TECHNISCHE UNIVERSITÄT MÜNCHEN

Fakultät für Medizin

Effects of perfluorocarbons on the neurological outcome after subarachnoid haemorrhage in mice

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Vollständiger Abdruck der von der Fakultät für Medizin der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Medizin

genehmigten Dissertation.

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Die Dissertation wurde am 28.01.2021 bei der Technischen Universität München eingereicht und durch die Fakultät für Medizin am 10.11.2021 angenommen.

Für
meine Eltern
meine Geschwister
und zahlreiche
große und kleine helfende Hände

Zusammenfassung:

Obwohl die SAB seit Jahrzehnten erforscht wird, konnte bisher keine effektive Behandlung gefunden werden, so dass die Mortalität und Morbidität weiterhin hoch sind. Da die SAB relativ junge Menschen trifft sind die sozioökonomischen Auswirkungen erheblich, auch wenn die Grundinzidenz niedrig ist. Daher sind die Folgen für die Patienten und ihre Familien erheblich.
Aufgrund der schweren und langanhaltenden Morbidität der Überlebenden hat sich die Wissenschaft bisher stark darauf fokussiert die zugrundeliegende Pathologie der neurologischen und funktionellen Langzeitschäden der SAB zu erforschen, sowie eine entsprechende Therapie zu finden. Lange Zeit fand man als einziges pathologisches Korrelat Vasospasmen der Gehirnarterien, die in der zweiten Woche nach der SAB zu beobachten waren. Therapien zur Unterdrückung dieser Spasmen konnten die Folgeerscheinung jedoch nicht konsistent verhindern und neurologische Einschränkungen traten auch ohne den Nachweis von Vasospasmen auf. Daher fokussierten sich manche Forscher auf ein besseres Verständnis der zugrundeliegenden Pathophysiologie, was zur Formulierung einer neuen Theorie einer multifaktoriellen Genese der Folgeerscheinungen führte. Neben Vasospasmen spielen dabei Mechanismen wie Mikrothrombosen, Entzündungsreaktionen und Cortical Spreading Ischaemia eine Rolle in der Ausbildung der Langzeitfolgen. Eine sich früh entwickelnde zerebrale Ischämie spielt dabei eine Schlüsselrolle in der Ausbildung dieser verschiedenen Prozesse.
Mit verschiedenen Perfluorocarbonen konnte experimentell die Gewebeoxygenierung verbessert werden, indem sie die Sauerstofftransportkapazität von Hämoglobin ergänzten oder komplett ersetzten. In Experimenten an neurologischen Erkrankungen mit ähnlicher Pathophysiologie zur SAB, wie ischämischer Schlaganfall und Schädel-Hirn-Traum, konnte die Behandlung mit Perfluorocarbonen die zerebrale Sauerstoffversorgung verbessern und die neurologischen Langzeitschäden lindern. Daher wird in dieser Arbeit untersucht, ob eine Behandlung mit dem Perfluorocarbon Oxycyte im Mausmodell die neurologischen Langzeitfolgen lindern kann.
Nachdem eine zerebrale Ischämie nach SAB frühzeitig auftritt wurde Oxycyte direkt nach Auslösung der Blutung verabreicht und mit einer Sauerstofftherapie von 90vol% kombiniert. Um den Einfluss der beiden Behandlungen zu trennen wurden die Experimente um entsprechende Kontrollgruppen ergänzt und mit Gruppen von Schein-SAB-Auslösung verglichen. Zur Differenzierung des Einflusses der Behandlungen auf die Prozesse, die während der ersten 72 Stunden nach Auslösung der SAB ablaufen wurden die Tiere zusätzlich nach drei verschiedenen Zeiträumen ausgewertet. Um Unterschiede zwischen den Gruppen zu objektivieren wurde die sensorische, motorische und reflektorische Funktionalität in einem Test überprüft, sowie das Ausmaß des Gehirnödems mittels der Feucht-/Trockengewicht Methode gemessen.
Die Auswertung der Experimente zeigt, dass die kombinierte Behandlung aus Oxycyte und Sauerstofftherapie die neurologischen Einschränkungen bei Tieren, die von der SAB schwere funktionelle Einschränkungen davontragen, im Vergleich zu Kontrollen tendenziell verschlimmert. Tiere mit leichtgradigen Einschränkungen könnten dagegen von der Therapie profitiert haben. Dieser Unterschied könnte durch eine unterschiedliche Beeinträchtigung des intrinsischen Radikal-Abwehrsystems und nachfolgenden Fehlfunktion der Stickstoffmonoxid-vermittelten Vasodilatation zustande kommen. In der Konsequenz könnte die zusätzliche Behandlung mit Sauerstoff zu einer vermehrten Radikalproduktion geführt haben.
In der Zukunft könnte es daher interessant sein die Behandlung mit Perfluorocarbonen ohne zusätzliche Sauerstofftherapie durchzuführen und/oder die Behandlung um einen Radikalfänger oder Stickstoffmonoxid zu ergänzen.

Abstract:

Although SAH is investigated since decades, the efforts to find an effective treatment were not very successful and the mortality and morbidity is still important. Since SAH hits a quite young collective of patients, the socioeconomic consequences are important, even though comparatively the incidence is not very high. Consequently, patients and their families are severely affected.

Because of the severe and long-lasting morbidity of the survivors, a lot of research focused on understanding the pathology of and finding a treatment against the neurologic and functional sequels that persist after the initial haemorrhage. For a long time, the most important pathological finding after SAH were cerebral arterial vasospasms, occurring in the second week after the initial impact. However, treatments to suppress these spasms failed to reliantly prevent the sequels and neurologic impairments were also found in the absence of spasms. Thus, some researchers focused on a better understanding of the pathophysiology, leading to a new theory of a multifactorial genesis. Besides arterial vasospasms, processes like the formation of microthrombi, inflammation and cortical spreading ischaemia are involved and contribute to the formation of neurological impairments. Cerebral ischaemia, occurring early after SAH, plays a key role in the formation of these different processes.

Different types of perfluorocarbons were able to experimentally improve tissue oxygenation by increasing or replacing the oxygen carrying capacity of haemoglobin. When investigated in neurologic diseases with a similar pathophysiology to SAH, like ischemic stroke or traumatic brain injury, a treatment with perfluorocarbons was able to improve cerebral oxygenation and ease the neurological sequels. Consequently, this work investigates if the perfluorocarbon Oxycyte was able to improve the neurological outcome after SAH in mice.

As cerebral ischaemia was shown to occur early after SAH, Oxycyte was applied directly after the haemorrhage induction and combined with a hyperoxygenation of 90vol%. To be able to differ the effects of both treatments different control as well as sham groups were investigated. Additionally, the animals were observed for three different time periods to analyse the influence of the treatments on the processes taking place during the first 48 hours after SAH. To objectify differences between the different groups, the sensory, motor and reflex functionality of the animals were tested in a neurological score and the quantity of brain oedema was measured via the wet/dry weight method.

The analysis of the experiments shows that the combined treatment of Oxycyte and highly-concentrated oxygen tends to result in a worse outcome in animals that are more severely affected by the haemorrhage compared with controls. However, animals with less severe affection might profit from the treatment. This effect is possibly caused by a different level of functionality of the intrinsic radical scavenging systems and subsequently a disfunction of nitric oxygen mediated vasodilation. Therefore, the additional treatment with highly-concentrated oxygen could have led to more radical production.

Consequently, in the future it might be interesting to investigate the effect of perfluorocarbons without further oxygenation and/or the additional treatment with a radical scavengers or nitric oxide.

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List of abbreviations:

ACA	Anterior cerebral artery
ADL	Activities of daily living
BBB	Blood-brain barrier
BOXs	Bilirubin oxidation products
CBF	Cerebral blood flow
CBV	Cerebral blood volume
CCA	Common carotid artery
CMRO2	Cerebral metabolic rate of oxygen
CPP	Cerebral perfusion pressure
CSD	Cortical spreading depression
CSF	Cerebrospinal fluid
CSI	Cortical spreading ischaemia
CSWS	Cerebral salt wasting syndrome
DBI	Delayed brain injury
DCI	Delayed cerebral ischaemia
EBI	Early brain injury
EC	Extracellular
ECA	External carotid artery
eNOS	Endothelial nitric oxide synthase
ET-1	Endothelin-1
GOS	Glasgow outcome scale
HIF-1α	Hypoxia-inducible factor 1α
IADL	Instrumental activities of daily living
ICA	Internal carotid artery
ICP	Intracranial pressure
LDF	Laser-Doppler flowmetry
MABP	Mean arterial blood pressure
MCA	Middle cerebral artery
mNSS	Modified neurologic severity score
nNOS	Neuronal nitric oxygen synthase
NO	Nitric oxygen
NS	Not significant
OER	Oxygen extraction ratio
PFC	Perfluorocarbon
PPA	Pterygopalatine artery
PTSD	Posttraumatic stress disorder
QALY	Quality adjusted life years
QOL	Quality of life
RBC	Red blood cell
rCBF	Regional cerebral blood flow
ROS	Reactive oxygen species
SAH	Subarachnoid haemorrhage
SPC	Slow potential change
TNFα	Tumour necrosis factor alpha

1. Introduction

The purpose of this work is to first take a closer look at the pathophysiology of aneurysmal subarachnoid haemorrhage, next to propose a new approach to tackle the multifactorial causes for functional impairments as sequels of this illness and finally to present the results of experiments investigating this theory.

1.1. Subarachnoid haemorrhage

1.1.1. Definition

Subarachnoid haemorrhage (SAH) is defined as a spontaneous bleeding into the subarachnoid space, a room which is formed by the arachnoid membrane and the pia mater, the inner layer of the meninges. By this definition 2-10% of all strokes are due to SAH. (Feigin et al., 2009; Qureshi et al., 2007)

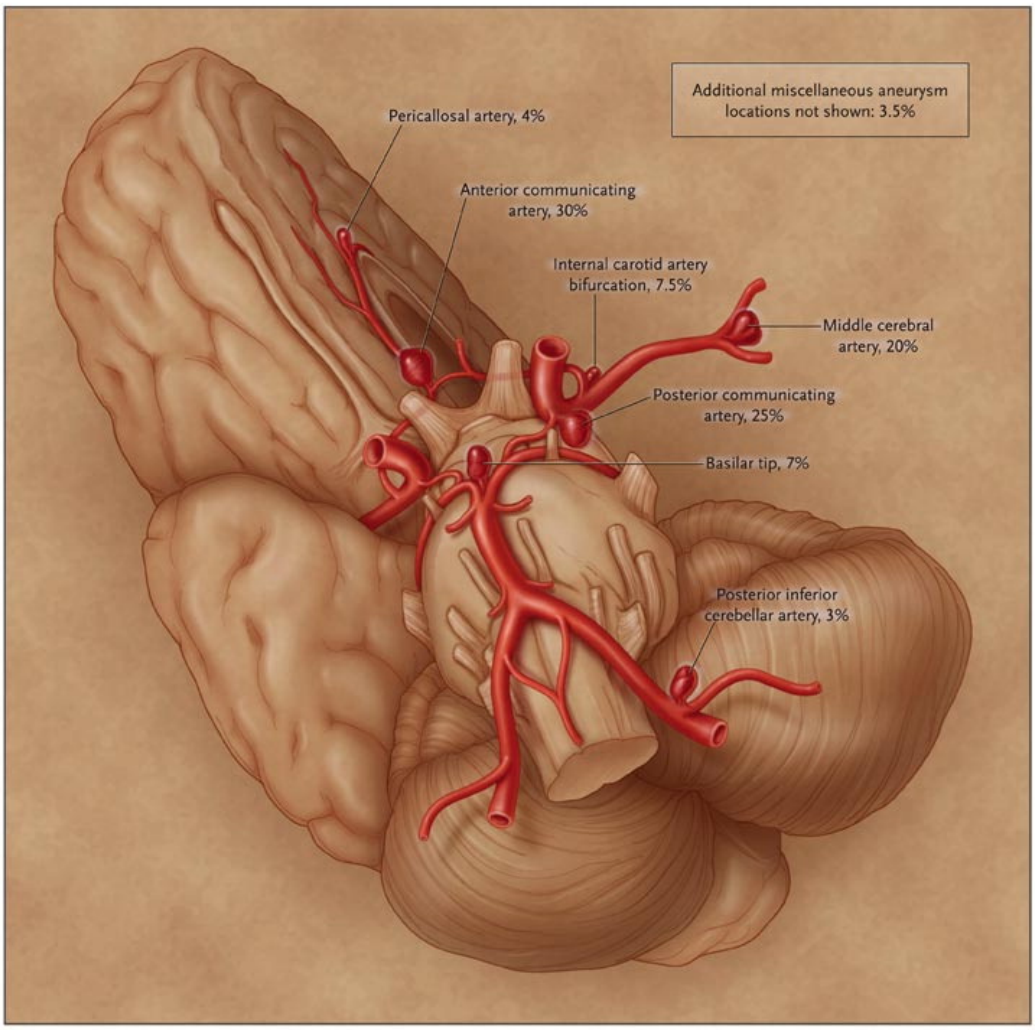


Figure 1:
Quantity of cerebral arterial aneurysms at different locations of the circle of Willis. The anterior communicating artery is the most common site with 30% of aneurysms, followed by the posterior communicating artery (25%) and the middle cerebral artery (20%). Overall, 75% of cerebral aneurysms occur in these three locations. (Reproduced with permission from Brisman et al. (Brisman et al., 2006), Copyright Massachusetts Medical Society.

By aetiology SAH can be divided into two groups, aneurysmal SAH and non-aneurysmal SAH or atypical SAH. With 85% of cases the rupture of an aneurysm is the most common reason for SAH. (Chen et al., 2014) Other reasons are in about 10% non-aneurysmal perimesencephalic haemorrhage and a heterogeneous group consisting amongst others of inflammatory diseases and malformations of blood vessels, coagulation disorders, neoplasia and drug abuse. (Cuvinciuc et al., 2010) Due to the predominant occurrence of aneurysmal SAH, in this work SAH is used as equivalent for aneurysmal SAH.
The first symptom of SAH is often a sudden, sharp and very intense headache, also referred to as "thunderclap headache" and categorized by patients as the worst pain ever experienced. Other symptoms that can occur additionally but as well exclusively, are vomiting, loss of consciousness, neck stiffness, focal neurological deficits and epileptic seizures. (van Gijn et al., 2001) However, these symptoms can be transient, develop over time and are not always present.

1.1.2. Epidemiology

In the general population unruptured aneurysms of the intracranial arteries are present in 2-3% with an increasing prevalence with age. However, the risk of rupture is not always the same. Aneurysms smaller than 10mm account for around 90% of the unruptured aneurysms and present only a rupture rate of 0,7% per year, opposed to those bigger than 10mm which present a rupture rate of around 4%. (Rinkel et al., 1998) The general incidence of SAH ranges around 6-9/100.000 worldwide, with important differences between high- and low-income countries on one side, and between single countries of comparable wealth on the other. (de Rooij et al., 2007; Feigin et al., 2009) Furthermore, in contrast to ischaemic stroke, the incidence of SAH did not seem to change over the past 40 years. (Feigin et al., 2009) With most cases between an age of 40 and 60 years (mean age 55 years), SAH strikes a relatively young part of the population, affecting more women, who present a 1.24 times higher risk for SAH than men (figure 2). (de Rooij et al., 2007; Rinkel et al., 2011) There is evidence in literature that this gender differences may be related to hormone status in women. (Longstreth et al., 1994)

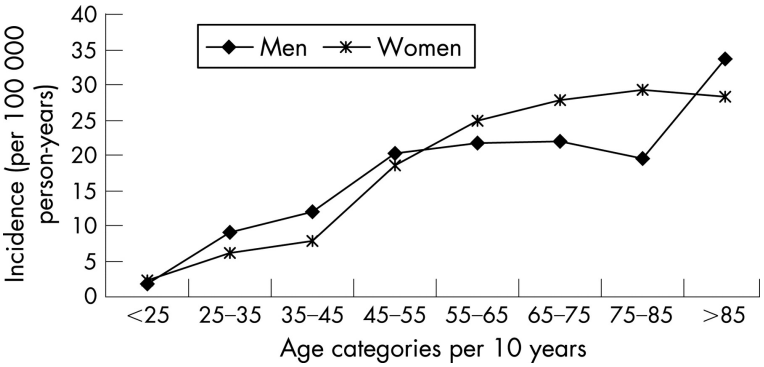


Figure 2:
Relative incidence of SAH for both sexes in different age categories. The risk for an SAH is rising with increasing age. (Reproduced with permission from de Rooij et al. (de Rooij et al., 2007))

Although most deaths following SAH are due directly to the initial haemorrhage (Broderick et al., 1994), the outcome is also influenced by various complications occurring afterwards. In the first 24h the most important is the risk of rebleeding from the aneurysm. Rebleeding occurs in 4-14% of SAHs, with most cases in the first 12 hours, causing a worse outcome and accounting for 22% of all fatalities (Broderick et al., 1994; Connolly et al., 2012). The other mayor complication following SAH is cerebral infarction, most commonly iatrogenic following an intervention or due to delayed cerebral ischaemia (DCI). (Vergouwen et al., 2011c) DCI, a term in the past often solely attributed to vasospasm, nowadays is considered to have a multifactorial aetiology. (Cossu

et al., 2014) (See 1.1.3.) Unfortunately, only recently a multinational expert group gave a consensus definition for the use of the term DCI. (Vergouwen et al., 2010) Before, many studies differed in the definition and, in consequence, comparability of most existing data vary. Nonetheless, DCI is considered to occur in about 20-30% post-SAH, also depending on whether patients were treated with calcium antagonists. (Dorsch, 2011; Schmidt et al., 2008) Most incidents were found to occur between day 4 and 10 post-SAH. (Brilstra et al., 2000) In contrast to rebleeding, DCI is not as deadly. In fact, around 25-30% of patients experiencing DCI decease, contributing a proportion of around 5% to all fatalities. (Dorsch, 2011; Lantigua et al., 2015) More importantly, DCI is the most important factor responsible for impairment and disability post-SAH, which results in around 25-45%. (Dorhout Mees et al., 2012; Dorsch, 2011) Further common complications of SAH which contribute as well to its high case fatality rate and morbidity are hydrocephalus, systemic inflammatory response syndrome, cardiac and pulmonary diseases, ionic changes and seizures. (Connolly et al., 2012; Lantigua et al., 2015)

Overall case fatality is considered to range between 30% to 50%, which reflects, in most cases, the 30-days fatality rate. (Lovelock et al., 2010; Nieuwkamp et al., 2009). 55% of deaths are due to the initial haemorrhage (Lantigua et al., 2015), and 12% already die before receiving medical attention (Huang et al., 2002). About one to two decades ago, half of all fatalities happened in the first 24 hours (Stegmayr et al., 2004), while in a recent publication this number was reduced to a third. (Lantigua et al., 2015) Therefore SAH still remains a devastating disease, although studies reveal that fatality rates have decreased at least by 17% over the past 3 decades (Nieuwkamp et al., 2009), without showing a coherent increase in morbidity (Lovelock et al., 2010).

But, even if SAH does not result in death, the prognosis is not bright. To assess morbidity and mortality after brain damage, the Glasgow Outcome Scale (GOS) was introduced by Jennett and Bond in 1975 (Jennett et al., 1975) and, since then, broadly used in studies about SAH (table 1). The scale graduates brain damage by five levels, which are: (1) death; (2) persistent vegetative state (unconsciousness); (3) severe disability (dependency on daily support); (4) moderate disability (clear disability but independent); and (5) good recovery (minor neurologic impairments might persist). By counting categories 2-4 as morbidity, a mayor study, conducted in the 1980s, has shown a total morbidity rate of around 15%, with almost half of concerned patients remaining severely disabled or unconscious. In contrast, around 60% were considered to have recovered well (Kassell et al., 1990) Unfortunately, more recent large-scale studies have not focused on overall morbidity, even though this is of major interest from a patients' perspective. Only a small study with 92 patients recently found a good recovery in 47%, moderate disability in 36% and severe disability in 10%. (Karic et al., 2016) However, since not all aspects of daily living were considered, newer studies show as well that a good recovery does as well result in impairment. At least 40-50% of surviving patients show cognitive dysfunction, psychological disturbances and a reduced quality of life (QOL) at follow-

Glasgow Outcome Scale (GOS)	
1	death
2	persistent vegetative state / unconsciousness
3	severe disability / dependence on daily support
4	moderate disability / mostly independent
5	good recovery / minor neurologic impairments

Table 1:
Glasgow outcome scale (GOS). Lower score shows worse outcome.

up evaluations. This includes, amongst others, problems with attention, concentration, learning, memory and speech, a sensation of reduced energy, depression, sleeping problems and anxiety, a reduced capacity of mobility and employment as well as social problems to an important extent. (Al-Khindi et al., 2010; Wermer et al., 2007) Although some authors also report an improvement in these areas in later follow-up evaluations, an important number of patients still describe some sort of limitation. (Greebe et al., 2010; Powell et al., 2002) Even one to five years after the initial SAH, 28-62% of patients achieved pathological results in complex choice reaction tasks, for example affecting short-term memory in 53% and long-term memory in 21%. (Hutter et al., 1993) Considering the relatively young age of persons affected by SAH and the high percentage of mortality, disability and long-term impairment of most survivors, one must consider the socioeconomic consequences.

