversion
Liu et al., 2023	Rats	cVNS	Ischemic stroke - transient	MCAO	30 s; 20 Hz; 0,5 mA	VNS initiated 30 min after ischemia; 30 s stimulation every 4 min for 1 h	1, 2, 3, 5 days	Infarct volume, neurobehavioral assessment; iNOS, TNFs, TGF β , TLR4, MyD88, NF κ B, Arg1, IL-10, IL-1 β , CD206, CD86	Reduced the frequency of SDs in the cortical peri-infarct area compared to sham VNS, without affecting relative blood flow changes, blood pressure, heart rate or breathing rate
Lindemann et al., 2020	Rats	cVNS	Ischemic stroke - permanent	MCAO	30 s; 25 Hz; 0,5 mA	VNS initiated 30 min after ischemia; 30 s stimulation every 5 min for 1 h	3 days	Infarct volume, behavioral tests (Garcia), pulsoxyimetry, blood pressure measurement	Reduced the frequency of SDs in the cortical peri-infarct area compared to sham VNS, without affecting relative blood flow changes, blood pressure, heart rate or breathing rate
Lindemann et al., 2020	Rats	tcVNS	Ischemic stroke - permanent	MCAO	2 min; 25 Hz; mA unknown	VNS initiated 30 min after ischemia; VNS for 2 min repeated once after 15 min	3 days	Infarct volume, behavioral tests (Garcia), pulsoxyimetry, blood pressure measurement	

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Table 1 (continued)

Article	Animal model	VNS type	Stroke type	Stroke model	Stimulation parameters (time; frequency; el. current)	Stimulation description	Timepoint	Markers	Effect
Lindemann et al., 2020	Rats	cVNS	Ischemic stroke - transient	MCAO	30 s; 25 Hz; 0,5 mA	VNS initiated 30 min after ischemia; 30 s stimulation every 5 min for 1 h	3 days	Infarct volume, behavioral tests (Garcia), pulsoxymetry, blood pressure measurement	Cortical stroke volume was reduced; stroke volume in the basal ganglia remained unchanged; motor outcome was improved 2 days after transient MCAO
Lindemann et al., 2020	Rats	tcVNS	Ischemic stroke - transient	MCAO	2 min; 25 Hz; mA unknown	VNS initiated 30 min after ischemia; VNS for 2 min repeated once after 15 min VNS initiated 30 min before occlusion and just after MCA reperfusion; 3 s on, 3 s off for total of 10 min	3 days	Infarct volume, behavioral tests (Garcia), pulsoxymetry, blood pressure measurement	Cortical stroke volume was reduced; stroke volume in the basal ganglia remained unchanged; motor outcome was improved 2 days after transient MCAO, but was similar to sham VNS animals 3 days after ischemia
Liu et al., 2016	Rats	cVNS	Ischemic stroke - transient	MCAO	3 s; 20 Hz; 1 mA	VNS initiated 30 min after ischemia; 30 s stimulation every 5 min for 1 h	16 days	Behavioral experiments (Morris water maze)	Improved spatial memory; improved fear memory
Lu et al., 2017	Rats	cVNS	Ischemic stroke - permanent	MCAO	30 s; 20 Hz; 0,5 mA	VNS initiated 30 min after ischemia; 30 s stimulation every 5 min for 1 h	24 h	Infarct volume; neurological score; protein levels (a7nAChR, p-JAK2, p-STAT3)	Improved neurological function and reduced cerebral infarct volume; enhanced levels of a7nAChR, p-JAK2, and p-STAT3
Ma et al., 2016	Rats	taVNS	Ischemic stroke - transient	MCAO Occlusion of the bilateral common carotid arteries	30 s; 20 Hz; 0,5 mA	VNS initiated 30 min after ischemia; 30 s stimulation every 5 min for 1 h	24 h; 3 days; 7 days	GDF11, ALK5; neurobehavioral function; EC proliferation	Improved neurobehavioral recovery, upregulated cerebral GDF11 and downregulated splenic GDF11; increased expression of ALK5 in ECs and stimulated proliferation of ECs
Masada et al., 1996	Gerbils	cVNS	Ischemic stroke - transient	Occlusion of the bilateral common carotid arteries	3 min; 40 Hz; 0,1 or 0,2 mA	VNS for 3 min during ischemia	5 days	Neuronal density	Reduced neuronal cell death in hippocampus VNS during ischemia rescued hippocampal neurons from ischemic insult; VNS affects both glutamate release and CBF in ischemia.
Miyamoto et al., 2003	Gerbils	cVNS	Ischemic stroke - transient	Occlusion of the bilateral common carotid arteries	5 min; 40 Hz; 0,4 mA	VNS for 5 min during ischemia VNS initiated 30 min after ischemia; 30 s stimulation every 5 min for 1 h	7 days	Glutamate release, hippocampal blood flow, hyperemia	VNS significantly improved the survival rate; VNS significantly improved the survival rate; VNS significantly improved the survival rate
Sun et al., 2012	Rats	cVNS	Ischemic stroke - transient	MCAO	30 s; 20 Hz; 0,5 mA	VNS initiated 30 min after ischemia; 30 s stimulation every 5 min for 1 h	24 h	Infarct volume, neurological score	Ischemic lesion volume was smaller; better functional scores at 24 h
Sun et al., 2012	Rats	cVNS	Ischemic stroke - permanent	MCAO	30 s; 20 Hz; 0,5 mA	VNS initiated 30 min after ischemia; 30 s stimulation every 5 min for 1 h	24 h	Infarct volume, neurological score	Ischemic lesion volume was smaller; no statistically significant differences in the neurobehavioral scores
Suzuki et al., 2019	Mice	tcVNS	SAH	Intracranial Aneurysm Model - elastase injection	2 min; 25 Hz; mA unknown	VNS initiated 24 h after SAH; 2 min stimulation repeated twice 5 min apart once daily until euthanasia VNS initiated 30 min after ischemia; 30 s stimulation every 5 min for 1 h	21 days or severe signs	mRNA (IL1B, IL6, iNOS, MMP9, MCP1); neurological examination	In lower SAH grades VNS significantly reduced aneurysm rupture rate; in higher SAH grades VNS significantly improved the survival rate; VNS significantly improved the survival rate; VNS significantly improved the survival rate
Tang et al., 2022	Rats	cVNS	Ischemic stroke - transient	MCAO	30 s; 20 Hz; 0,5 mA	VNS initiated 30 min after ischemia; 30 s stimulation every 5 min for 1 h	3 days	NLRP3 inflammasome; neurological score; TUNEL;	VNS inhibited the expression of pyroptosis-related molecules and decreased the number of