As stated above, most patients experiencing SAH are 40 to 60 years old, hence they represent an important part of the working population, being responsible for the economic sustentation and care of their families. Most could have continued working for another 15-20 years, which shows the important impact of a comparatively rare disease like SAH to the life of various individuals. (Ogden et al., 1993) Instead, they lose an average of 10 quality adjusted life years (QALY), which, for example, summed up to a total of 74807 QALYs lost due to SAH in the UK in 2005. (Rivero-Arias et al., 2010) Phillips et al. showed a reduction of 7-year life expectancy from >80% in controls to 40% post-SAH. (Phillips et al., 1980) SAH accounts only for 2-10% of all strokes, but is responsible for 27% of all stroke-related mortality before 65 and is, in this dimension, comparable to ischaemic stroke (38%) and intracranial haemorrhage (34%). (Johnston et al., 1998) Although there is evidence that the amount of totally dependent survivors is relatively low, (Fogelholm et al., 1993) after one year, there are still 4-10% who need support in activities of daily living (ADL), as for example eating, cleaning, personal hygiene and dressing. (Hackett et al., 2000; Noble et al., 2008a) However, these are only the most basic needs of self-care. Once we look at the instrumental activities of daily living (IADL), comprehending more complex tasks like going shopping, doing laundry and housekeeping, and managing finances, the results do not look so bright anymore. Powell et al. evaluated patients 9 to 18 months after their haemorrhage and found 50% were limited and dependant in these activities. (Powell et al., 2002, 2004) Considering this and the importance of cognitive dysfunction after the haemorrhage mentioned above, it is not surprising that employment is one of the areas most affected in patients. After one year, there are 50-80% who can return to their previous occupation. (Hackett et al., 2000; Pritchard et al., 2001) However, studies evaluating employment one to three years post-SAH found only 40-60% were still working to a similar extent than before the SAH. (Carter et al., 2000; Schuiling et al., 2005) There are few studies looking into the long-term development of employment. These publications report that, 5 to 9 years later, there are no more than 50% of patients who can work to a similar extent than before the haemorrhage. (Hellawell et al., 2001; Wermer et al., 2007) Additionally, there is another 20-35% who return to work with reduced hours and responsibility, due to fatigue and cognitive deficits. (Passier et al., 2011; Wermer et al., 2007) Nonetheless, even those returning to their job need an average of 9 months to recover. (Wermer et al., 2007) Moreover, apart from their professional activity, patients report having difficulties in interpersonal relationships and social and leisure activities. After 12-16 months 50-60% percent state being impaired in their social interaction, feeling disturbed by groups of people, small children and normal sound levels, and/or not being able to enjoy things they did before. (Morris et al., 2004; Vilkki et al., 2004) What concerns partnerships, one study could find 7% of patients being divorced because of the SAH (Wermer et al., 2007) and other publications

report increased tension and less affection in relationships. (Buchanan et al., 2000; Lezak, 1988) Still, this is only the patients' side to the outcome. For their families, in especially the partners, SAH is a life-changing event. In those cases, where patients are not able to care for themselves, family and relatives act in 75-100% as informal carers. In 75% it is the partner who cares for his significant other, hence as well being the person being affected the most emotionally and in life style besides the patient. (Mezue et al., 2004; Pritchard et al., 2001) Besides caring, they also play an important role in rehabilitation and social support (Low et al., 1999) as patients lacking this support were found to retain more sequels. (Toomela et al., 2004) This means an important amount of emotional and social strain for the partner. About 50% experience either of both, with half of them indicating to feel completely overwhelmed and 40% finding it easier coping with the initial crisis than the return to home of their partner. (Mezue et al., 2004; Pritchard et al., 2001) On the emotional side, 26% were found to fulfil diagnostic criteria for posttraumatic stress disorder (PTSD), three times as much as the general population (Noble et al., 2008b), whereas psychological stress was generally and sustainably increased. (Buchanan et al., 2000; Low et al., 1999) Furthermore, they showed an increased rate in psychiatric disorders, like anxiety and depression, and sleep disturbances. (Berry et al., 1997; Hop et al., 2001) Besides, the social and professional life is affected as well. (Hop et al., 2001) Carers reported suspended holiday plans, less visits from common friends and family, loss of friendships, social isolation, no time for own privacy and about half of them stated a negative impact on their working life, with some of them getting in trouble financially or even losing their job. (Lezak, 1988; Mezue et al., 2004) In the long run, this resulted on average in a reduction of 10% in quality of life (Hop et al., 2001) and more than 25% of carers requiring medication themselves (Pritchard et al., 2001). After the initial exaltation about the survival had passed, even a fourth of carers, in addition to the same number of patients, would go as far as saying dead would have been the better alternative compared to the outcome experienced. (Buchanan et al., 2000) Financial consequences come alongside the personal implications. After the first year, costs resulting from the SAH were estimated to be in total £23500 in the UK (Rivero-Arias et al., 2009) and €38300 in Germany (Dodel et al., 2010). Direct costs accounted for two thirds of the spendings, with expenses of inpatient treatment summing up to the vast amount of them (Roos et al., 2002), for example $65000 in the US (Qureshi et al., 2007), 23000£ in the UK (Rivero-Arias et al., 2010) and €22500 in Germany (Dodel et al., 2010). Taylor et al estimated lifetime costs to be $228000 in the US with indirect costs accounting for 60% in the long-term view. (Taylor et al., 1996) Examples for these indirect costs are £42 million for informal care and £300 million due to lost productivity in total per year. (Rivero-Arias et al., 2010) Altogether, SAH sums up to €437 million and £510 million annually costs in Germany and the UK, respectively (Dodel et al., 2010; Rivero-Arias et al., 2010), and to $5.6 billion lifetime costs in the US. (Taylor et al., 1996) Compared to ischaemic stroke, although way more rarely occurring, SAH produces significantly higher costs in each patient and, by this, contributes in an important quantity to overall stroke costs. (Taylor et al., 1996) Rivero-Arias et al. showed as well that total costs were higher in patients experiencing DCI (Rivero-Arias et al., 2009) and Taylor et al. predicted health care costs will not be reduced by better survival rates, but only by a better functional status post-SAH (Taylor et al., 1996).

In conclusion, the long-term implications of SAH on individuals, relatives and society highlight the need for new treatment options.

1.1.3. Pathophysiology

Ever since the first observation of spasms in arteries after a SAH in 1951 (Ecker et al., 1951), it was generally assumed that a crucial lack of blood supply, caused by the vessel narrowing, was almost exclusively responsible for the delayed morbidity and mortality. (Kassell et al., 1990). In consequence, this assumption turned the term vasospasm into a synonym of DCI. (Vergouwen et al., 2010) Indeed, early studies found an association between vasospasm and cerebral infarction, clinical deterioration, neurologic deficits and mortality (Allcock et al., 1963; Fisher et al., 1977), which was affirmed by later publications. (Crowley et al., 2011; Rabinstein et al., 2004) The link between vasospasm and poor outcome was established because, on the one hand, the appearance of vasospasm on angiography and the development of DCI followed a similar time course, being present most importantly in the second week after SAH, with a maximum incidence on days 6 to 8. (Dorsch, 1995) On the other hand, several authors showed that the severity of vasospasm goes along with an increased number of cerebral infarctions. (Crowley et al., 2011; Weidauer et al., 2007) Furthermore, vasospasm was identified as a prognostic factor for worse neurologic outcome (Crowley et al., 2011), and cerebral perfusion was shown to be reduced in regions supplied by arteries with severe spasms. (Dankbaar et al., 2009)

However, the concept of vasospasm being the sole cause of DCI has been doubted in the last decade. First, the incidence of vasospasms is not proportionate to the one of DCI or cerebral infarction. (Dorsch et al., 1994; Vergouwen et al., 2011c) While vasospasm is angiographically present in around 70%, only a third of all patients finally develop DCI. (Dorsch et al., 1994) Besides, even severe vasospasm not always results in DCI (Etminan et al., 2013) and, the other way around, DCI occurs as well in the absence of vasospasm (Frontera et al., 2009). Likewise, there is evidence that areas of infarction do not always overlap with the irrigation territory of a spastic vessel (Rabinstein et al., 2004), and vasospasm does not necessarily reduce cerebral blood flow (CBF) to the threshold for ischaemic levels (Dhar et al., 2012; Sviri et al., 2006). Also, CBF can be reduced without any correlation to spasm (Dhar et al., 2012). Finally, there are findings that outcome can be improved without a decrease in vasospasm, mainly by nimodipine, (Petruk et al., 1988; Pickard et al., 1989) and, contrariwise, a reduction of vasospasm by medical or angiographical treatment does not lead to a better outcome in SAH patients (Etminan et al., 2013). In any case, poor outcome is directly linked to the presence of DCI and cerebral infarction. (Vergouwen et al., 2011b). Since investigation into vasospasm could not hold up to the expectations, the pathophysiological processes responsible for the occurring ischaemia need to be identified. Nowadays a multifactorial explanation is favoured. (Vergouwen et al., 2011c) This includes pathological processes following early after SAH, also called early brain injury (EBI), and delayed damage to tissue, termed delayed brain injury (DBI) (figure 3). Vasospasm still is considered to play an important role, but processes like impaired cerebral microcirculation, inflammation and cerebral spreading ischaemia (CSI) are considered to be involved. (Foreman, 2016)

This chapter gives an overview of the mechanisms involved after a SAH. It is presented how these mechanisms are interlinked, to what extent studies have shown an influence on DCI and outcome and tries to identify key processes and molecules that could be tackled therapeutically.

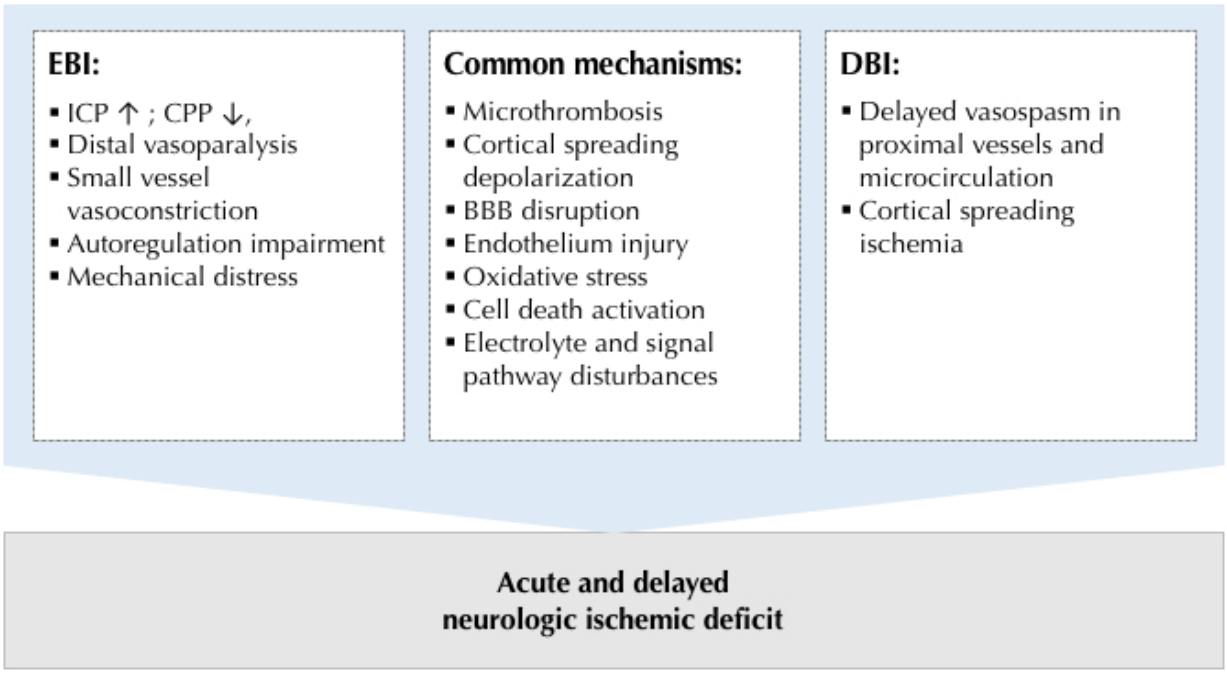


Figure 3:
Different mechanisms are involved in the pathophysiology of SAH. Some are exclusively for early brain injury (EBI) or delayed brain injury (DBI), respectively. However, most mechanisms can be found early after the haemorrhage as well as at a later stage. (Caner et al., 2012)

1.1.3.1. Early events

If we take a closer look at the events taking place in the first minutes and hours after a SAH, a chain reaction can be observed, with reciprocal activation of pathophysiological mechanisms, which leads to ongoing and sustainable damage to cerebral cells. However, the consequences of this damage on the outcome often only appear later in time. (Pulsinelli et al., 1982) So, what happens after the rupture of an aneurysm?

Many authors have described an almost immediate and sharp increase in intracranial pressure (ICP) up to levels between the diastolic and systolic blood pressure. Next, in most patients, ICP remains high for seconds to a few minutes and then returns to a level somewhat above baseline (figure 4) (Nornes et al., 1972; Trojanowski, 1982b). This pattern is considered to represent a physiological reaction in order to stop the bleeding from a ruptured aneurysm, since the high ICP serves as a sort of "brain tamponade", staying increased as long as coagulation takes place, and dropping afterwards. (Grote et al., 1988) Therefore, only a small haemorrhage volume can be found. (Nornes et al., 1972) In some patients ICP remains sustainably increased for several hours which is explained by mass effects due to expanding haematoma, oedema and acute hydrocephalus, as the haemorrhage and cerebral blood volume (CBV) found are significantly bigger, and leads to high mortality due to missing reperfusion. (Nornes et al., 1972)

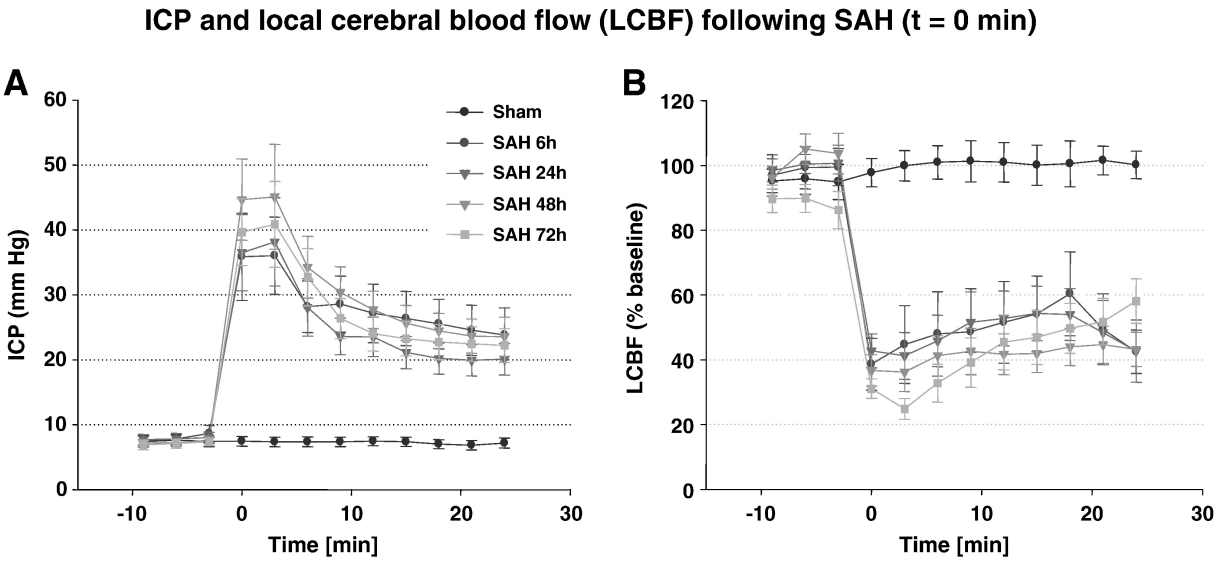


Figure 4:
Early after SAH, intracranial pressure peaks to a level between 40 to 120mmHg and then returns to levels slightly above baseline within minutes. While cerebral perfusion pressure rises to levels around 60mmHg, CBF steeply declines to 5-10% of baseline and only slowly recovers to around 30-40% of baseline in the first hour. (Bederson et al., 1995) (Figure shows the development of the ICP and the local cerebral blood flow during the first 30 minutes in experiments with different periods of observation after SAH, reproduced with permission from Schöller et al. (Scholler et al., 2007))

The spillage of blood into the subarachnoid space contributes to these events in the following ways: firstly, clots of blood plug the outflow and resorption of cerebrospinal fluid (CSF) leading, thus, to acute hydrocephalus. (Kosteljanetz, 1984) Secondly, the high pressure of the blood jet and the volume of the haemorrhage lead to direct mechanical trauma, compression of arteries and increased ICP. (Arutiunov et al., 1974; Schwartz et al., 2000) Furthermore, substances resulting from the destruction of blood cells produce acute proximal vasoconstriction and, by vasoparalysis, distal arteriolar vasodilatation. (Grote et al., 1988; Simeone et al., 1968) Together, these factors determine the importance and duration of the ICP increase.
The resulting intracranial hypertension causes a temporal drop in cerebral perfusion pressure (CPP) (Czosnyka et al., 2005; Marbacher et al., 2012) which, together with acute vasoconstriction (Bederson et al., 1998; Friedrich et al., 2012a), hypovolemia (Solomon et al., 1984), and compromised cerebral autoregulation (Czosnyka et al., 2005; Rasmussen et al., 1992), leads to diminished or interrupted CBF (figure 4 and 5) (Westermaier et al., 2009b). (Bederson et al., 1995) This condition, termed no-reflow phenomenon (Ames et al., 1968; Asano et al., 1977), has to endure as long as coagulation needs time to ensure arrest of aneurysm bleeding. (Grote et al., 1988) However, CBF should recover as fast as possible since, meanwhile, global cerebral ischaemia and capillary thrombosis spread (figure 5). (Brinker et al., 1992; Grote et al., 1988; Hill et al., 2015; Miranda et al., 2006) Since CPP recovers earlier than CBF, the extent of the no-reflow phenomenon is, most importantly, influenced by the intensity and amount of acute vasoconstriction which, again, depends on the severity of bleeding. (Bederson et al., 1998; Westermaier et al., 2009a)

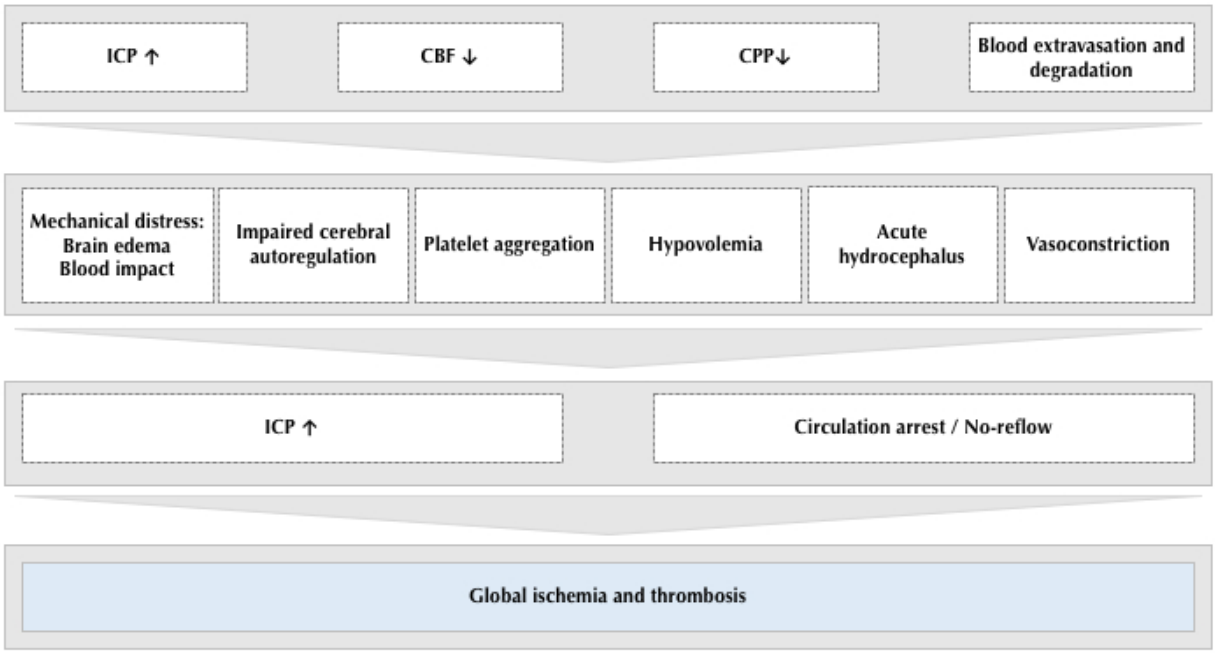


Figure 5:
In consequence to the initial impact of the SAH, the intracranial pressure (ICP) rises and the cerebral blood flow (CBF) as well as the cerebral perfusion pressure (CPP) is diminished. Thereby, a series of further pathomechanisms is initiated, finally leading to a circulation arrest and the no-reflow phenomenon with the consequence of global ischaemia and thrombosis. (Sehba et al., 2006)

The magnitude of the pathophysiological mechanisms, like altered cerebral metabolism (Samuelsson et al., 2009), initiation of inflammation (Fassbender et al., 2001; Graetz et al., 2010) and disruption of blood-brain barrier (BBB) (Trojanowski, 1982a), hereby depends on the degree of the ICP increase, the duration of transient ischaemia (Iadecola et al., 2001), the extent of reperfusion injury (Hallenbeck et al., 1990; Steinberg et al., 1994) and the quantity of subarachnoid blood. (Ishikawa et al., 2009) Especially the reduction of CBF during the first hour is crucial for the early outcome, as a reduction to 40% or less comes along with a 100% mortality whereas reduction to a lesser extent correlates with the grade of consciousness. (Bederson et al., 1998; Honda et al., 2012; Jakobsen, 1992) Furthermore, perfusion deficits seen on computed tomography increase significantly the odds for DCI. In the long term, as they depend on each other, initial ICP increase (Nagel et al., 2009; Soehle et al., 2007), haemorrhage volume (Lagares et al., 2015), sustainability of intracerebral hypertension (Hayashi et al., 1977; Nornes et al., 1972) and perfusion deficits (Mir et al., 2014) predict the outcome and the development of DCI. Approximately 48h after the initial insult (Jackowski et al., 1990), CBF overshoots the pre-SAH baseline resulting in an increased CBV. (Asano et al., 1977; Kuyama et al., 1984). This indicates a state of luxury perfusion, since CBF becomes detached from oxygen metabolism which is diminished in this situation (Westermaier et al., 2009b). (Jakobsen et al., 1990) This impairment of autoregulation is thought to be due to high lactate levels caused by anaerobe metabolism in the absence of sufficient oxygen supply in the early phase. (Lassen, 1966; Voldby et al., 1985b)

1.1.3.2. Early brain injury and Delayed brain injury

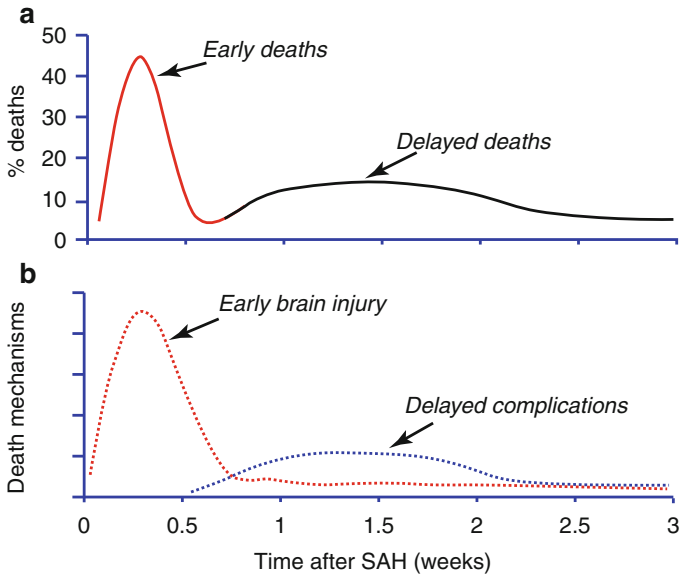


Figure 6:
a: As shown in 1.1.2. most deaths occur in the first days after the haemorrhage; b: Early brain injury is defined to occur during the first 72h after the insult which matches with the increased numbers of deaths. Consequently, the damage from EBI appears to be more severe. (Reproduced with permission from Sehba et al. (Sehba et al., 2015))

Initiated by these early events, the different pathophysiological ways set off. (Bederson et al., 1998) Generally, they are summarized under the terms EBI and DBI, often claiming the same mechanism for both terms (figure 3). In fact, EBI and DBI are solely differentiated in time, highlighting events taking place before and after the beginning of delayed vasospasm, which typically starts after 72 hours (figure 6). (Cahill et al., 2006) Nonetheless, there are events usually occurring early, like disruption of the cerebral autoregulation, interruption of the blood-brain barrier and brain oedema (Kusaka et al., 2004), and others rather associated with the later phase, like delayed vasospasm and cortical spreading ischaemia (Macdonald, 2014). Nonetheless, all these mechanisms are extensively interconnected, as early ones result in later ones, and can take place at different points in time. In summary, both terms share similar mechanisms and together are responsible for occurring ischaemia and cerebral infarction, hence DCI (figure 3). For an overview see these reviews. (Chen et al., 2014; Macdonald, 2014; Sabri et al., 2013; Sehba et al., 2012)

1.1.3.2.1. Mechanical stress

Mechanical trauma following SAH includes direct damage from the resulting blood jet and secondary effects from early pathomechanisms, like formation of hydrocephalus, global brain oedema and haematoma, together contributing to an early and a delayed rise in ICP, resulting in shift, herniation and compression of brain tissue (figure 7). (Hayashi et al., 1977; Macdonald, 2014)

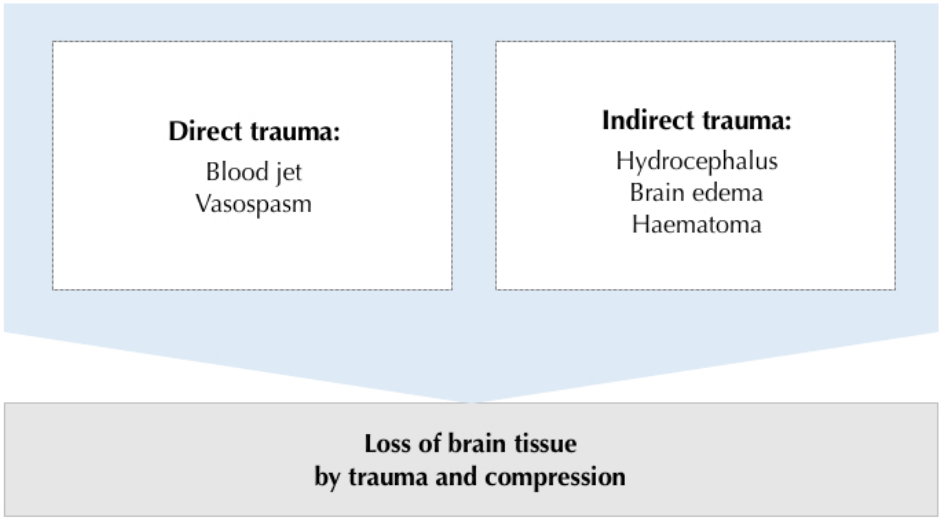


Figure 7:
The mechanical stress resulting in brain damage can by classified in two categories: direct and indirect trauma. Direct trauma results from the impact of the blood jet and the vessel deformation by vasospasm. Indirect trauma results from pressure onto brain tissue by expanding processes.

The blood leaves the artery in a high-pressure spurt leading to stretching, pulling and disruption of surrounding structures like arteries, nerves and brain tissue. This mechanical impact on vessels and connective tissue leads to artery spasm. However, spasms from pure mechanical trauma were shown to only last for 15 to 20 minutes. (Arutiunov et al., 1974; Kapp et al., 1968)
Acute hydrocephalus is present in around 20% of patients and can also be found in the subacute and delayed period, although to a lesser extent. As a result of its expandatory nature and, therefore, increased ICP, development of hydrocephalus accounts for a greater risk of neurologic impairment and mortality. (Demirgil et al., 2003; Milhorat, 1987; Suarez-Rivera, 1998)
Global brain oedema is observed in 8% of patients at admission and develops lately in another 12%. (Claassen et al., 2002) Two mechanisms contribute to its formation. (Barry et al., 2012; Ostrowski et al., 2006a; Westermaier et al., 2012) On the one hand, ischaemia, leading to cell death due to energy failure, inflammation (see 1.1.3.2.5.) and toxic effects from blood breakdown products (see 1.1.3.2.2.) are responsible for the cytotoxic component. On the other hand, breakdown of the blood-brain barrier and impaired autoregulation (see 1.1.3.2.2.), resulting from ischemic injury and decreased CPP, result in vasogenic oedema formation. Global brain oedema was shown as well to lead to increased mortality and morbidity. (Claassen et al., 2002)
Altogether, the injury produced by mechanical trauma entails a vicious circle, leading to early and delayed brain injury. (Johshita et al., 1990)

1.1.3.2.2. Blood breakdown products

When brain tissue is exposed to fresh blood in the absence of cerebral hypertension, vasoconstriction, formation of microthrombi, disruption of the BBB and egression of platelets are the consequences. (Doczi et al., 1986) Theses processes are mediated by haemoglobin and its breakdown products, most importantly oxyhaemoglobin, iron, bilirubin and bilirubin oxidation products (BOXs) (figure 8). Following the order of metabolism, they peak at different moments post-SAH, contributing by this to early and late brain injury. (Foreman, 2016) This is facilitated

by ineffective cerebral mechanisms of haematoma clearance, allowing these substances to persist over a long time, hereby potentiating their pathogenicity. (Galea et al., 2012)
Degradation of haemoglobin results in oxidative stress by formation of radicals. This results in oxidation of bilirubin and lipid membranes, changes in the structure of vessel wall architecture, and altered cell metabolism (figure 8).

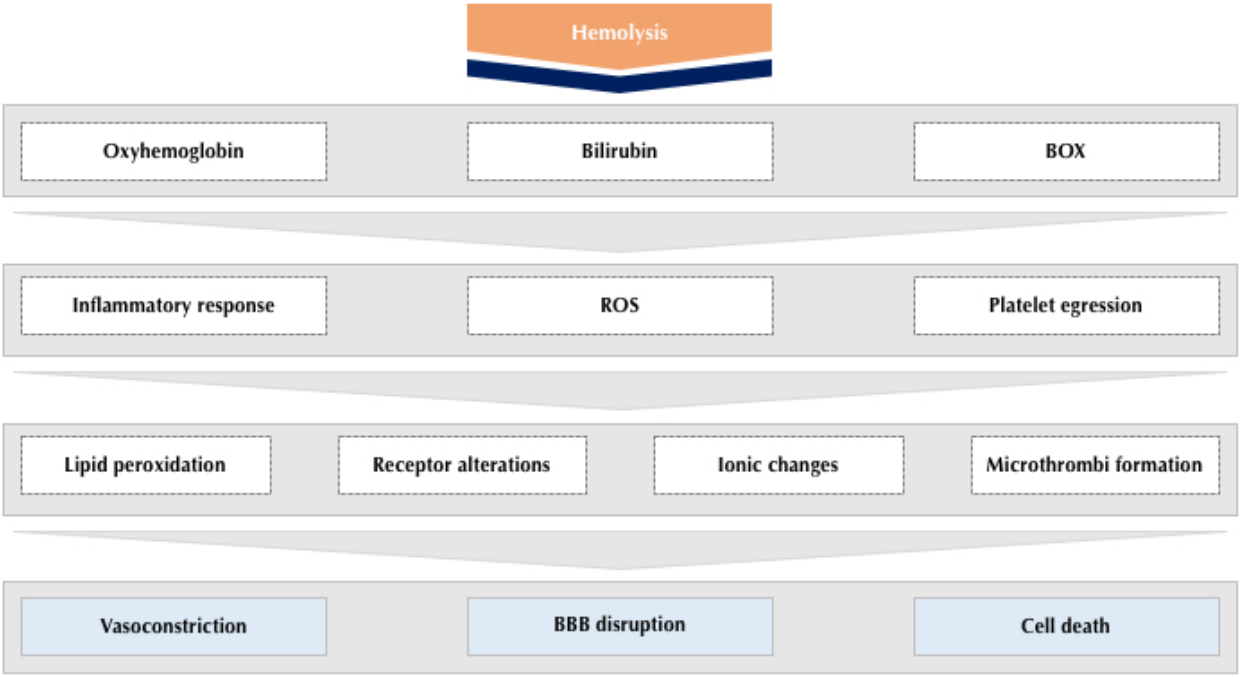


Figure 8:
The lysis of red blood cells liberates components that are responsible for the activation of different mechanisms which finally lead to vasoconstriction, blood brain barrier disruption and cell death, amongst others. BOX = bilirubin oxidation products; ROS = Reactive oxygen species; BBB = blood brain barrier.

Consequently, ionic disequilibrium and DNA damage lead to apoptosis and necrosis of neuronal, astrocytic, endothelial and smooth muscle cells. Which is, in turn, the reason for disruption of the BBB (figure 8). (Pluta, 2005; Sehba, 2015) The altered metabolism contributes as well to the development of vasospasm. First, the equilibrium between nitric oxide (NO) and endothelin-1 (ET-1) is turned in favour of the second by direct scavenging of NO, stimulation of ET-1 production, destruction of neuronal nitric oxide synthase (nNOS) and alteration of endothelial nitric oxide synthase (eNOS). (Pluta, 2005) Vasoconstriction, respectively, vasospasm is the consequence (figure 9). (Pluta et al., 2009) However, following the pathophysiological pattern of delayed vasospasm, solutions of blood and CSF have a stronger vasospasmogenic effect if they are applied to arteries after an incubation time of seven days. (Sonobe et al., 1978)
Accordingly, high CSF-levels of haemoglobin breakdown products were shown to correlate with increased mortality and occurrence of DCI. (Lee et al., 2010; Li et al., 2014; Pyne-Geithman et al., 2005; Pyne-Geithman et al., 2013)

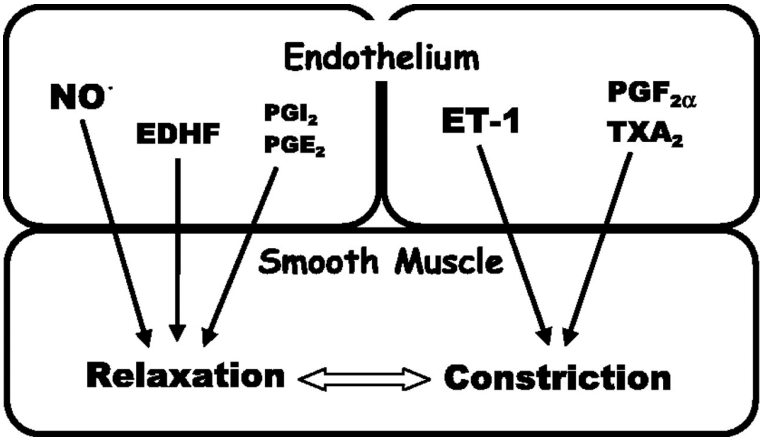


Figure 9:
Vasoactive agents are released from blood cells. Physiologically, NO is an important factor for vessel relaxation. On the other hand, ET-1 triggers vasoconstriction. After SAH the level of NO is reduced due to scavenging and ET-1 is released from damaged blood cells. (Reproduced with permission from Andresen et al. (Andresen et al., 2006))

1.1.3.2.3. Dysfunctional microcirculation

It has been shown that the early events after SAH lead to a subacute diffuse microangiopathy of the brain. (Neil-Dwyer et al., 1994) This microangiopathy consists of different elements: impaired autoregulation of perfusion, breakdown of the BBB, spasm of the microvasculature, decrease of capillary density and a hypercoagulable state. Common mechanisms are mechanical compression and damage to the vessel wall, having impaired endothelial function and exposure of smooth muscle cells as a result, activation of inflammation, disturbances in the coupling within the neurovascular unit and an altered reactivity to signalling molecules (figure 10). (Cossu et al., 2014; Sabri et al., 2012; Sun et al., 2009) It is assumed that the result is an increased heterogeneity in capillary transit time leading to an activation of salvatory mechanisms and ultimately to a decrease in CBF and tissue oxygen pressure. However, when these mechanisms decompensate, cortical spreading depolarisation (CSD) and ischaemia are the consequences. (Dankbaar et al., 2009; Ostergaard et al., 2013) Even if, so far, there is no prove of the details, there is no doubt that CSD, inflammation and oxidative stress are involved in dysfunctional microcirculation, thrombosis and early ischaemia.

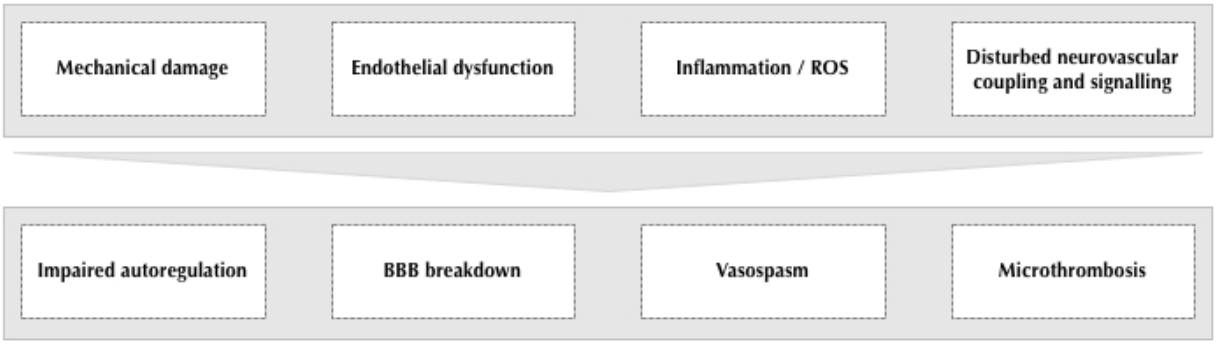


Figure 10:
Different mechanisms contribute to the malfunction of the microcirculation. Initially, there are mechanical damage, endothelial dysfunction, consequences of inflammation and radical production, and disturbed signaling, which, combined, promote impairment of autoregulation, blood brain barrier breakdown, vasospasm and microthrombosis. In sum, these alterations result in a reduced microcirculatory blood supply.

1.1.3.2.3.1. Impaired autoregulation

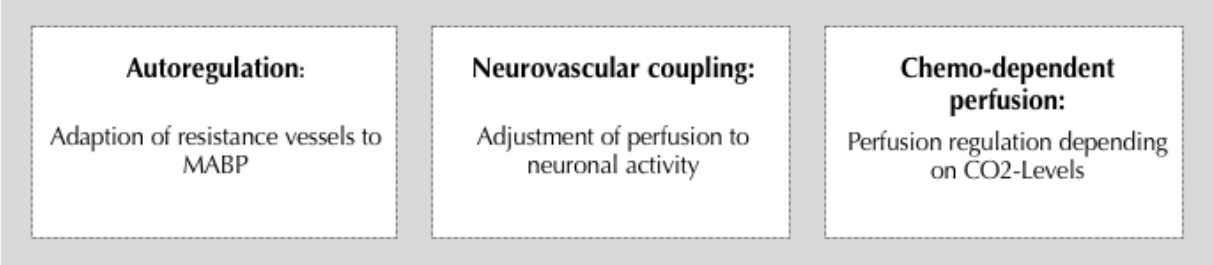


Figure 11:
Blood flow in cerebral vessels depends on three feedback-regulated mechanisms: Adaption of resistance vessels to the mean arterial blood pressure (MABP), change of blood flow according to neuronal activity, and regulation of perfusion corresponding to cerebral CO2-levels.

Physiologically, cerebral perfusion is strictly regulated by three different mechanisms (figure 11) (Guo et al., 2016): firstly, autoregulation, in a narrow sense, is the adaption of cerebral resistance vessels to changes in the mean arterial blood pressure (MABP). In the brain, vascular resistance is mediated to 70% by small vessels. (Faraci et al., 1990) Accordingly, dysfunctional microcirculation affects autoregulation decisively. Secondly, perfusion changes as response to neuronal activity are mediated by neurovascular coupling. (Attwell et al., 2010)(see 1.1.3.2.7.) And thirdly, CBF is adjusted to CO_2-levels which is termed chemo-dependent perfusion. This mechanism was shown to be regulated by NOS activity. (Iadecola et al., 1994) Together, they rely on the functionality of endothelial, smooth muscle and neuronal cells, and their reaction to physical stress and signalling molecules. (Paulson et al., 1990)

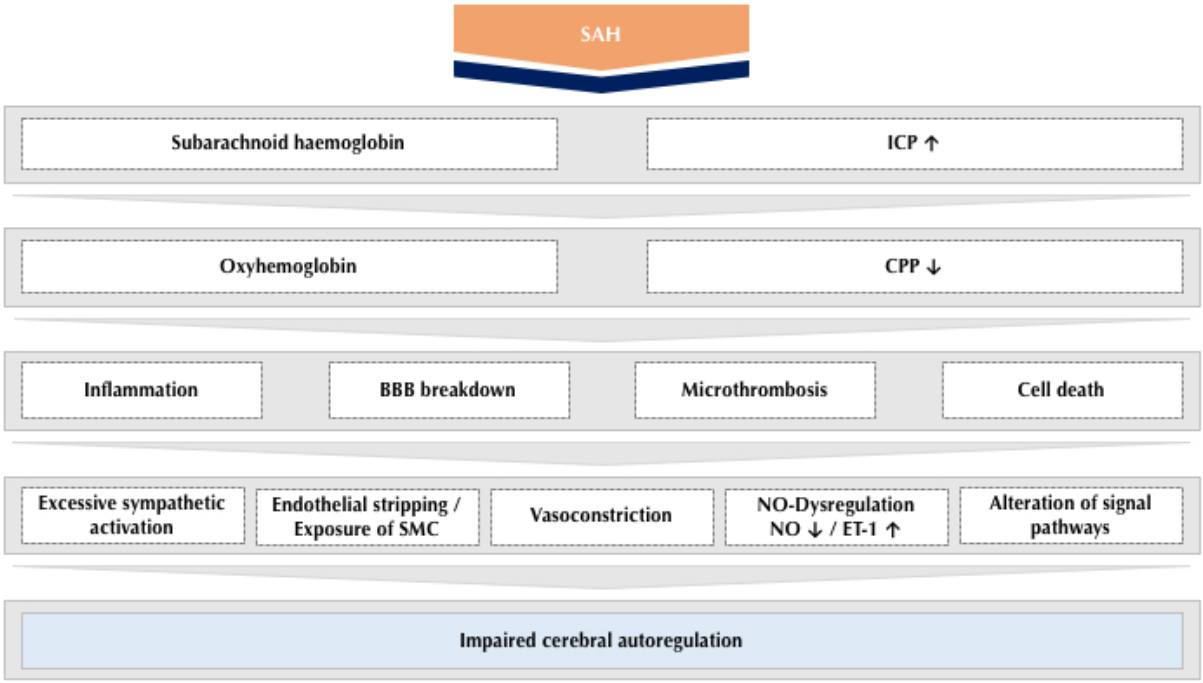


Figure 12:
Triggered by blood breakdown products and the elevated intracranial pressure, processes like inflammation, blood brain barrier breakdown, microthrombosis and cell death lead to a loss of endothelium, a sustained vasoconstriction, a dysregulation of the balance between vasoconstrictors and vasodilators, and an alteration of signal pathways involved in autoregulation mechanisms. Hereby results an impairment of the cerebral autoregulation.