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Table 1 (continued)

Article	Animal model	VNS type	Stroke type	Stroke model	Stimulation parameters (time; frequency; el. current)	Stimulation description	Timepoint	Markers	Effect
Xiang et al., 2015	Rats	cVNS	Ischemic stroke - transient	MCAO	30 s; 20 Hz; 0,5 mA	VNS initiated 30 min after ischemia; additional stimulation at 12 h, 1, 2, 3, 7, 14 days after surgery; 30 s stimulation every 5 min for 1 h	12 h; 1 d, 2 d, 3 d, 7 d, 14 d	inflammatory cytokines Infarct volume, neurological score, pro-inflammatory cytokines (TNF α , IL6)	pyroptotic cells and membrane pores Reduced infarct volume, improved neurological function, and decreased the expression levels of tumor necrosis factor- α and interleukin-6 Restored cognitive and motor impairments;
Xu et al., 2018	Rats	cVNS	Ischemic stroke - transient	MCAO	30 s; 20 Hz; 0,5 mA	VNS initiated 30 min after ischemia; 30 s stimulation every 5 min for 1 h	1 d, 3 d, 7 d, 10 d, 14 d, 21 d	Infarct volume, neural apoptosis, inflammatory secretion and signaling pathway	significantly neurological deficits; infarct volume, neural apoptosis and inflammatory cytokines release Significantly smaller infarct sizes; VNS protected vascular tight junction proteins from disruption in microvessels, and reduced expression of MMP-2/9
Yang et al., 2018	Rats	tcVNS	Ischemic stroke - transient	MCAO	2 min; 25 Hz; mA unknown	VNS initiated 30 min after ischemia; total of 5, 2 min stimulations, one every ten min	24 h	Infarct volume, BBB leakage	The expression of L-PGDS increased; better neurological deficit scores, reduced infarct volume, and decreased neuronal apoptosis
Zhang et al., 2017	Rats	cVNS	Ischemic stroke - transient	MCAO	30 s; 20 Hz; 0,5 mA	VNS initiated 30 min after ischemia; 30 s stimulation every 5 min for 1 h VNS initiated 30 min after ischemia; 30 s stimulation every 5 min for 1 h	24 h; 3 days	Neurological score; infarct volume; neuronal apoptosis (Bax, Bcl-2, cleaved caspase-3; L-PGDS)	Promotion of M2 microglia polarization; inhibition of M1 microglia polarization; inhibition of TLR4/MyD88/NFkB
Zhang et al., 2021a	Rats	cVNS	Ischemic stroke - transient	MCAO	30 s; 20 Hz; 0,5 mA	VNS initiated 30 min after ischemia; 30 s stimulation every 5 min for 1 h VNS initiated 30 min after ischemia; 30 s stimulation every 5 min for 1 h	3 days	Infarct volume; neurological score; TLR4, MyD88, NFkB	Decreased infarct volume and suppresses neurological; inhibited autophagy and apoptosis Promotion of the M1-to-M2 phenotype conversion to alleviate inflammatory response and brain injury through inhibition of TLR4/MyD88/NF-kB pathway
Zhang et al., 2021b	Rats	cVNS	Ischemic stroke - transient	MCAO	30 s; 20 Hz; 0,5 mA	VNS initiated 30 min after ischemia; 30 s stimulation every 5 min for 1 h	24 h	Infarct volume, neurological deficit, autophagy, and apoptosis	Decreased infarct volume, improved neurological outcomes, reduced apoptotic neurons; promoted microglial M2 polarization
Zhang et al., 2022	Rats	cVNS	Ischemic stroke - transient	MCAO	30 s; 20 Hz; 0,5 mA	VNS initiated 30 min after ischemia; 30 s stimulation every 5 min for 1 h	3 days	Infarct volume; neurological score; TLR4, MyD88, NFkB	Promoted the secretion of acetylcholine, inhibited the secretion of interleukin-1 β , interleukin-6, and tumor necrosis factor- α , and reduced connexin 43 phosphorylation
Zhao et al., 2019	Mice	tcVNS	Ischemic stroke - transient	MCAO	2 min; 25 Hz; mA unknown	Unknown when was VNS initiated; 2 min stimulation every 10 min for 1 h	3 days	Neurological score; infarct volume; neuronal apoptosis (Bax, Bcl-2)	
Zhao et al., 2022	Rats	taVNS	Ischemic stroke - transient	MCAO	30 min; 10 Hz; 1 mA	VNS initiated 24 h after ischemia; 30 min stimulation once per days for a total of 7 days	24 h; 7 days	Ach, IL1B, IL6, TNF α ; connexin 43	