After SAH, CPP decreases, endothelial function and morphology become impaired, NO and ET-1 signalling is modified and constriction and thrombosis of vessels take place, together resulting in an altered mechano- and chemoregulation (figure 12). (Foreman, 2016; Sehba et al., 2012) The Autoregulation is impaired during the first 72h, and again during the period of delayed vasospasms, with a temporal recovery in between. (Lang et al., 2001; Ratsep et al., 2001) Chemoregulation is as well impaired during the first four days and can recover afterwards. Absence of recovery was shown in patients who later develop DCI. (Carrera et al., 2010) In general, impairment of CBF-regulation correlates with neurological deficits, poor recovery and development of DCI. (Budohoski et al., 2012; Jaeger et al., 2012)

1.1.3.2.3.2. Blood-brain barrier disruption

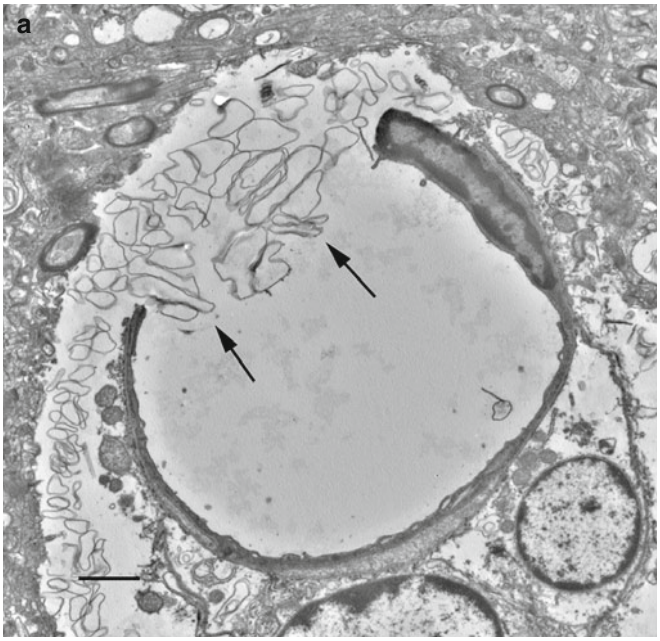


Figure 13:
The arrows show destruction of the vessel wall after SAH and, hence, disruption of the blood brain barrier. (Reproduced with permission from Sehba et al. (Sehba et al., 2013))

BBB disruption is observed 30-60 minutes after the haemorrhage and affects small vessels first, progressing inhomogenously. (Doczi, 1985; Trojanowski, 1982a) It shows a biphasic development with the first peak within 48h and a second peak several days later. (Cahill et al., 2006) 40% of patients are affected during the first five days, while only 11% develop BBB disruption afterwards. (Doczi, 1985) During the second peak larger vessels are involved to an important extent and apoptosis is the predominant cause for BBB breakdown. (Gules et al., 2003) Besides, upregulation of lytic enzymes, signalling molecules and transmembrane channels, as well as the effects of hemodynamic disturbances, radical liberation, cerebral hypertension and cerebral spreading ischaemia are involved (figure 14). (Gursoy-Ozdemir et al., 2004; Johshita et al., 1990; Ostrowski et al., 2006a) They result in a degradation of the endothelium and the basal lamina, leading to an increased permeability, amongst others for inflammatory cells, and cerebral oedema (figure 13 and 14). (Yatsushige et al., 2006; Yatsushige et al., 2007)
In consequence, BBB disruption results in exposure of smooth muscle cells, which contributes to thrombosis and vasoconstriction, and facilitates inflammation. Accordingly, it was found to correlate with clinical grade and outcome. (Park et al., 2004; Said et al., 1993; Sarrafzadeh et al., 2012)

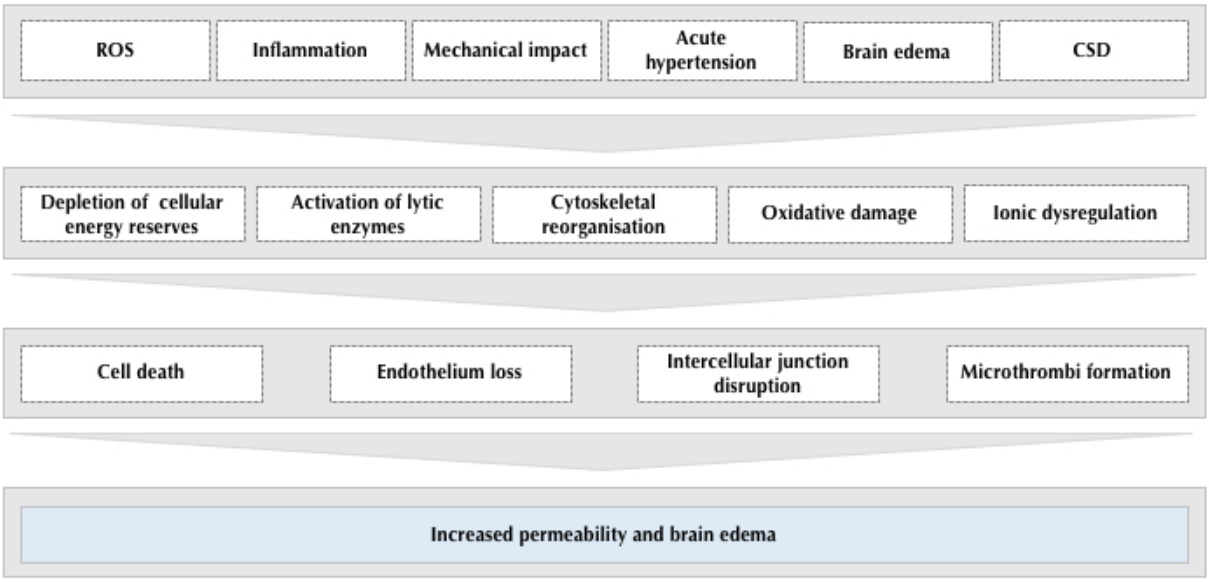


Figure 14:
The processes initiated by a SAH evoke a direct damage to the endothelium by mechanical and inflammatory stress, deprive it from a sufficient energy supply and produce a dysregulation of ionic homeostasis. An increased permeability and, in consequence, a breakdown of the blood brain barrier results, facilitating the brain oedema formation. (Fujii et al., 2013; Pun et al., 2009)

1.1.3.2.3.3. Microthrombosis

Formation of thrombi starts within minutes after SAH, is seen in a majority of patients and often can be found in constricted vessels. (Friedrich et al., 2012a; Sehba et al., 2005) Their appearance can also be biphasic with patients dying during the first four days and those dying between the fourth and the seventh days post-SAH showing the highest quantity. (Stein et al., 2006) During the second peak, effects of delayed vasospasm play an important role, as injury to the endothelium leads to activation of coagulation. (Ohkuma et al., 1991) Both peaks are due to an hypercoagulative state, which is the result of endothelial dysfunction and injury, leading to exposure of wall tissue, impairment of fibrinolysis by oxygen radicals, and high serum and CSF levels of pro-coagulative substances. Furthermore, decreased levels of NO, an inhibitor of coagulation, and increased expression of selectins are involved (figure 15). (Ostergaard et al., 2013; Sabri et al., 2012; Vergouwen et al., 2008)
Microthrombosis early after SAH seems to play an important role in the development of ischaemia, as high levels of pro-coagulative substances in the first 72h correlate with DCI and poor outcome. Furthermore, clinical symptoms of ischaemia correlate with the amount of microthrombi and in patients with DCI a significant higher thrombi burden was found in regions with cerebral infarction. (Frijns et al., 2006; Stein et al., 2006) A possible explanation would be microthrombi causing the no-reflow phenomenon, and thereby ischaemia. (Friedrich et al., 2010) Further effects of early thrombi formation are denudation of the endothelium, which produces more coagulation and favours the release of vasoconstrictive substances and BBB-degrading enzymes by activated platelets. (Fernandez-Patron et al., 1999; Sehba et al., 2005)

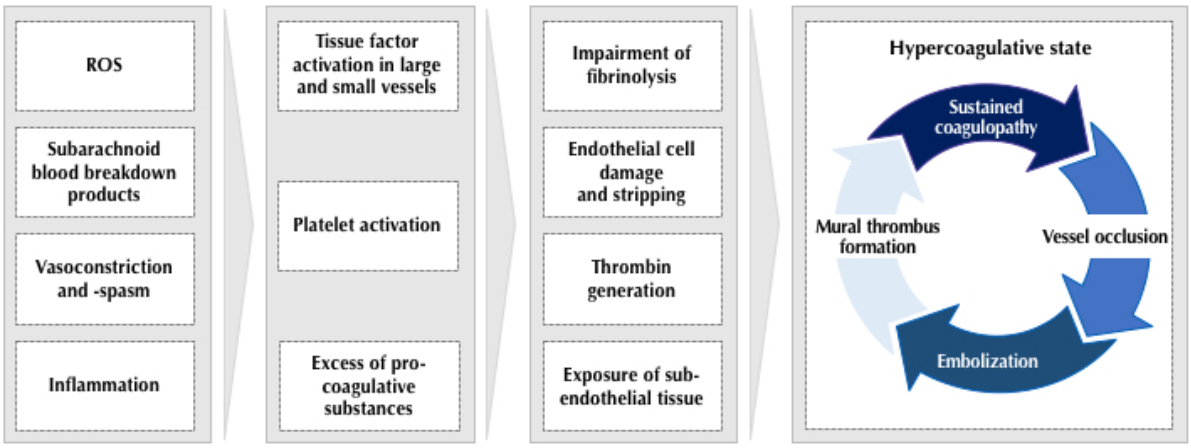


Figure 15:
After a SAH, radical production, products from haemolysis, vasospasm and inflammation are responsible for an excess of procoagulative substances and trigger the activation of platelets and tissue factor. Consequently, due to a procoagulative state, fibrinolysis gets impaired and more thrombin is generated. Furthermore, damaged endothelium and exposure of subendothelial structures promote thrombosis. In sum, these mechanisms result in an hypercoagulative state constituted by a self-sustaining thrombotic loop. (Pun et al., 2009; Stein et al., 2006))

1.1.3.2.3.4. Acute vasospasm

In contrast to the traditional theory, vasospasm already takes place in the early phase. So far, in humans only early constriction of pial and parenchymal arterioles was shown, whereas in animals also larger vessels show constriction within minutes after SAH. (Bederson et al., 1998; Sehba et al., 2007) In general, these early spasms are present between three and 72h post-SAH. (Uhl et al., 2003) Since small vessels seem quantitively and qualitatively more constricted, this affects also vascular resistance and autoregulation, and explains the observed decrease of capillary density. (Friedrich et al., 2012a) These vessels seem to be affected earlier since, in comparison to larger vessels, their endothelium and basal membrane gets dysfunctional and apoptotic much earlier. (Friedrich et al., 2012b; Park et al., 2001) The result is a decreased response to vasodilators like NO, which is, amongst others, reduced in quantity by Oxyhaemoglobin. In addition, vessels become more sensitive to vasoconstrictors, which are elevated in the CSF. (Debdi et al., 1992; Hatake et al., 1992) In conclusion, acute vasoconstriction contributes to the significant CBF decrease, which may result in ischaemia. (Friedrich et al., 2012a)

1.1.3.2.4. Cell Death

The impact of the SAH results in death of neuronal, endothelial, glial, and smooth muscle cells. As underlaying processes apoptosis as well as necrosis are involved and can occur simultaneously (figure 16). (Cahill et al., 2006; Prunell et al., 2005) Signs for both are found within minutes after the haemorrhage and multiple brain regions are affected within 24 hours. (Friedrich et al., 2012b; Marbacher et al., 2012) High rates of apoptosis in patients dying on days 2-11 after SAH show the ongoing nature of cell death, maintained by the damage produced by other pathomechanisms. (Nau et al., 2002) Since apoptosis is usually energy-dependent, the severity of the impact to the cells and the tissue tolerance to ischaemia determine which of both

processes is activated. (Yuksel et al., 2012) In consequence, severe injury, with rapid developing ischaemia and rapid energy consume results in necrosis, and thus in more inflammation and brain injury. (Cahill et al., 2006; Ogihara et al., 2001)
Responsible are processes that increase oxygen and energy consumption, like exotoxicity of elevated glutamate, ionic imbalances and CSD, on the one hand, (Ohta et al., 2001) and processes leading to direct damage to the cell or activation of cell death pathways, on the other. The different pathways of apoptosis are activated through ischaemia, which is mediated by hypoxia-inducible factor 1α (HIF-1α), high intracellular calcium levels, oxidative stress and tumour necrosis factor α (TNFα), which is elevated due to inflammation. (Hasegawa et al., 2011; Ostrowski et al., 2006a) In necrosis, the impact of oxyhaemoglobin, reactive oxygen species and energy failure play a predominant role, but elevated intracellular calcium and tumour necrosis factor can be involved as well (figure 16). (Chen et al., 2014)

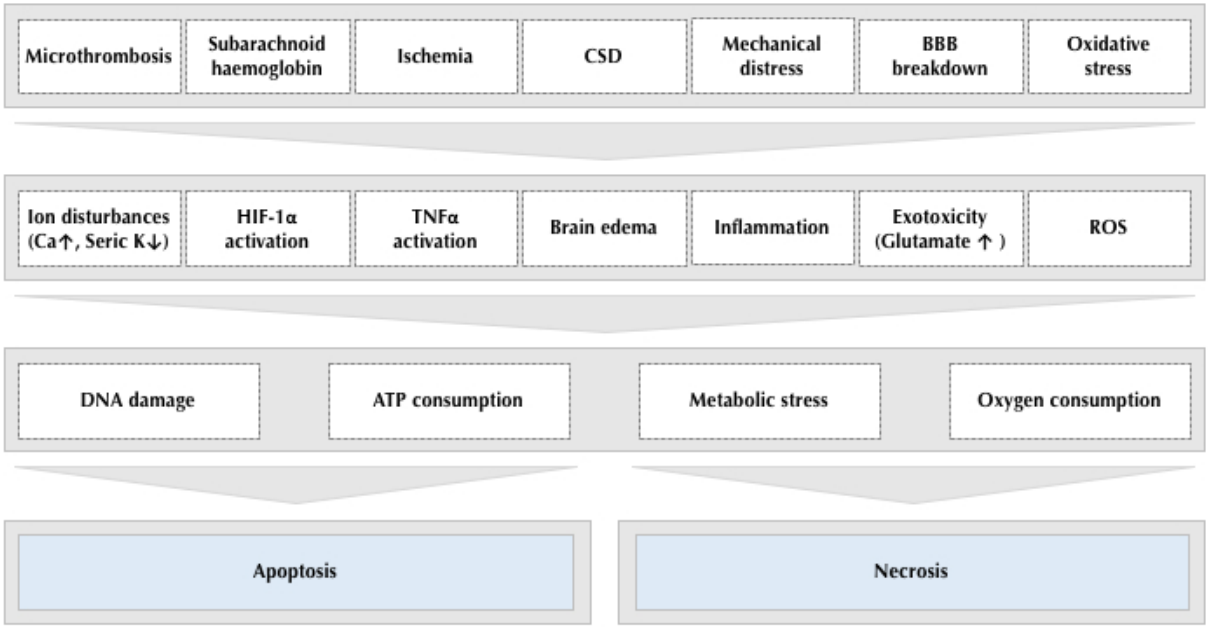


Figure 16:
The pathophysiological processes, initiated by the haemorrhage, cause a hostile environment for cells. Mechanical, radical and inflammatory stress, ischaemia and dysregulation of electrolytes and neurotransmitter entail the upregulation of HIF-1α and TNFα. Damage to the DNA and metabolic stress come along with increased energy and oxygen consumption up to a point where cell death is inevitable. Whether apoptosis or necrosis is initiated hereby depends on surrounding factors.

Not surprisingly, endothelial destruction results in vasospasm and BBB breakdown. Furthermore, neuronal death correlates with the haemorrhage magnitude and DCI, as well as, together with glial death, with clinical grade and outcome. (Mabe et al., 1991; Oertel et al., 2006; Park et al., 2004)

1.1.3.2.5. Inflammation

The inflammatory response after a SAH is induced by products from haemolysis, effects of radicals, the elevated ICP and ischaemia. (Barone et al., 1999; Graetz et al., 2010) It serves the purpose to eliminate toxics products and its magnitude depends on their amount. Leukocytes and macrophages are activated and extravasated within 24 hours alongside with an activation of the complement system. Simultaneously, cytokines, chemokines and adhesion molecules peak and lead to stimulation of proinflammatory transcription factors, high intracellular calcium levels,

secretion of TNFα and ET-1, depletion of NO and cell death. (Helbok et al., 2015; Provencio et al., 2005; Sercombe et al., 2002) Furthermore, the activation of leukocytes and their interaction with the endothelium contribute to the obstruction of blood vessels and the degradation of the blood-brain barrier, whereby more leukocytes are attracted (figure 17). (Hamann et al., 1995; Mazzoni et al., 1996) By these mechanisms, inflammation contributes to cerebral infarction, intracranial hypertension, thrombosis, BBB breakdown and vasoconstriction. (Iadecola et al., 2001; Levi et al., 2004; Yang et al., 2011)

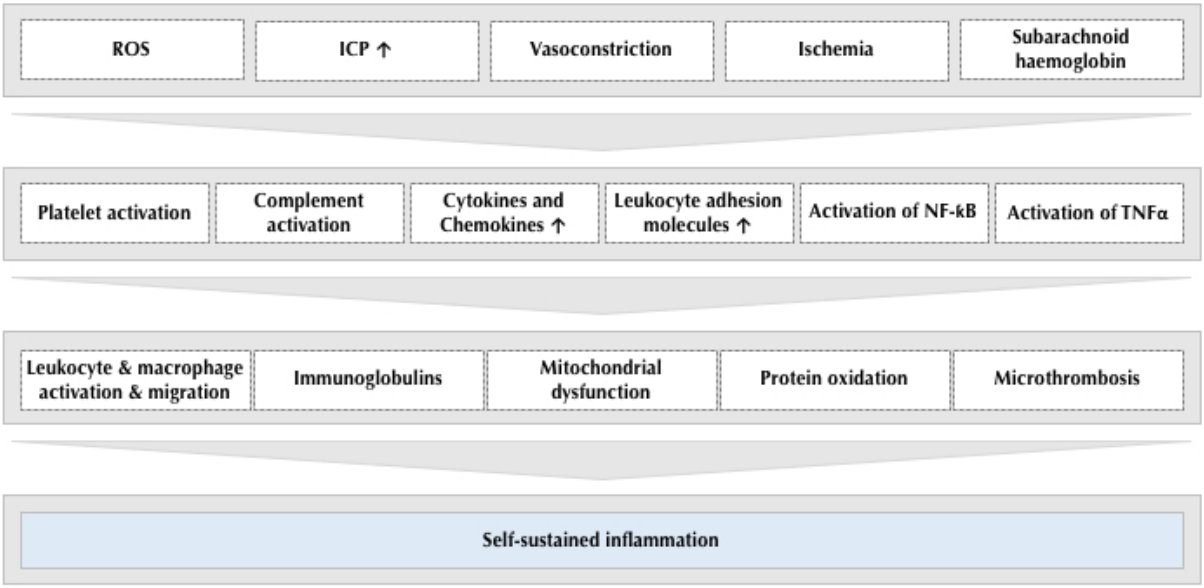


Figure 17:
Mechanical stress, ischaemia, and products of haemolysis create a proinflammatory state. Consequently, cyto- and chemokines, as for example TNF-α and the nuclear factor NF-κB, and, subsequently, immunoglobulins are liberated, the complement system and platelets are activated and leukocytes are mobilised. Due to a overstimulation, the tissue gets damaged, thrombosis is facilitated and the mitochondrial function fails. Hereby, the inflammatory system is even more stimulalted and finally sustains itself. (Pacher et al., 2007))

Consequently, it was shown that high levels of proinflammatory mediators correlate with clinical grade, low CPP, development of ischaemia, vasospasm and DCI, and poor outcome. (Helbok et al., 2015; Sehba et al., 2012) Similarly, a high white blood cell count during the first 5 days post-SAH is associated with a poor clinical grade and predicts clinical deterioration, vasospasm, DCI and mortality. (Neil-Dwyer et al., 1974; Srinivasan et al., 2016) Nonetheless, modulation of the inflammatory response leads to both, negative and positive effects, as it its crucial for remodelling of the damaged tissue. (Iadecola et al., 2011)

1.1.3.2.6. Delayed Vasospasm

In the past, the term vasospasm after SAH referred to delayed developing spasms which occur 3 to 14 days after the haemorrhage with a maximum on days 6 to 8. (Wilkins, 1990) These spasms are present in two-thirds of patients. (Dorsch, 2011) On angiography, vasospasms affect predominantly larger proximal vessels (figure 18). (Cossu et al., 2014) Despite this, impaired microcirculation was shown to be responsible for reduced regional CBF, even if none or only mild spasms of vessels observable by angio- or sonography are present. (Ohkuma et al., 2000)

In contrast to physiological conditions, after SAH multiple vasoconstrictive agents are released by cells and the blood clot, e.g. oxyhaemoglobin, prostaglandins, serotonin and ET-1. (Nishizawa et al., 2005) Therefore, the volume of the haemorrhage and the clot elimination rate are the most important predictors for the severity and the duration of the spasms. (Reilly et al., 2004) Furthermore, the dysfunction of the endothelium, the mechanical impact on the vessels, the effects of blood breakdown products and the inflammatory response contribute to the formation of delayed vasospasms (figure 19). Again, the development of delayed vasospasms is associated with the presence of early spasms. (Moussouttas et al., 2014) Processes taking place during EBI were shown to be responsible for the upregulation and desensitization of vasoconstrictive receptors, hereby leading to increased and sustained contractility and facilitating the development of later spams. (Kikkawa et al., 2010; Povlsen et al., 2013)

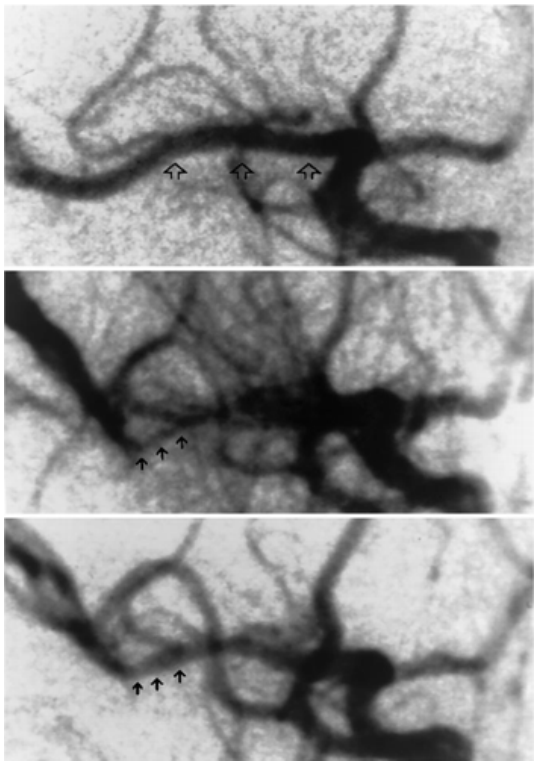


Figure 18:
Arrows indicate a localized vasospasm (Reproduced with permission from Afshar et al. (Afshar et al., 1995))

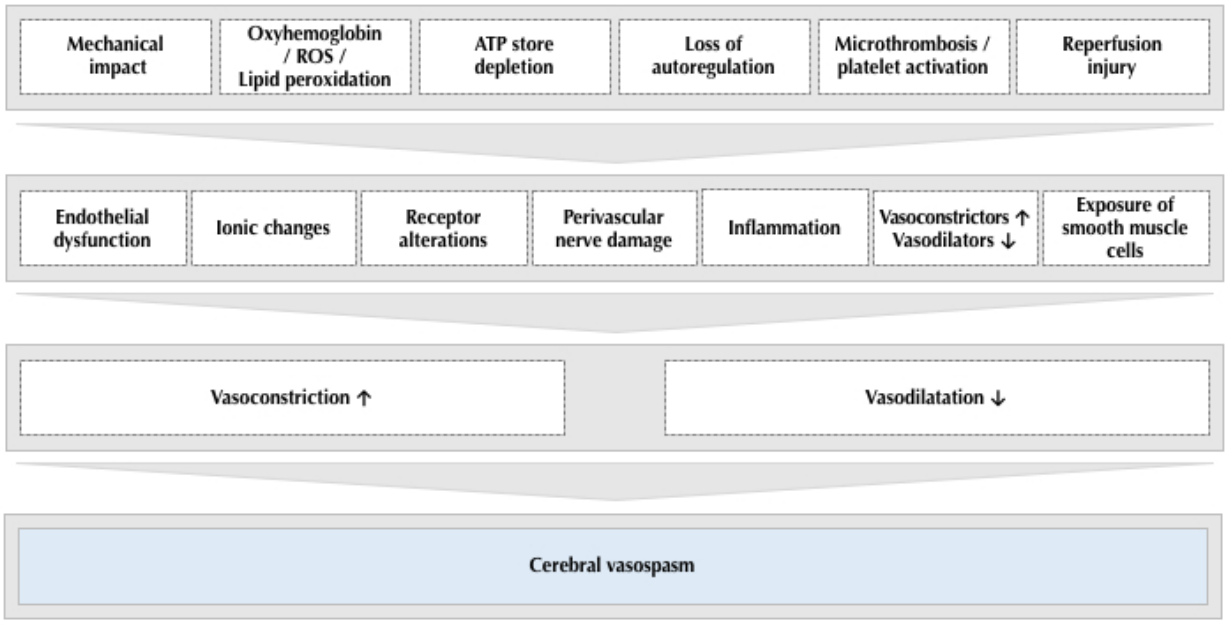


Figure 19:
A variety of mechanisms induced by a SAH alter the functionality of the neurovascular unit. Endothelium gets lost due to cell death, leaving smooth muscle cells exposed to the blood stream. Receptors get modified and ions and signalling molecules prevail in altered concentrations. All together, these changes shift the balance in favour of vasoconstriction and facilitate vessel spasms. (Cossu et al., 2014)

Significant effects on outcome could only be found for the presence of severe spasms, which are in most cases defined by a reduction of CBF to less than 50%. These spasms can impair autoregulation, reduce the CBF to ischaemic levels and are still the most important factor responsible for infarction. (Brown et al., 2013; Ohkuma et al., 2000; Voldby, 1988) However, also moderate spasms were shown to lead to more neurological worsening, cerebral infarction, poor outcome and mortality. (Vergouwen et al., 2011a) This is explained by a second hit theory where the extent of loss of autoregulation, the presence of collateral flow and anastomoses, the present metabolic demand and the genetic predisposition determine if the additional impact of vasospasms results in DCI. (Macdonald, 2014)

1.1.3.2.7. Cortical Spreading Ischaemia (CSI)

The phenomenon termed Cortical Spreading Depolarization (CSD) is a progressive wave of neuron and astrocyte depolarization that leads to loss of all electric activity, disturbed neurotransmitter distribution and cellular ion gradients and cellular swelling due to cytotoxic oedema (figure 20). The consequences are a depletion of energy reserves and an increase in glucose and oxygen demand. CSD can take place in healthy brain tissue and is then followed by a hyperaemic response mediated by neurovascular coupling. As a result of the increase in CBF and energy supply, the brain tissue normally quickly recovers. (Attwell et al., 2010; Wellman et al., 2013)

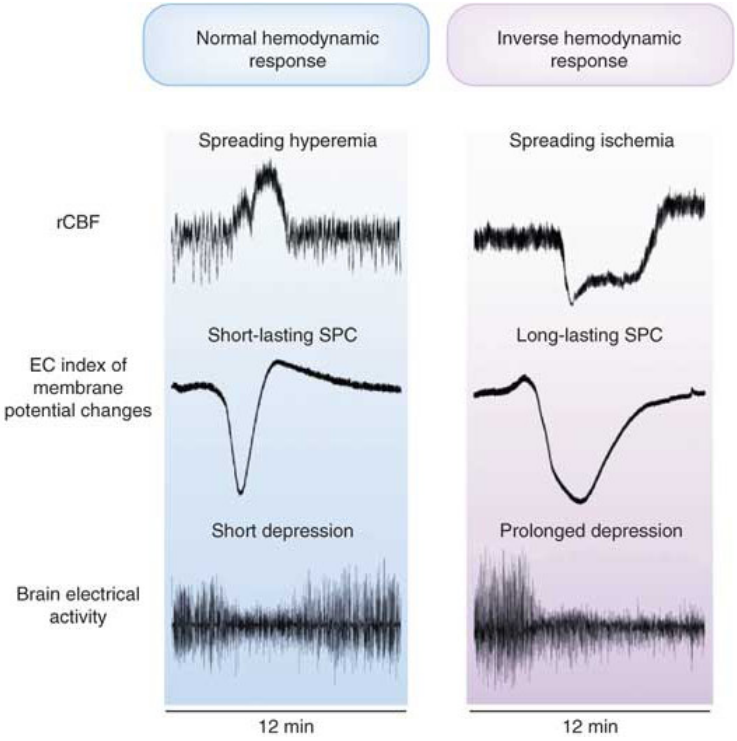


Figure 20:
The Illustration shows the haemodynamic responses to cortical spreading depression under physiological conditions (left side), and the inversed haemodynamic response occurring in SAH. The regional cerebral blood flow (rCBF) is lowered and the impaired functionality of electrolyte transporters caused by ischaemia causes a delayed repolarization shown by the extracellular (EC) derived slow potential change (SPC). In consequence, the electro-encephalogram indicates a prolonged depression of brain electrical activity. (Reproduced and modified with permission from Dreier et al., 2011 (Dreier, 2011))

After SAH, CSDs can occur as single incidences or clusters and affect 70-80% of patients. There are two peaks, on day 0 and day 7, with 75% of CSDs happening between days 5 and 7. (Bosche et al., 2010; Dreier et al., 2012) Besides spontaneous appearance, in SAH hypoglycaemia, oxyhaemoglobin, vasospasms, microcirculatory dysfunction, microthrombi, ET-1 and high sodium levels act as inductors. (Dreier et al., 2000; Dreier et al., 2002; Leng et al., 2011) Contrary to conditions in the healthy brain, after SAH the haemodynamic response can become inversed because of the depletion of NO, increased contractility of myocytes and vasoconstriction due to

pathologic calcium and sodium signalling. (Dreier et al., 1998; Wellman et al., 2013) Accordingly, a wave of depolarization is followed by severe hypoperfusion that results in failure of energy supply and possibly cell death (figure 21). Therefore, this phenomenon was called cortical spreading ischemia (CSI) and linked to the widespread cortical necrosis found after SAH. (Dreier et al., 2009; Neil-Dwyer et al., 1994)

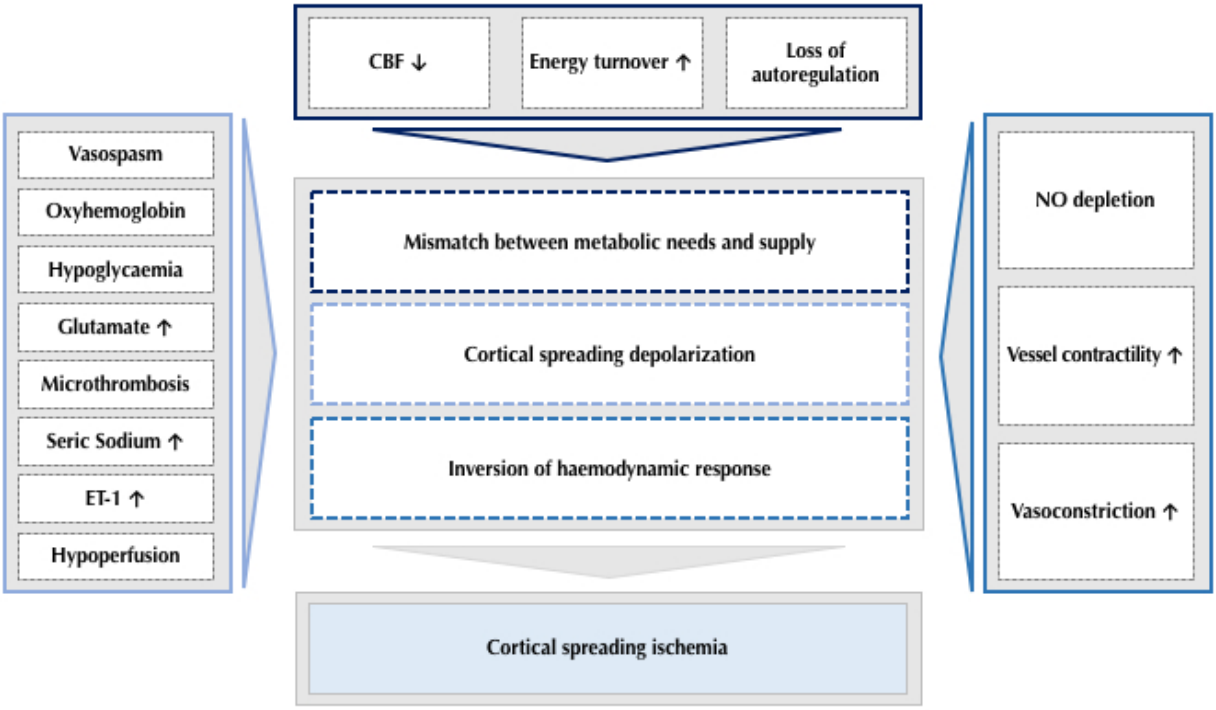


Figure 21:
Three conditions contribute to the phenomenon of cortical spreading ischemia after SAH. Firstly, cortical spreading depolarization (CSD) occurs more often after SAH due to various factors. Secondly, because of nitric oxygen (NO) consumption and increased vessel contractility the regular hyperaemia in reaction to a CSD is inversed to hypoperfusion. Finally, lost autoregulation, reduced cerebral blood flow, and increased energy consumption cause a mismatch between metabolic needs and energy and oxygen supply. Therefore, the increased number of CSDs aggravates the ischemia. (Cossu et al., 2014)

Clusters of CSDs lead to significantly longer hypoperfusion periods than single waves and can last for hours. Additionally, auto-amplification takes place, as severe perfusion deficit leads to progression of CSI and prolongation of hypoperfusion periods and, consequently, to hypoxia and infarction. (Dreier et al., 2009) This was shown for clusters lasting longer than 3 minutes or reducing CBF to 40-70%. (Chen et al., 2014; Hartings et al., 2013) Clusters of CSDs were observed at the beginning of clinical deterioration and a higher quantity of CSDs were associated with worse outcome and development of DCI. Moreover, the late peak of CSDs and the development of DCI show the same time course, and CSDs correlated significantly better with DCI than vasospasm and occurred also in its absence. (Dreier et al., 2012; Dreier et al., 2006; Woitzik et al., 2012)

1.1.4. A new approach to the prevention of DCI

As the previous chapter has shown, very complex processes contribute to the pathophysiology of SAH. They overlap in the different pathways and, thereby, are interdependent. By activating and promoting each other, they lead to a vicious circle, ending up in cerebral infarction and clinical deterioration. So, in contrast to most pre-existent treatments who focused on a single pathomechanism, a treatment for the prevention of DCI would ideally influence multiple mechanisms, interfere early and stop the development of a vicious circle.
Ischaemic cell death is basically a failure of energy metabolism due to missing oxygen and glucose supply. During resting state metabolism the brain is responsible for 20% of all energy usage, most of which is needed for ensuring cell homeostasis. (Attwell et al., 2010) This energy demand is increased by processes like inflammation or CSD. In SAH the brain tissue has been proven to be ischaemic during the early period of hypoperfusion. The cerebral metabolic rate of oxygen (CMRO2) is decreased while the oxygen extraction ratio (OER) increases. In consequence, the brain tissue oxygenation is severely decreased during the first two hours and 60% of patients show decreased oxygen tension during the first 24 hours. (Frykholm et al., 2004; Helbok et al., 2015; Westermaier et al., 2009b) Experiments on animals have shown that lactatacidosis develops within minutes after SAH and correlates with vasoparalysis, while high-energy phosphates are reduced in patients within hours after the haemorrhage and further decline during the following days. (Gewirtz et al., 1999; Voldby et al., 1985a; Yoshimoto et al., 1993) On the one hand, on a cellular level oxygen shortage produces loss of cell function which implicates loss of autoregulation, sustained disturbance of ionic homeostasis and failure in oxygen-dependent synthesis of messengers, like NO. (Attwell et al., 2010) In addition, the energy extraction from the remaining oxygen is decreased as progressive mitochondrial dysfunction was shown after SAH. (Jacobsen et al., 2014) On the other hand, lack of oxygen causes cell death which produces BBB breakdown and release of toxic products and, in consequence, vasospasm, thrombosis, inflammation and more cell death. Consequently, patients with severe ischaemia show lower levels of energy substrates, a higher lactate/pyruvate ratio and increased neuronal injury markers compared to patients without clinical ischaemia (figure 22). (Helbok et al., 2015; Schulz et al., 2000) This is more pronounced in patients with acute development of neurologic deficits, a group with high mortality, but is still considerable in patients with DCI. (Sarrafzadeh et al., 2002) Finally, patients with higher oxygen consumption and lower brain tissue oxygenation develop DCI more often and have overall a worse outcome. (Badjatia et al., 2011; Jaeger et al., 2012)

These arguments speak in favour of inadequate oxygen supply playing a central role in the pathogenesis of SAH. Therefore, improving oxygenation of brain cells early after SAH could be an elegant and effective way to lessen neurologic damage and prevent DCI. There is already evidence for positive effects of hyperbaric oxygen after SAH, however, as this treatment requires a rather complex infrastructure, it does not seem to be suitable for clinical practice. (Ostrowski et al., 2006b) Yet, the application of perfluorocarbons (PFC) early after SAH might be an applicable alternative, which to the date of these experiments had not been investigated.

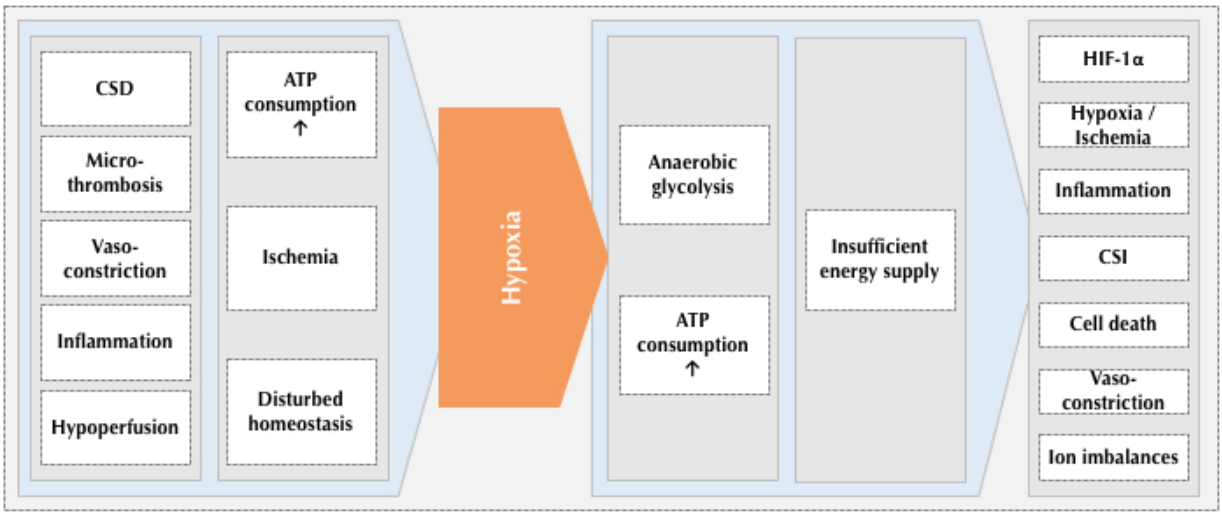


Figure 22:
Hypoxia is a central aspect in the different pathomechanisms developing after a SAH. Because of ischaemia and a disturbed homeostasis, metabolism is increased and energy reserves are consumed. Due to a lack of oxygen, cells change to anaerobic glycolysis with less efficient energy production, leading to a faster consumption of high-energy phosphates (ATP) that ends in an insufficient energy supply of the brain tissue. In consequence, the pathomechanisms initiated by the haemorrhage result in more ischaemia and brain tissue loss. (Cossu et al., 2014)

1.2. Perfluorocarbons

PFCs are a family of substances that were developed in the 1940s during the invention of the atomic bomb as a material for the storage of highly radioactive material. In the 1960s they experienced a comeback when medical research became interested in them. Since then, investigation in them took place in as different fields as respirational fluids, blood substitutes, decompression sickness, imaging, targeted drug therapy and neuroprotection. Their multifunctionality is due to their unique properties. (Spiess, 2009)

1.2.1. Properties

PFCs are developed by a complete substitution of fluoride for hydrogen in hydrocarbons. The amount of carbon atoms and their arrangement in a chain or circle decides on the physical properties. The bond between carbon and fluoride is one of the strongest known in chemistry and in PFCs it is responsible for their outstanding chemical properties. PFCs are almost completely chemically and biologically inert. The molecule structure is highly non-polar and, as a result, PFCs are hydrophobic and lipophobic at the same time. In consequence, PFCs are not miscible in aqueous liquids and are only poorly solvable by emulsification in lipids. Furthermore, their compound is responsible for the highest solubility of non-polar gases in any known liquid. This includes, amongst others, oxygen, carbon dioxide, NO and nitrogen, who all show high solubility-coefficients in PFCs but only negligible direct binding. Therefore, their solubility in PFC-liquids depends directly and linearly on the prevailing partial pressure (figure 23). (Spiess, 2009)

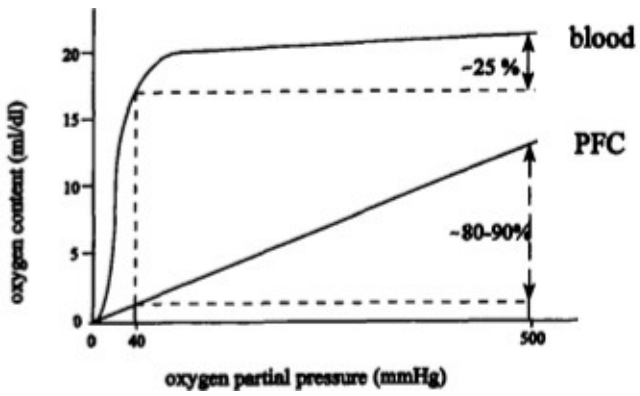


Figure 23:
While only 25% of oxygen stored in haemoglobin is available at a normal capillary oxygen partial pressure of 40mmHg, 80-90% of oxygen stored in a PFC-molecule are released due to the linear dynamic of oxygen binding. (Reproduced with permission from Habler et al., 1997 (Habler et al., 1997))

For the use of PFCs in humans, it is important to note that the plasma is an aqueous liquid and gas embolism represents a serious threat. Hence, a PFC for intravenous application must be emulsified and must not vaporise at body temperature. Most PFCs for intravenous application are nanoparticles consisting of the PFC encapsulated in micelles with a size of generally less than 0,4 µm. In physiological conditions the human body has a metabolic oxygen demand corresponding to 5 vol% free available oxygen on a capillary level, which is also the quantity that can be released from haemoglobin oxygenated with 21 vol%, hence breathing air. (Schneider et al., 2014) In comparison, the maximum amount of oxygen dissolvable in PFC

products for biomedical application so far is 40-50 vol%. However, the quantity critically depends on the oxygen partial pressure (figure 23). Then again, in haemoglobin the oxygen is bond to the molecule and not freely available for metabolic purposes, unlike oxygen dissolved in PFCs. Therefore, a pure PFC-solution currently can carry slightly more oxygen as the identical volume of blood with a haematocrit of 45%. (Schneider et al., 2014) Besides plasma and red blood cells (RBC), PFCs form a third space for oxygen transport without altering the capacity of haemoglobin for carrying oxygen. Additionally to raising the total amount of oxygen, PFCs increase the oxygen supply to cells by further mechanisms. Firstly, PFCs raise the total amount of free oxygen, whereby the diffusion pressure is increased and oxygen can penetrate deeper into the tissue. (Keipert et al., 1994) Secondly, the small molecule size of PFCs offers a large surface for gas exchange, which, in consequence, leads to an accelerated oxygen release from RBCs. Thirdly, in small vessels RBCs are known to flow in the middle, divided from the endothelium by a plasma gap. Plasma has a poor solubility for oxygen, so this gap slows the oxygen release from haemoglobin and the diffusion into the target tissue. The PFC nanoparticles can fill up this gap and bridge the oxygen transport to the tissue (Eggleton et al., 1998; Zhou et al., 2008) This effect can accelerate diffusion up to 50 times (figure 24). (Schroeder et al., 2008)