in the treatment of stroke (and other pathologies where inflammation plays a central role) could focus on.

Firstly, even though hemorrhagic stroke accounts for approximately a quarter of all stroke cases, VNS was only used by Suzuki et al. (2019) in SAH. To our knowledge, there are no publications that would attempt to use either direct or indirect VNS in intracerebral hemorrhage. It could be beneficial to focus more on VNS as a treatment option for hemorrhagic

stroke.

The stimulation parameters and design also vary significantly. Initiation of VNS ranges between 30 min after ischemia to 3 days. The time of one uninterrupted stimulation varies between 30 s and 30 min. The electrical parameters range between 10 and 40 Hz and 0,1 and 1 mA (some studies included voltage as a parameter, but we do not deem this to be correct because voltage is dependent on current as well as

resistance, and resistance of the tissue can be variable with every experimental unit). Thus, more standardized stimulation parameters could help with reproducibility.

A period of 1 h of interrupted stimulation (30 s of stimulation, 5 min apart for 1 h) was most commonly used. However, it is possible that the VN gets depleted after such period and that desired anti-inflammatory effects mediated by CAP are no longer present. The depletion of the nerve would have to be studied.

Vagus nerve stimulation is undoubtedly intriguing method for modulating inflammatory response. Nevertheless, there are certain discrepancies between experimental and clinical studies. It is possible that in highly inflammatory states (such as stroke) VNS will not be a suitable therapeutic option, because promising results of experimental studies have not yet been successfully translated into the clinical practice. Thus, VNS could pose as a supplementary treatment option and be combined with other treatment strategies.

7. Conclusion

Vagus nerve stimulation is an interesting approach to modulating inflammatory response. It has been focused on repeatedly in the stroke context. In this review, we have described vagus nerve anatomy, its role in the immune system, and mechanisms of the cholinergic anti-inflammatory pathway. We have provided a summary of available experimental literature focusing on vagus nerve stimulation as a therapeutic option in ischemic and hemorrhagic stroke and added ideas for future research directions.

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Declaration of Competing Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Data availability

No data was used for the research described in the article.

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5. Conclusion

Subarachnoid hemorrhage (SAH) is predominantly caused by the rupture of a cerebral aneurysm, and it represents a severe medical condition associated with high morbidity and mortality. There are several life-threatening complications associated with SAH, like acute hydrocephalus, rebleeding, or delayed cerebral ischemia (DCI).

DCI is a severe late complication, which affects approximately 30% of patients with SAH and significantly contributes to worsening outcomes. Cerebral vasospasm was considered the only cause of DCI however, DCI may occur independently of vasospasm development. The concept of early brain injury (EBI) was elaborated to explain the pathophysiology of DCI. EBI includes pathologies like transient global cerebral ischemia, oxidative stress, blood-brain barrier disruption, brain edema, endothelial/neuronal apoptosis, and inflammation.

Inflammation plays a key role in the pathophysiology of SAH, interconnecting all EBI-related pathologies. Inflammation is also associated with both DCI and cerebral vasospasm. One of the most extensively studied proinflammatory markers associated with SAH is IL-6. IL-6 is associated with severity of SAH as well as with outcome. However, there is a high variability in levels of IL-6 which is a problem for effective therapy targeting inflammation after SAH. Vagus nerve stimulation is a promising way to activate CHAIP and control inflammation without a risk of immunosuppression.

6. Full list of the author's publications in chronological order (publication highlighted in bold are commented above)

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