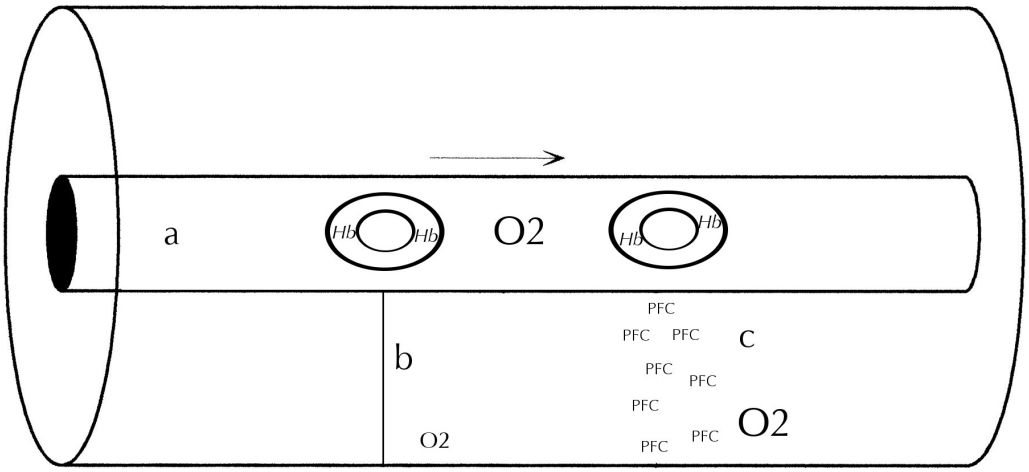


Figure 24:
In a small blood vessel, the stream of erythrocytes concentrates in the middle (a). Thereby oxygen must reach the tissue by diffusion, a slow and inefficient way of transport (b). PFC-molecules fill the space between the erythrocytes and the vessel wall, can free the oxygen more effectively from hemoglobin, and accelerate the transport to the tissue, supplying it thereby with a greater amount of oxygen (c). (Spiess, 2009)

Another advantage of PFCs is apparently the molecule size in comparison to the 7 µm diameter of RBCs. If blood flow gets clogged because of thromboembolism or tissue swelling, the resulting capillary diameter is often too narrow for RBCs to penetrate. However, down to a diameter of their molecule size, PFCs can still supply the corresponding tissue with oxygen and (re-)establish aerobic metabolism. (Faithfull, 1992) Additionally, PFCs were also shown to upregulate mitochondrial function, which further increases aerobic energy supply. (Daugherty et al., 2004) Even if blood flow is severely decreased or completely halted, PFCs still better the oxygen delivery to the tissue. (Spiess, 2010; Symons et al., 1999) In consequence, PFCs were shown to increase oxygen partial pressure in a vast variety of tissues and to enhance OER especially in situations with critical perfusion. (Faithfull, 1992) Moreover, organs can be successfully oxygenated with PFCs and, in haemodilution experiments, animals survived an extreme substitution of blood by PFC-emulsion. (Spiess, 2009)

Besides these effects on oxygen and other gases, PFCs have other biological implications that might influence their impact on SAH pathophysiology. Each injection of PFCs represent a form of haemodilution that results in a lower blood viscosity. Additionally, PFCs constitute a physical barrier for interaction between blood cells and endothelium. (Spiess, 2010) Both contribute to a better blood flow. The second protects the endothelium as well and might especially influence inflammatory processes. Accordingly, a reduced neutrophil activation, chemotaxis, recruitment and infiltration after PFC-treatment was found in lung injury. (Heard et al., 2000; Rossman et al., 1996) Furthermore, this might also explain antithrombotic effects of PFCs. (Lowe, 2000) Some products also can increase arterial blood pressure (Daugherty et al., 2004) which could be due to osmotic elevation of intravascular blood volume. But, effects on gene regulations are also described. Hyperoxic PFC-treatment downregulated HIF-1α and its target genes, mediators known to be important in the pathophysiology after SAH. (Calvert et al., 2006) In general, PFCs are considered safe and have not led to major adverse-effects so far. (Abutarboush et al., 2016) A fact probably also due to its elimination by exhalation through the lung. However, the application of PFCs together with increased oxygen inspiration has shown effects that might negatively influence the processes in place during EBI. Firstly, if both treatments are applied together, the formation of oxygen radicals is promoted (Yao et al., 2015). Secondly, whether the treatment results in vasodilation or –constriction seems to depend on oxygen level and PFC-dosage. (Abutarboush et al., 2016; Moon-Massat et al., 2014) The rise in free carbon dioxide and the drainage of NO by increased solubility is probably involved in the regulation (Rafikova et al., 2004; Spiess, 2010). (Spiess, 2009)
So far, there have been two generations of biomedical applicable PFC-products. While the first generation was successfully approved for some indications in humans, it was finally dismissed because of troublesome production, storage and practical use. Besides, substances showed a long-lasting persistence in organs and the artificial emulsifiers were associated with hypotension, complement release and white cell depression. Second generation substances have a higher PFC to emulsifier volume ratio in common and therefore can carry more oxygen. Furthermore, they are based on biological emulsifiers and are no longer needed to be stored frozen. However, substances from this generation only obtained approval in Russia and Mexico, as the most promising candidate, Oxygent, was stopped because of various side effects during a phase III human trial of haemodilution. However, later the study protocol was criticised for being too aggressive. In this work, the PFC-emulsion Oxycyte was used. (Castro et al., 2010; Spiess, 2009)

1.2.2. Oxycyte

Oxycyte is a third-generation PFC. It consists of an emulsion from egg-yolk phospholipids and 60% perfluoro-tert-butylcyclohexane, which boils at 147°C. The substance is stable at room temperature and can be stored at 4°C. Exposed to the appropriate oxygen tension, it has a maximum capacity of 43ml oxygen per 100ml, which is five times the capacity of haemoglobin. The size of the nanomolecules is approximately 0,2 µm, about 35 times smaller than RBCs. After a single injection, it remains in the circulation for about 20 to 24 hours, then its lipid content is metabolized in the liver, before the PFC part is eliminated through the lung. (Bullock et al., 2013) After injection, Oxycyte increases the total oxygen content in blood and the oxygen delivery to the parenchyma. (Yang et al., 2008) Besides the effects described in the previous chapter, it was shown to lessen reperfusion injury and to increase brain tissue oxygenation in a model of selective cerebral artery occlusion. (Cabrales et al., 2007; Woitzik et al., 2007) The substance

successfully passed phase I human safety testing and, furthermore, is not linked to vasoconstriction, impaired microvascular function or alterations of haemodynamic or metabolic parameters. (Cabrales et al., 2004; Nolte et al., 2000) Anyway, there are isolated reports of reduced platelet function, but severe affection of the coagulation could not be found. (Cronin et al., 2016; White et al., 2009)

1.2.3. Neuroprotective evidence

In addition to the positive effects found for hyperbaric oxygen after SAH, it appears interesting to investigate the effect of PFCs on the outcome after SAH, as there is already evidence for neuroprotection in brain pathologies. In animal studies, promising effects were found for both systemical and topical application. Often the investigated substance was Oxycyte. For ischaemic stroke, a topical application of PFC via a ventriculo-cisternal catheter was found to be neuroprotective. (Bell et al., 2002) Whereas systemical infusion decreased ischaemic brain damage after transient ischaemia, and neuronal electric activity decreased less and regenerated faster during and after ischaemia. (Sutherland et al., 1984) In a model of traumatic brain injury, animals treated with Oxycyte had a better functional outcome in cognitive testing and reduced neuronal cell death, while the oxygen consumption was increased. (Zhou et al., 2008) In spinal cord injury, the topical application via a lumbar catheter had neuroprotective effects and the systemic application lead to a better integrity of the white matter and spinal cord anatomy, as well as to a reduction of cell loss and damaged zones. (Osterholm et al., 1984; Yacoub et al., 2014) Ultimately, in a model of subdural haematoma, brain oxygenation was increased and brain damaged reduced. (Kwon et al., 2005) These findings were essentially ascribed to a better brain perfusion and the oxygen tension increase by PFCs. (Daugherty et al., 2004; Schroeder et al., 2008) Therefore, a treatment with PFCs might also result in neuroprotective effects after SAH.

2. Aims of the study

As described in chapter one, a complex pathophysiology consisting of various reciprocal depending mechanisms is responsible for the detrimental effects that result in high mortality and morbidity early and later after SAH. The initiation of the pathomechanisms takes place during the first 72 hours and can be subsumed under the term EBI. The development of hypoxia plays an important role in the pathophysiology and the severeness of the disease, it evolves due to the early events after SAH and causes a reinforced and sustainable progress of the different pathomechanisms (see fig. 22). PFCs are capable of preventing hypoxia by the improvement of tissue oxygenation due to their high oxygen carrying capacity and small molecule size and have proven neuroprotective effects in different settings of neurological damage. Therefore, PFCs might influence EBI positively and lead to a neuroprotection after SAH.

The principal aim of this study was to investigate the effect of the third generation PFC Oxycyte on the neurological outcome after SAH in an experimental setting in mice. As described above, the neurological outcome is determined by the magnitude of EBI. Therefore, Oxycyte was planned to be injected directly after SAH to be able to intervene early and influence the mechanisms underlying EBI positively from the beginning. Consequently, the influence of the treatment on the development of neurological damage and functionality was intended to be analysed at different time points during the period of EBI. Since neuroprotective evidence for Oxycyte was shown in other brain pathologies when the treatment was combined with hyperoxygenation (Yacoub et al., 2014; Zhou et al., 2008), the treatment with Oxycyte was planned to be studied in the combination with postinterventional exposure to 90vol% O2. Furthermore, since preliminary experiments in our laboratory, included in the dissertation of Sebastian Hambauer (Hambauer, 2017), suggested a worse neurological outcome for a Oxycyte dose of 10ml/kg, the object in this study was to investigate the treatment with a lower dose of 5ml/kg.
Due to the mentioned preliminary experiments, a secondary aim of this study was to differ the effect of the treatment with Oxycyte from the influence of the hyperoxygenation. Otherwise, it would not have been possible to distinguish an additional benefit or harm due to the Oxycyte application. Therefore, in order to compare the combined therapy of Oxycyte and hyperoxygenation to hyperoxygenation alone further control groups were added.

3. Materials and methods

The following experiments were performed at the research laboratory of the department for neurosurgery at the university hospital Klinikum rechts der Isar, Technische Universität München, between April 2012 and July 2013. The experiments and methods described in the following chapters were registered at and performed according to the guidelines of the responsible authority, the District Government of Upper Bavaria, and officially approved (reference number 55.2-1-54-2532.2-1-12).

3.1. Animals

For the SAH model of intravascular perforation male C57BL/6N mice (Charles River Laboratories, Research Models and Services Germany GmbH, Sulzfeld, Germany) with 20-22g body weight were used. Animals were kept in an air flow cabinet (Uni-Protect, Ehret, Emmendingen, Germany)), with free access to water and nutrition (Altromin 1324 (15000 I.U. Vit. A/kg; 600 I.U: Vit. D/kg; 75 mg Vit E./kg), Altromin Spezialfutter GmbH & Co. KG, Germany). Environmental conditions were 45-60% air humidity, 24°C room temperature, and a 12-hour circadian rhythm.

3.2. Work station

The operational area for the induction of the SAH was equipped with the following instruments: A microscope (Olympus SZX7, Olympus Deutschland GmbH, Hamburg, Germany) combined with an external flexible adjustable cold-light source (Olympus KL 1500 compact, Olympus Deutschland GmbH, Hamburg, Germany) was used during microsurgical preparation. Animal body temperature was kept stable by a feedback-looped control unit (TC-1000 Temperature Controller, CWE Inc., Ardmore, USA) equipped with corresponding sensor and heat pad. The ventilation of intubated mice was secured by a ventilator (MiniVent Type 845, Hugo Sachs Elektronik, March, Germany) and surveyed with a capnograph (Micro-Capnograph, Columbus Instruments, Columbus, USA). For ICP registration, two different systems were used during the experiments (1. Codman Microsensor Basic Kit + ICP-Express, both Codman, Norderstedt, Germany; 2. Samba 201 + Samba Preclin 420 LP, both Samba sensors, Gothenburg, Sweden).

3.3. Experimental execution

3.3.1. Preparations

Prior to operations, instruments and equipment was tested and calibrated daily, and/or, if necessary, replaced. Mice were weighed (572, d=0,01g, Kern & Sohn GmbH, Balingen, Germany) and examined for potential injuries.

3.3.2. Anaesthesia

To secure sufficient anaesthesia, analgesia and muscle relaxation a combination of Fentanyl (0,05 mg/kg; Fentanyl-Janssen, Janssen-Cilag GmbH, Neuss, Germany), Midazolam (5 mg/kg; Midazolam-ratiopharm, ratiopharm GmbH, Ulm, Germany), and Medetomidine (0,5 mg/kg; Dorbene vet., Pfizer GmbH, Berlin, Germany) was injected intraperitoneally via insulin syringes

(Omnican 100, B. Braun Melsungen AG, Melsungen, Germany) prior to operation. In cases where the initial dosage did not secure a sufficient anaesthesia or if the operation exceeded 60 minutes a second injection containing a quarter of the initial dosage was applied to secure sufficient anaesthesia for the time of the intervention. (Thal et al., 2007)

3.3.3. Monitoring

During the anaesthesia, adequate oxygen intake was guaranteed through mechanical ventilation, normothermia was assured by the feedback-controlled temperature regulator and the ICP was monitored by subdural probe placement. This monitoring was only interrupted temporally if animals had to be turned during operations.

3.3.3.1. Intubation

After establishment of effective anaesthesia, animals were intubated for mechanical ventilation. A 20-gauge intravenous catheter (Introcan Safety, 20 G, B. Braun Melsungen AG, Melsungen, Germany) served as an endotracheal tube after being shortened. (Zausinger et al., 2002) Mice were attached by their incisors onto a cord, thereby fixing them to a cardboard and bringing the body axis into a 45° angle to the ground, which eventually offered a direct view on the vocal cords from above. This area then was externally illuminated by the light source. Thus, the endotracheal tube could be forwarded in between the vocal cords under direct vision through the microscope. Next, the animal was fixated on the heat pad in ventral position. The tube then was connected to the ventilator, delivering 100 vol% oxygen at a rate of 0,1 l/min, and the capnograph probe was advanced through a small hole on the side of the tube. Thereby, successful intubation and adequate oxygen supply could be guaranteed.

3.3.3.2. Temperature-regulation

The heating pad was ought to keep body temperature stable at 37,0 °C. Therefore, temperature had to be registered internally, which occurred by rectal placement of the probe. To protect the mice from irritations and injuries resulting from probe placement an ointment was used at insertion (Bepanthen Augen- und Nasensalbe, Bayer Vital GmbH, Leverkusen, Germany).

3.3.3.3. ICP-monitoring

Exact monitoring of the ICP is important in the endovascular perforation model of SAH induction. But, subdural placement of the probe can also mechanically damage the brain parenchyma. Therefore, during the experiments, the initial system was replaced by another kit with a smaller probe to minimize the mechanical injury. Prior to system exchange, equivalence of ICP baseline values from both systems were compared to warrant the continuity of results. However, as the probe from the Samba-system is very small and fragile, its way of insertion and fixation had to be modified to not damage it in the process of insertion and retrieval. Anyway, the preceding preparation was identical for both methods: skin was incised above the right temporal muscle, which then was separated at the origin and further dissected laterally. Thereby, the relatively thin temporal bone became accessible and a hole of the size of the ICP-probe could be drilled (Proxxon Micromot 50/E; Proxxon GmbH, Niersbach, Germany) at the transition between the temporal and parietal bone. (Friedrich, 2010).

3.3.3.3.1. Method 1

During the 4h and 24h groups, ICP was measured by the Codman-system. However, as this system is designed to be used in the human, its probe (⌀1,2mm) is relatively big in comparison to the anatomical dimensions of the mouse skull. On the other hand, this size makes it sturdier. So, subsequent to insertion, the probe can be secured by dental cement (Poly-F Plus Bondex, Dentsply DeTrey GmbH, Konstanz, Germany) and left in place during turns of the animal. (Friedrich, 2010)

3.3.3.3.2. Method 2

Due to the reasons mentioned above, in the 48h groups the Samba-system was used. It uses a probe a third in size in comparison to the Codman-system (⌀0,42mm). Unfortunately, this causes fragility and if secured directly with dental cement it could take damage at the removal. Therefore, during its use, a thin plastic tube with an internal diameter the size of the probe was introduced in the drill hole and secured with dental cement. In consequence, the probe had to be carefully retrieved and replaced prior to and after each turn of the animal.

3.3.4. Dissection

For the investigation of SAH, the way of haemorrhage induction should simulate the processes occurring after the rupture of an aneurysm as accurate as possible. Induction by intraluminal artery perforation seemed to suit this purpose best. This method was originally established in rats (Bederson et al., 1995; Longa et al., 1989) and later implemented in mice. (Feiler et al., 2010; Kamii et al., 1999) After having turned the animal to a dorsal position, dissection started by uncovering the left carotid bifurcation, thereby granting access to the common carotid artery (CCA), the internal carotid artery (ICA) and the external carotid artery (ECA). In the next step, the ECA was ligated close to the bifurcation and the CCA and ICA were clipped. Thereby, flow in the bifurcation was interrupted, an important precondition for limiting blood loss. Now, microsurgical scissors were used to cut a small hole into the ECA proximal to the ligation. Through this hole, a filament (Prolene 5-0, Ethicon, Norderstedt, Germany) was introduced into the ECA and forwarded into the ICA. Another suture secured the entrance of the filament to the ECA, so the clips could be removed with minimal to zero blood loss. Removal of the CCA clip is important to guarantee a natural blood pressure during SAH induction. Now the ECA could be severed between both ligations to allow an easy motion of the filament. This permits a straight advance of the filament in the ICA. This is important for two reasons. Firstly, while advancing the filament, the outlet to the pterygopalatine artery must be avoided, and, secondly, if the filament comes to rest upon the base of skull instead of entering through the carotid canal it cannot induce a SAH.

3.3.5. SAH-induction

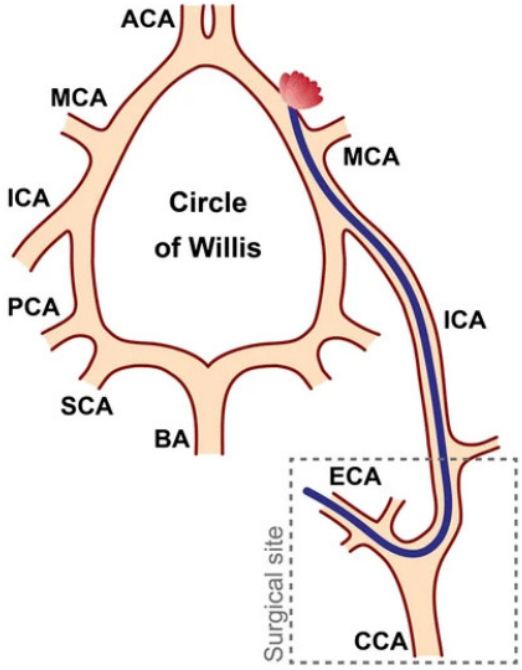


Figure 25:
The suture was introduced in the external carotid artery (ECA), advanced through the internal carotid artery (ICA) and perforated the vessel intrasubarachnoidal at the branch-off into the anterior cerebral artery (ACA) and the middle cerebral artery (MCA); BA: basilar artery, CCA: common carotid artery, PCA: posterior cerebral artery, SCA: superior cerebellar artery. (Reproduced with permission from Schüller et al./Bühler et al. (Buhler et al., 2014; Schuller et al., 2013))

Once placed in the ICA, the filament was advanced straight and without delay up to the ramification of the ICA into the medial and anterior cerebral artery where it penetrated the vessel wall and induced the haemorrhage (figure 25). (Bederson et al., 1995) The moment of perforation was indicated by a sudden and sharp increase in ICP. Afterwards, the filament was immediately withdrawn to not block the blood-flow and thereby influence the haemorrhage volume. To prevent any bleeding from the ECA-stump, the remaining ligation was retightened. Within minutes the ICP fell and stabilized at about 20 mmHg. The ICP-probe was retrieved, the drill hole in the skull was closed with fibrin glue (Histoacryl, Braun Aesculap, Tuttlingen, Germany) and the skin sutured.

3.3.6. Antagonisation

After finishing the operation, the mice were extubated and the remaining probes withdrawn. The effect of anaesthesia was then antagonised by applying Atipamezole (2,5mg/kg, Alzane, Pfizer GmbH, Berlin, Germany), Flumazenil (0,5mg/kg, Flumazenil Inresa, Inresa Arzneimittel GmbH, Freiburg, Germany) and Naloxone (Naloxon Inresa, Inresa Arzneimittel GmbH, Freiburg, Germany) intraperitoneally.

3.3.7. Analgesia

Since animals were ought to survive up to 48h after the induction of the haemorrhage, an adequate analgesia was indispensable and applied subsequent to the antagonisation. Animals with 24h survival were injected with Buprenorphine (0,15 µg/kg, Temgesic, Reckitt Benckiser Healthcare, UK) intraperitoneally. However, since this substance is known to possibly have a neuroprotective effect (Ozden et al., 2004) and to have a shorter duration of action, Carprofen (5 mg/kg, Rimadyl, Pfizer GmbH, Berlin, Germany) was used instead for the series with 4h and 48h

survival. In animals with 48h survival, the injection was repeated after 24h. To check if the results with both analgetics were comparable, brain water content of sham-groups treated with both substances were compared and showed no significant differences.

3.3.8. Observation

After the operation, animals were observed for a minimum of 4h in a separate box, tempered to 37°C, while having free access to food and water. To treat animals with oxygen, the gas could be insufflated, thereby reaching a concentration of 90 vol%. Once the treatment or observation time was over, animals were moved to cages in the air flow cabinet, but kept separated from non-operated mice. 24h after the induction of the haemorrhage their body temperature and weight were checked. If these controls revealed a weight loss of ≥20%, signs of inadequate body temperature control or other signs of severe suffering (apathy, denial of food, akinesia, seizures, bleeding, obvious pain), the mice were euthanised by cervical dislocation. Otherwise, animals were killed after reaching the intended survival time and their brain was collected for further investigation.

3.4. Drug delivery

To investigate the influence of Oxycyte (Oxycyte, Oxygen Biotherapeutics Inc., Morrisville, NC, USA), the substance had to be applicated intravenously. A volume of 240 µl, containing a dosage of 5 mg/kg were injected into a tail vein by an insulin syringe (Omnican 100, B. Braun Melsungen AG, Melsungen, Germany). In the control groups, the animals were treated with the same volume of saline solution. In groups with 48h survival, the treatment was repeated after 24h.

3.5. Brain water content

By measuring the brain water content, it is possible to quantify the extent of brain oedema developing after SAH (Claassen et al., 2002; Katzman et al., 1977). The extent, again, correlates with the severity of the SAH and the neurologic outcome (Claassen et al., 2002; Doczi, 1985). The brain water content can be determined by comparing the wet with the dry weight of the brain (Katzman et al., 1977). Prior to balancing, the olfactory bulb and the cerebellum were removed and the hemispheres were separated from each other. Wet weight was obtained directly after harvesting of the brain (ABT 320-4M, d=0,1mg, Kern & Sohn GmbH, Balingen, Germany) and dry weight after 24h of dehydration at 110°C. The Brain water content then was calculated as follows: [(wet weight – dry weight) / wet weight] x 100. (Feiler et al., 2010) If the water content of the whole brain is displayed, it results from the addition of both hemispheres.

3.6. Neuroscore

The Modified Neurological Severity Score was used to assess the neurologic functionality. This score integrates the results from multiple individual tests and thereby generally indicates the neurologic status (table 2). Its composition and realisation were modified over the years by various authors. (Chen et al., 2001; Pfeilschifter et al., 2012). The version used in this work included four individual tests, covering different areas of motor and sensory functions. Some of the tests are used in literature to analyse contralateral hemisymptomatic after unilateral cerebral lesion. However, since SAH affects both hemispheres, symptoms from both sides of the body were counted.

In the first test the extent of paresis was examined. (Bederson et al., 1986; Chen et al., 1996) The mouse was lifted by its tail and hold close to the table top. A healthy animal tries to reach for the ground with its forepaws. Impaired motor function is analysed by three criteria: Paresis of a forepaw, paresis of a hindpaw and inability to raise the head above 10° in the vertical axis. Each criterion was considered independently and awarded with one point if present. Points were added, allowing a maximum score of three for this subdomain.
In the second test the gait was inspected. (Bederson et al., 1986; Longa et al., 1989) For this purpose mice were allowed to move freely. In contrast to the first test, the most correct grading was chosen, as higher scores included symptoms of lower ones. Animals not able to walk straight were awarded one point, those who were circling in one direction two points and mice that were falling or rolling to one side three points. Thus, this subdomain again had a maximum score of three.

<table>
<tr><td colspan="5">mNSS - Modified neurological severity score</td></tr>
<tr><td>Extent of paresis (points added)</td><td>Gait (most suitable)</td><td>Coordination (most suitable)</td><td>Reflexes (points added)</td><td></td></tr>
<tr><td rowspan="2">Flexion of a forepaw (1)</td><td rowspan="2">Inability to walk straight (1)</td><td>Grabs sides of the beam (1)</td><td rowspan="3">Corneal reflex absent (1)</td><td rowspan="5">Maximum score (14)</td></tr>
<tr><td>One paw is not used or falls down while balancing (2)</td></tr>
<tr><td rowspan="2">Flexion of a hindpaw (1)</td><td rowspan="2">Circling to one side (2)</td><td>Both paws fall down, Mouse spins on beam, or falls down (>30 sec.) (3)</td></tr>
<tr><td>Mouse attempts to balance but falls down (>20 sec.) (4)</td><td rowspan="3">Pinna reflex absent (1)</td></tr>
<tr><td rowspan="2">Ability to vertical raise the head (1)</td><td rowspan="2">Rolling or falling to one side (3)</td><td>Mouse attempts to balance but falls down (>10 sec.) (5)</td></tr>
<tr><td>Mouse does not attempt to balance and/or falls down (<10 sec.) (6)</td></tr>
</table>

Table 2:
Modified neurological severity score (mNSS) adapted for SAH in mice. Maximum score is 14, higher score shows worse outcome. (Modified from Pfeilschifter et al. (Pfeilschifter et al., 2012)

The third test focused on coordination (Chen et al., 1996; Germano et al., 1994). Mice had to balance on a metal beam (⌀ 1,5 cm; length 50 cm; altitude 20 cm) covered in tape (Leukoplast, BSN medical GmbH, Hamburg, Germany) for better grip. Mice were granted a maximum of six points, again according to the most correct grading. One point signified the mouse had to grab the sides of the beam to keep the balance. If one paw was hanging down paretic and not used for balancing, the animal obtained two points. Three points meant both fore- or hindpaws were

hanging down, the mouse was spinning around the beam or falling off when more than 30 seconds had passed. Falling of the beam after 20 to 30 seconds had passed was awarded with four points. Accordingly, 10 to 20 seconds was awarded with five points and with six points if the animal showed no attempt to balance at all or fell of the beam in less than 10 seconds.
Finally, the sensory function was tested by checking the corneal and the pinna reflexes on both sides. (Germano et al., 1994) The first one can be induced by touching the eyes with a cotton bud. To test the second one, a small wooden rod was introduced into the external auditory canal. If the pinna reflex was present, the stimulation of the canal walls induced a shaking of the head. The absence of each reflex was counted as one point.
So, in total, the best result for the whole score was 0, if neurologic functionality was completely preserved, and 14 in the worst case. Results could be classified roughly into four groups. Scores from 0 to 2 meant the animals were almost not affected. Mice with a score of 3 or 4 had slightly decreased neurologic functionality. 5 to 8 already implied severe impairment and animals with scores of 9 and above nearly did not show reactions to the tests at all.

3.7. Statistical analysis

The acquired data was analysed with IBM SPSS Statistics (IBM, Armonk, New York, USA) and Microsoft Excel (Microsoft Corporation, Albuquerque, USA). Diagrams and tables were created with Microsoft PowerPoint (Microsoft Corporation, Albuquerque, USA) or with Origin (Origin Lab Corporation, Northampton, USA). If not further specified, stated numbers are mean values ± standard deviation. Results from brain water content and mNSS were checked for normal distribution and homogeneity of error variances. If the data complied with these preconditions a student's t-test was performed to show significant differences between two groups and a one-way ANOVA with a confidence interval of 95% was performed to test for significant differences between more than two groups, followed by a post-hoc Tukey's test. To test for significant differences between two groups with normally distributed data but missing homogeneity of error variances the Welch's test was used. If normal distribution of data or homogeneity of error variances were not given, a Kruskal-Wallis and post-hoc Dunn's test was used for analyses between more than two groups. To analyse differences in the neuroscore in the 48h-groups the method of Winer and Shirley was used, corresponding to a mixed-ANOVA in ranks. In graphs, significant results are highlighted by an asterisk, with p at least <0.05 considered as significant difference (*). Mortality amongst animals was analysed by a chi-square test.

3.8. Series of experiments

Prior to the onset of the experiments, the intervention was practised to guarantee comparable conditions before starting the application of the treatment in the different groups. The animals belonged to ten different groups which investigated three different survival periods. The criteria for inclusion were as follows: animals had to survive the intended observation time, intravenous injection of drugs had to be successful and the presence of a SAH had to be confirmed macroscopically when brains were extracted.
To ensure the best possible randomisation and blinding, the operation was started in all animals without assigning the animal to a group already at this point. However, all experiments for a determined survival period were performed contiguously before the experiments for a different survival period were started. The operation was carried out uniformly until directly before the induction of the haemorrhage. Only then the animal was assigned to a group by manually

drawing lots. Therefore, during the SAH-induction and the intravenous injection the surgeon was not blinded anymore. Afterwards, the animal was labelled without association to the treatment group but in order to retrace the order of the experiments. For the assessment of the neuroscore the animals were identified by their label and evaluated by the surgeon without further blinding measures. After euthanising the animals, the hemispheres were collected in small glass containers labelled with random numbers.

3.8.1. 24h Oxycyte

As mentioned above, there already existed data in our laboratory for a survival of 24 hours included in the dissertation of Sebastian Hambauer (Hambauer, 2017). This data showed that animals who receive a high dose of Oxycyte (10 ml/kg) together with a treatment of 24 hours of 90 vol% oxygen show a tendency to increased brain water content and worse mNSS compared to a treatment with saline solution at ambient air. To investigate further into these findings, this work includes three groups with a survival of 24 hours. On the one hand, a SAH-group and a sham-group were treated with NaCl at ambient air. Additional to being controls, these groups served the purpose to compare the results with the findings from Hambauer's work. On the other hand, in the third group SAH animals were treated with saline solution and 24 hours of 90 vol% oxygen to investigate the influence of the high oxygen treatment.

3.8.2. 4h Oxycyte

The relative short period of 4 hours was chosen to investigate the short-term influence of Oxycyte on the neurologic outcome. For this purpose, three groups were compared. The first one was a SAH-group treated with Oxycyte and 90 vol% oxygen. However, animals obtained a Oxycyte dosage of only 5 ml/kg. This dosage has proven effectiveness as well and might reduce possible damage from oxygen toxicity. (Zhou et al., 2008) The other groups were controls: both included SAH-animals injected with saline solution, but in one animals were exposed to 90 vol% oxygen and, in the other, they were breathing ambient air. By this, consistency to the results from the 24h-groups was aimed to be granted.

3.8.3. 48h Oxycyte

To assess the influence of Oxycyte on long-term development of neurologic damage four different groups were investigated over a period of 48 hours. These groups received the treatment twice (see 2.4.). Due to the negative experience with the exposure of highly concentrated oxygen over a long time from Hambauer's work (Hambauer, 2017), animals received the oxygen treatment only for a total of 8 hours. The first 4 hours were applied directly after the first treatment injection and the second 4 hours after the second treatment injection. In the first group, SAH was followed by a treatment with 5 ml/kg Oxycyte and 90 vol% oxygen. Group two and three were SAH-controls injected with saline solution, one with a 90 vol% oxygen treatment and the other one breathing ambient air. The fourth group contained sham-operated animals who were also injected with saline solution and held at ambient air.

4. Results

4.1. General observations

For this work, 125 of the 166 operated mice were included in the analysis. A total of 41 animals were excluded from the analyses: 25 died during the observation period, in 11 animals the operation could not be completed successfully and 5 animals were excluded because intravenous injection could not be achieved. Therefore, overall mortality was 17% (25/150).
In the included experiments the operation took a mean of 62±8 min, while SAH was induced after 45±8 min. If only SAH-groups are considered, mean operation time is 63±8 min and time to SAH-induction is 45±8 min, compared to 60±7 min and 42±6 min in the sham-groups. If tested with student's t-test (no outliners excluded, data normally distributed, homogeneity of variances given), no significant differences could be found for the time to SAH-induction (mean time difference 3 min (95%-CI[-1, +7]), $t(123) = 1,39$, $p = 0,167$) or the total operation time (mean time difference 3 min (95%-CI[-1, +8]), $t(123) = 1,52$, $p = 0,131$) due to the intervention.
In the SAH-groups the ICP rose from a baseline of 9±2 mmHg by 72±25 mmHg to 81±25 mmHg after the haemorrhage induction. In contrast, in sham-animals the ICP rose only negligibly from 10±2 mmHg by 3±1 mmHg to 13±1 mmHg when the filament was placed inside the artery but did not penetrate the vessel. The statistical analysis with Welch's test (no outliners excluded, data normally distributed, homogeneity of variances not given) showed no significant differences for the ICP baseline (mean ICP difference -1 mmHg (95%-CI[-2, 0]), $t(20) = -1,66$, $p = 0,112$), but significant differences for the ICP increase (mean ICP difference 69 mmHg (95%-CI[64, 74]), $t(110) = 27,27$, $p < 0,001$) and the ICP maximum (mean ICP difference 69 mmHg (95%-CI[64, 74]), $t(107) = 27,76$, $p < 0,001$).

4.2. 24h-observation

Three groups were studied over a period of 24 hours to investigate a possible adverse effect of 90vol% oxygenation over 24h alone, thus none of them receiving Oxycyte.
The first group, which was oxygenated with 90vol%, (10/15 animals included, 2 animals excluded due to unsuccessful intravenous injection, 20% mortality) showed a mean operation time of 69±6min and a time to SAH of 50±7min. The ICP rose from 8±2mmHg by 56±17mmHg to 64±17mmHg. Animals were killed 24h21min±37min after SAH-induction. The right hemisphere contained 79,82±0,43% of water, the left 79,70±1,33%, and both together 79,76±0,96%. Results from mNRSS were as follows: 0 points no animal, 1-2 points one animal, 3-4 points three animals, 5-8 points five animals, 9-14 points one animal.
In the second SAH-group, exposed to ambient air, (5/6 animals included, 17% mortality) operation lasted 66±4min with SAH-induction after 48±2min. Prior to haemorrhage, the ICP was 9±2mmHg and increased after the perforation by 53±24mmHg to 62±23mmHg. Observation time was 24h43min±60min. Results from measuring the brain water content were 79,93±0,23% on the right side, 79,65±0,98% on the left, and 79,79±0,69% overall. Results from neuroscore were: 0 points no animal, 1-2 points one animal, 3-4 points three animals, 5-8 points one animal, 9-14 points no animal.
In sham animals (8/8 animals included, 0% mortality) the operation took 60±6min, with filament placement after 42±7min. ICP values stayed at 15±3mmHg. Animals were monitored for 24h28min±2h20min. The brain water content at the right side was 79,07±0,32%, on the left

78,76±0,47%, and 78,92±0,42% for the whole brain. In neurologic testing, all animals of this group scored 0. The results are presented in the figures 26-29.

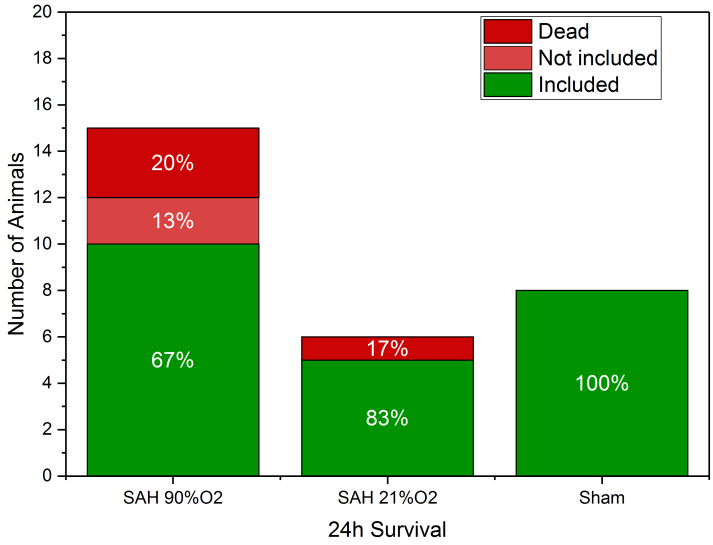
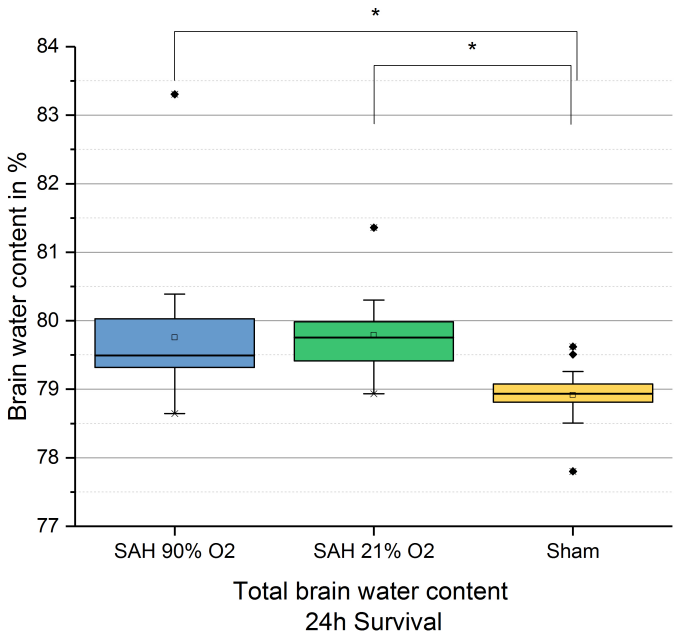


Figure 26:
Survival after 24 hours of observation; SAH 90%O2: 15 animals operated, 10 (67%) included, 2 animals (13%) excluded due to unsuccessful intravenous injection, 3 animals (20%) died before completing the 24 hours of observation; SAH 21%O2: 6 animals operated, 5 animals (83%) included, 1 animal (17%) died before completing the 24 hours of observation; Sham: 8 animals operated, 8 animals (100%) included.

A chi-square test was used to compare the mortality between the different groups (3 expected cell frequencies below 5; Monte Carlo method with 99% confidence coefficient) and showed no significant correlation ($\chi^2(2) = 1,81$, $p = 0,528$). The total operation time (no outliners excluded, data normally distributed, homogeneity of variances given) and the time to SAH (no outliners excluded, data normally distributed, homogeneity of variances given) was analysed with a one-way ANOVA. There was a significant difference in the total operation time between the 3 groups ($F(2, 20) = 4,38$, $p = 0,026$). A post-hoc analysis with a Tukey's test revealed a significant difference ($p = 0,02$) between the total operation time of the SAH 90% O_2 and the sham group (mean time difference 8 min, 95%-CI[-1, 16]). For the time to SAH no significant differences were found ($F(2, 20) = 3,36$; $p = 0,055$). A Kruskal-Wallis test (no outliners excluded, data not normally distributed) revealed no significant differences in the observation time between the different groups ($\chi^2(2) = 0,52$, $p = 0,773$). The ICP baseline (no outliners excluded, data normally distributed, homogeneity of variances given) was also analysed by a one-way ANOVA and showed a significant difference ($F(2, 20) = 5,60$, $p = 0,012$). A post-hoc analysis with a Tukey's test revealed a significant difference ($p = 0,09$) between the ICP baseline of the SAH 90% O_2 and the sham group (mean ICP difference -3 mmHg, 95%-CI[-6, -1]). The ICP raise (no outliners excluded, data normally distributed, no homogeneity of variances given) and the ICP maximum (no outliners excluded, data normally distributed, no homogeneity of variances given) were analysed by a Kruskal-Wallis test and showed significant differences for the ICP increase ($\chi^2(2) = 15,24$, $p < 0,001$) and the ICP maximum ($\chi^2(2) = 15,08$, $p = 0,001$). A post-hoc Dunn's test with Bonferroni correction showed a significant difference between the SAH 90% O2 and the sham group, but just a simple difference between the SAH 21% O2 group and the sham group for the ICP increase (SAH 90% O_2 vs. sham: $p_{corr} = 0,019$; SAH 21% O_2 vs. sham: $p = 0,006$, $p_{corr} = 0,681$) and the ICP Maximum (SAH 90% O_2 vs. sham: $p_{corr} = 0,027$; SAH 21% O_2 vs. sham: $p = 0,004$, $p_{corr} = 0,461$).

For the brain water content of the right hemisphere (no outliners excluded, data normally distributed, homogeneity of variances given) a one-way ANOVA found significant differences between the groups ($F(2, 20) = 12,76$, $p < 0,001$). A post-hoc Tukey's test revealed significant differences between both SAH groups and the sham group (SAH 90% O_2 vs. sham: mean difference 0,75%, 95%-CI[0,32, 1,17], $p = 0,001$; SAH 21% O_2 vs. sham: mean difference

Figure 27:
Total brain water content after 24 hours; SAH 90%O2: 79,82±0,96%; SAH 21%02: 79,79±0,69%; Sham: 78,92±0,42%; Significance: SAH 90%O2 vs. SAH 21%O2: NS, SAH 90%O2 vs. Sham: p=<0,001(*), SAH 21%02 vs. Sham: p=<0,002(*).



0,86%, 95%-CI[0,35, 1,38], p = 0,001). For the left hemisphere (no outliners excluded, data not normally distributed) a Kruskal-Wallis test also showed significant differences ($\chi^2(2) = 7,35$, p = 0,025). A post-hoc Dunn's test showed differences between the SAH groups and the sham group that were not significant after application of a Bonferroni correction (SAH 90% O_2 vs. sham: p = 0,012, $p_{corr} = 1,000$; SAH 21% O_2 vs. sham: p = 0,037, $p_{corr} = 1,000$).

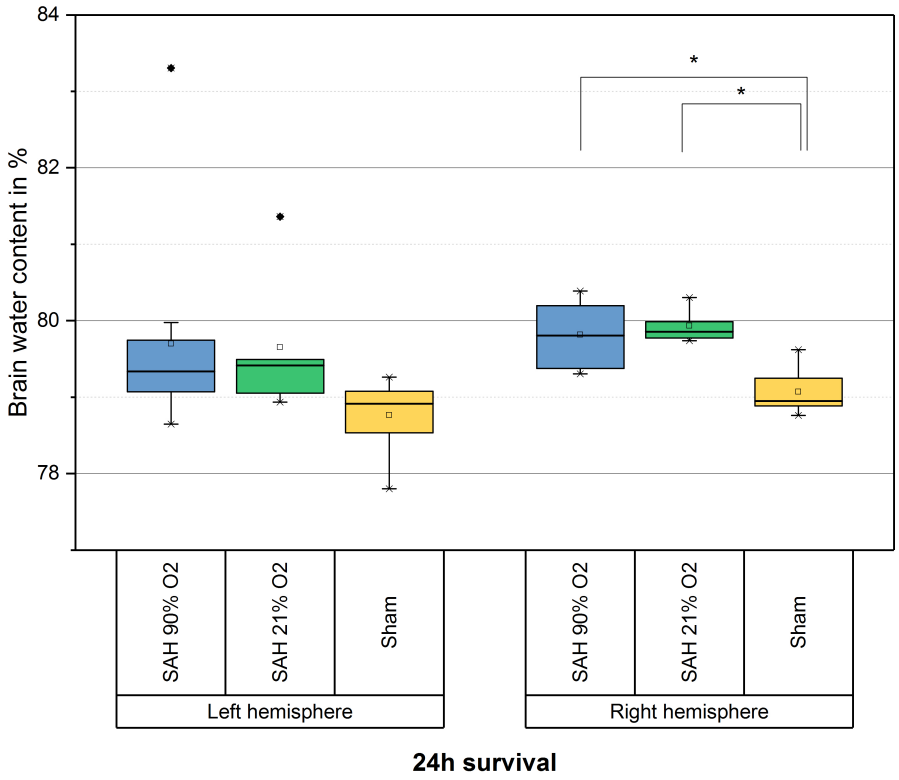


Figure 28:
Brain water content in the hemispheres after 24 hours survival; Left hemisphere: SAH 90%O2 79,70±1,33%, SAH 21%O2 79,65±0,98%, Sham 78,76±0,47%; Right hemisphere: SAH 90%O2 79,82±0,43%, SAH 21%O2 79,93±0,23%, Sham 79,07±0,32%; Significance left hemisphere: SAH 90%O2 vs. SAH 21%O2: NS, SAH 90%O2 vs. Sham: NS, SAH 21%O2 vs. Sham: NS; Significance right hemisphere: SAH 90%O2 vs. SAH 21%O2: NS, SAH 90%O2 vs. Sham: p=<0,001 (*), SAH 21%O2 vs. Sham: p=<0,001 (*).

A Kruskal-Wallis test showed a significant difference between the brain water content of the whole brain (no outliners excluded, data not normally distributed; ($\chi^2(2) = 17{,}24$, $p < 0{,}001$)). The post-hoc analysis with a Dunn's test adjusted with a Bonferroni correction showed significant differences between the SAH groups and the sham group (SAH 90% O_2 vs. sham: $p_{corr} = 0{,}001$; SAH 21% O_2 vs. sham: $p_{corr} = 0{,}002$).
For the results of the neuroscore (no outliners excluded, data not normally distributed) a Kruskal-Wallis test showed significant differences between the groups ($\chi^2(2) = 16{,}18$, $p < 0{,}001$). The post-hoc analysis with a Dunn's test showed differences between the SAH groups and the sham group, but only the difference between the SAH 90% and the sham group was significant after a Bonferroni correction (SAH 90% O_2 vs. sham: $p_{corr} = 0{,}010$; SAH 21% O_2 vs. sham: $p = 0{,}07$, $p_{corr} = 0{,}764$).

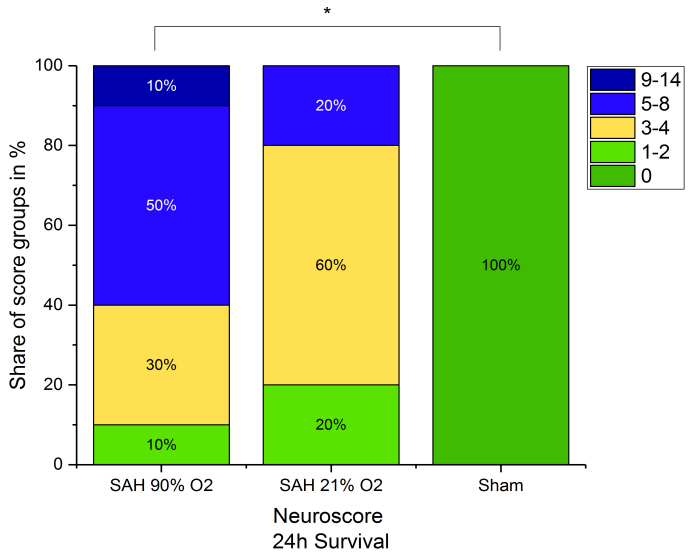


Figure 29:
Neuroscore after 24 hours survival; SAH 90%O2: 0 points no animal (0%), 1-2 points one animal (10%), 3-4 points three animals (30%), 5-8 points five animals (50%), 9-14 points one animal (10%). SAH 21%O2: 0 points no animal (0%), 1-2 points one animal (20%), 3-4 points three animals (60%), 5-8 points one animal (20%), 9-14 points no animal (0%); Sham: all animals scored 0 points (100%). Significance: SAH 90%O2 vs. SAH 21%O2: NS, but trend to better outcome in SAH 21%O2, SAH 90%O2 vs. Sham: p=<0,01 (*), SAH 21%O2 vs. Sham:NS

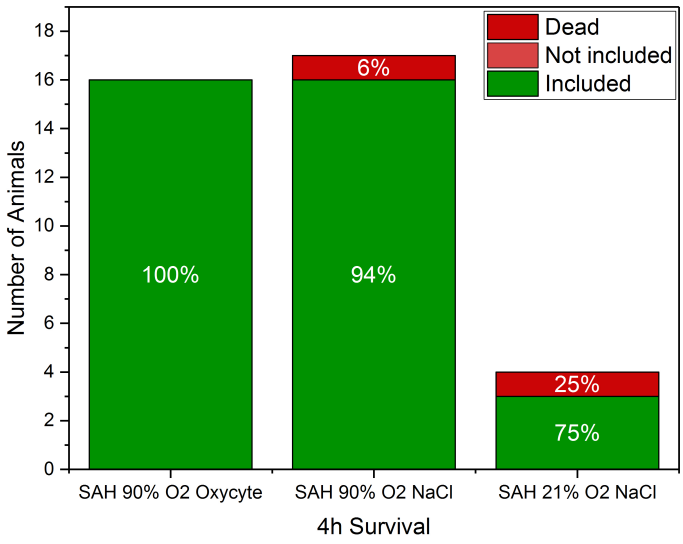
However, the results from the mNSS showed a trend to a worse neurologic score in the SAH-group treated with 90% oxygen (figure 29). Compared to the data from Hambauer (Hambauer, 2017), the mortality in the SAH groups was somewhat less (17% and 20% vs. 24%). The results from measuring the brain water content were similar, particularly in the SAH-groups oxygenated with 90 vol% oxygen and ambient air. Regarding the mNSS, the comparison between the data revealed that the poor outcome with high-dose Oxycyte in the work from Hambauer cannot be caused by the high oxygen levels alone and independently from Oxycyte, as, in this work, animals exposed to the same quantity of oxygen, but without Oxycyte-treatment, scored better.

In summary, in this experiment, no clear evidence for the toxicity of highly concentrated oxygen after SAH could be found. However, the results trend in this direction. Taken together with the results from Hambauer, applied with or without high-dose Oxycyte, long-term exposure to highly concentrated oxygen could not improve neurologic outcome.

4.3.4h-observation

In this series, three groups were studied for a period of 4 hours post-SAH.
The first group, treated with 5 ml/kg Oxycyte and 90 vol% oxygen, (16/16 animals included, 0% mortality) had a mean operation time of 65±11 min and SAH was induced after 47±13 min. The ICP increased from a baseline of 9±1 mmHg by 76±27 mmHg to 85±26 mmHg. Animals were monitored for 4h11min±30min. Brain water content was 79,88±0,43% on the right, 79,34±0,42% on the left and 79,61±0,50% in total. Results from mNSS were: 0 points no animal, 1-2 points two animals, 3-4 points five animals, 5-8 points nine animals, 9-14 points no animals.
For the SAH-control-group, oxygenated with 90 vol% oxygen (16/17 animals included, 6% mortality), operation time was 63±6 min and time to SAH 45±8 min. ICP rose from 9±1 mmHg by 74±22 mmHg to 83±23 mmHg at the beginning of the haemorrhage. The observation time was 4h16min±24min. The right hemisphere contained 79,60±0,41% water, the left 79,46±0,37% and both together 79,53±0,39%. Neurologic score results were as follows: 0 points no animal, 1-2 points three animals, 3-4 points four animals, 5-8 points five animals, 9-14 points four animals.
In SAH-animals treated at ambient air (3/4 animals included, 25% mortality) operation took 58±5 min with a haemorrhage induction after 38±2 min. Baseline-ICP was 9±1 mmHg, which increased after the vessel perforation by 93±43 mmHg to 103±42 mmHg. The observation time was 4h21min±20min. On the right side, the brain water content averaged 79,78±0,14%, on the left side 79,53±0,38% and in total 79,65±0,29%. The outcome of the mNSS was: 0 points no animal; 1-2 points one animal, 3-4 points no animal, 5-8 points no animal, 9-14 points two animals. The results are presented in the figures 30-33.

Figure 30:
Survival after 4 hours of observation; SAH 90%O2 Oxycyte: 16 animals operated, 16 animals (100%) included; SAH 90%O2 NaCl: 17 animals operated, 16 animals (94%) included, 1 animal (6%) died before completing 4 hours of observation; SAH 21%O2 NaCl: 4 animals operated, 3 animals (75%) included, 1 animal (25%) died before completing 4 hours of observation.



A chi-square test (4 expected cell frequencies below 5; Monte Carlo method with 99% confidence coefficient) showed no significant correlation between the mortality and the different groups ($\chi^2(2) = 3,93$, $p = 0,198$). A Kruskal-Wallis test showed no significant differences for the total operation time (no outliners excluded, data not normally distributed; $\chi^2(2) = 2,08$, $p = 0,354$) and the time to SAH (no outliners excluded, data not normally distributed; $\chi^2(2) = 3,09$, $p = 0,214$) between the groups. A one-way ANOVA (no outliners excluded, data normally

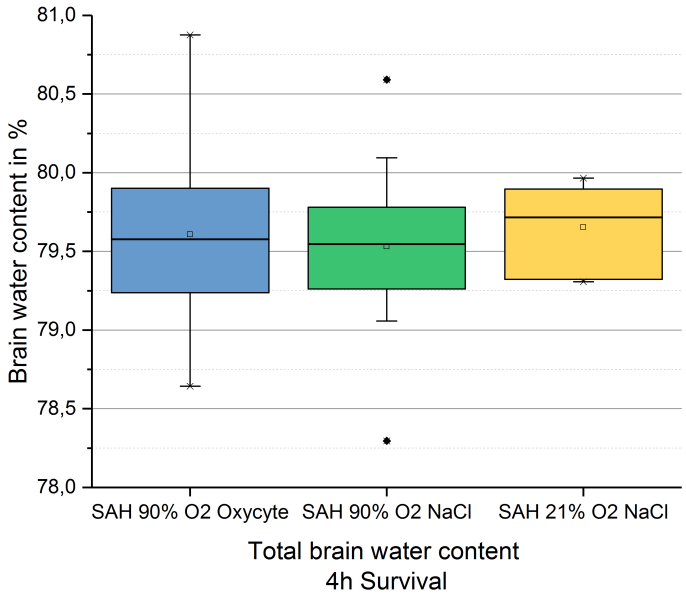
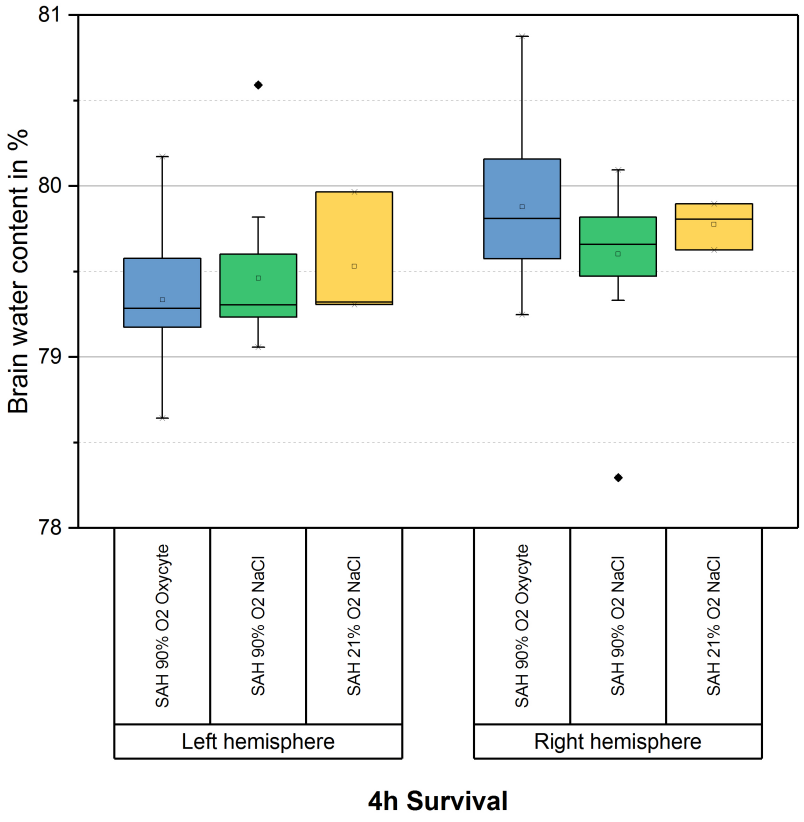


Figure 31::
Total brain water content after 4 hours survival; SAH 90%O2 Oxycyte: 79,61±0,50%, SAH 90%O2 NaCl: 79,53±0,39%, SAH 21%02 NaCl: 79,65±0,29%; Significance: SAH 90%O2 Oxycyte vs. SAH 90%O2 NaCl: NS, SAH 90%O2 Oxycyte vs. SAH 21% O2 NaCl: NS, SAH 90%02 NaCl vs. SAH 21% NaCl: NS.

distributed, homogeneity of variances given) revealed no significant differences in the observation time between the different groups ($F(2, 32) = 0,28$, $p = 0,760$). For the ICP baseline (no outliners excluded, data not normally distributed; $\chi^2(2) = 0,33$, $p = 0,847$), and the ICP raise (no outliners excluded, data not normally distributed; $\chi^2(2) = 0,49$, $p = 0,783$) a Kruskal-Wallis test showed no significant differences between the groups, as did a one-way ANOVA for the ICP maximum (no outliners excluded, data normally distributed, homogeneity of variances given; $F(2, 31) = 0,73$, $p = 0,488$).

Figure 32:
Brain water content in hemispheres after 4h survival; Left hemisphere: SAH 90%O2 Oxycyte 79,34±0,42%, SAH 90%O2 NaCl 79,46±0,37%, SAH 21%O2 NaCl 79,53±0,38%; Right hemisphere: SAH 90%O2 Oxycyte 79,88±0,43%, SAH 90%O2 NaCl 79,60±0,41%, SAH 21%O2 NaCl 79,78±0,14%,; Significance left hemisphere: SAH 90%O2 Oxycyte vs. SAH 90%O2 NaCl: NS, SAH 90%O2 Oxycyte vs. SAH 21%O2 NaCl: NS, SAH 90%O2 NaCl vs. SAH 21%O2 NaCl: NS; Significance right hemisphere: SAH 90%O2 Oxycyte vs. SAH 90%O2 NaCl: NS, SAH 90%O2 Oxycyte vs. SAH 21%O2 NaCl: NS, SAH 90%O2 NaCl vs. SAH 21%O2 NaCl: NS.



For the brain water content of the right hemisphere (no outliners excluded, data normally distributed, homogeneity of variances given; F(2, 32) = 1,86, p = 0,172), of the left hemisphere (no outliners excluded, data normally distributed, homogeneity of variances given; F(2, 32) = 0,57, p = 0,574), and for the total brain water content (no outliners excluded, data normally distributed, homogeneity of variances given; F(2, 67) = 0,34, p = 0,713) a one-way ANOVA found no significant differences between the groups.
For the results of the neuroscore (no outliners excluded, data not normally distributed) a Kruskal-Wallis test revealed no significant differences between the groups ($\chi^2(2) = 1,32$, p = 0,517). No significant differences between groups could be found, neither for the results of brain water content measuring's nor for neurologic functionality. The results for the total brain water content of the SAH 21% O2 NaCl was compared with the ones from the corresponding 24h-group with a student's t-test (no outliners excluded, data normally distributed, homogeneity of variances given) and showed no significant difference (mean difference -0,14% (95%-CI[-0,78, +0,50]), $t(14) = -0,46$, $p = 0,652$).

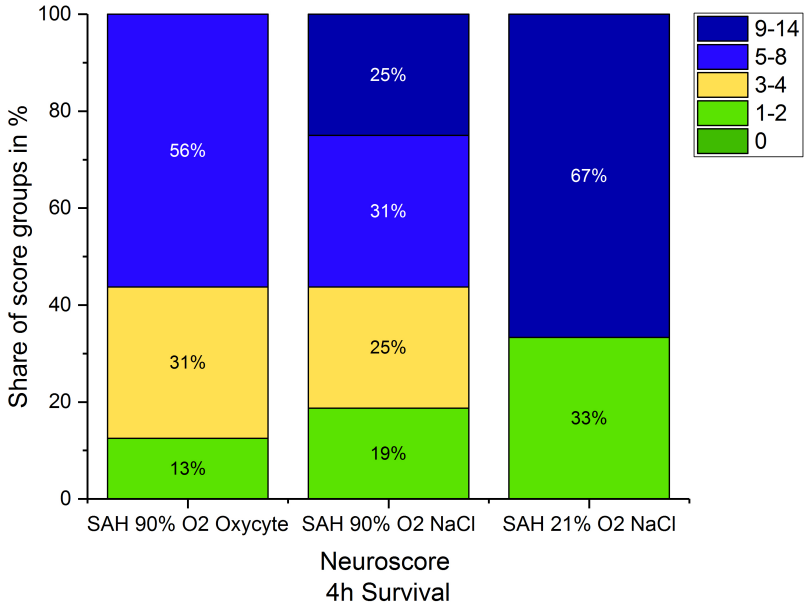


Figure 33:
Neuroscore after 4 hours survival; SAH 90%O2 Oxycyte: 0 points no animals (0%), 1-2 points two animals (13%), 3-4 points five animals (31%), 5-8 points nine animals (56%), 9-14 points no animals (0%). SAH 90%O2 NaCl: 0 points no animals (0%), 1-2 points three animals (19%), 3-4 points four animals (25%), 5-8 points five animals (31%), 9-14 points four animals (25%). SAH 21%O2 NaCl: 0 points no animal (0%); 1-2 points one animal (33%), 3-4 points no animal (0%), 5-8 points no animal (0%), 9-14 points two animals (67%). Significance: SAH 90%O2 Oxycyte vs. SAH 90%O2 NaCl: NS, but less animals with severe scores in SAH 90%O2 Oxycyte; SAH 90%O2 Oxycyte vs. SAH 21%O2 NaCl: NS, SAH 90%O2 NaCl vs. SAH 21%O2 NaCl: NS.

All in all, the low mortality in this series is noticeable, which can be explained by the short observation time. If results from brain water content measuring are compared, all three groups presented similar levels, without a trend to an increase in one of them. However, although not significant, in the diagram with mNSS-results (figure 33) the trend to a better outcome in the Oxycyte-group with no animal reaching the worst category of 9-14 score points at this very early time point of only 4h after SAH is worth mentioning, especially compared to the 90%O2-controls. Consistently, the 21% O_2-control-group showed the worst results in the neuroscore.

4.4.48h-oberservation

This series was composed of four groups.
In the group treated with 5 ml/kg Oxycyte (20/26 animals included, 23% mortality) the operation-time was 58±6 min, with a SAH-induction after 41±5 min. Due to the haemorrhage, the ICP rose from 10±2 mmHg by 70±26 mmHg to 80±26 mmHg. Following the intervention, the mice were oxygenated with 90 vol% oxygen for 4h5min±8min and transferred to normal cages afterwards. On average 24h15min±20min after the haemorrhage, the treatment with Oxycyte and 90 vol% oxygen was repeated for 4h7min±16min. The animals were killed and evaluated 48h44min±47min after the SAH. The results for the brain water measuring were 80,16±0,86% for the right hemisphere, 79,89±1,03% for the left hemisphere and 80,03±0,94% for both hemispheres together. 24h after SAH, the results from neurologic functional testing were: 0 points three animals, 1-2 points five animals, 3-4 points eight animals, 5-8 points three animals, 9-14 points one animal. Another 24h later, the results were: 0 points five animals, 1-2 points six animals, 3-4 points four animals, 5-8 points three animals, 9-14 points two animals.
In the second group, a control group treated with 90 vol% oxygen (20/27 animals included, 26% mortality), the intervention took 61±7 min, while the SAH was induced after 45±7 min. Prior to the haemorrhage, the ICP was 9±2 mmHg and increased by 76±19 mmHg to 85±19 mmHg as a result of the perforation. Subsequently to the operation, the animals were oxygenated with 90 vol% for 4h2min±9min. The treatment was applied for a second time over 3h59min±14min 24h7min±17min after the haemorrhage. In total, the surveillance time was 48h22min±22min. In the following analysis, the right hemispheres showed a water content of 79,69±0,58%, the left ones 79,37±0,54% and the whole brains 79,53±0,58%. The neuroscore after 24h was: 0 points one animal, 1-2 points nine animals, 3-4 points seven animals, 5-8 points two animals, 9-14 points one animal. After 48h the neuroscore was: 0 points two animals, 1-2 points eleven animals, 3-4 points five animals, 5-8 points two animals, 9-14 points no animal.
For the SAH-control group, surveyed at ambient air (17/25 animals included, 3 animals excluded due to failed intravenous injection, 20% mortality), the operation lasted 64±10 min, whereas the time to perforation was 48±8 min. The SAH was followed by an ICP-rise from 10±2 mmHg by 76±29 mmHg to 86±29 mmHg. For the following 4h3min±9min the animals could recover in the heated box. 24h2min±5min after the SAH the second saline injection was given, followed by another 4h3min±8min observation in the heated box. After 48h20min±25min the animals were killed. The brain water content averaged 79,97±0,65% on the right side, 79,69±0,80% on the left side and 79,83±0,73% in total. The first mNSS-testing, 24 hours after SAH, resulted in: 0 points three animals, 1-2 points two animals, 3-4 points six animals, 5-8 points five animals, 9-14 points one animal. The second testing, after 48h: 0 points four animals, 1-2 points six animals, 3-4 points five animals, 5-8 points one animal, 9-14 points one animal.
In the last group, which consisted of sham-operated animals (10/11 animals included, 9% Mortality), the filament was placed after 43±7 min, while the total intervention took 59±8 min. After the probe-insertion, the ICP averaged 9±2 mmHg and stayed around 13±2 mmHg during the rest of the operation, hence showing only a minimal raise of 3±1 mmHg. Afterwards, the animals were injected with saline and surveyed for 4h4min±11min. A second saline treatment and observation followed 24h9min±15min after the probe-insertion, which covered a period of 4h17min±29min. The total observation time for this group was 48h30min±21min. The analysis of the brain water content resulted in 79,08±0,57% for right hemispheres, 78,74±0,70% for lefts

hemispheres and 78,91±0,65% for the whole brain. Both inspections of neurologic functionality, after 24h as well as after 48h, resulted in a mNSS of 0 for all animals in this group.
The results are presented in the figures 34-37.

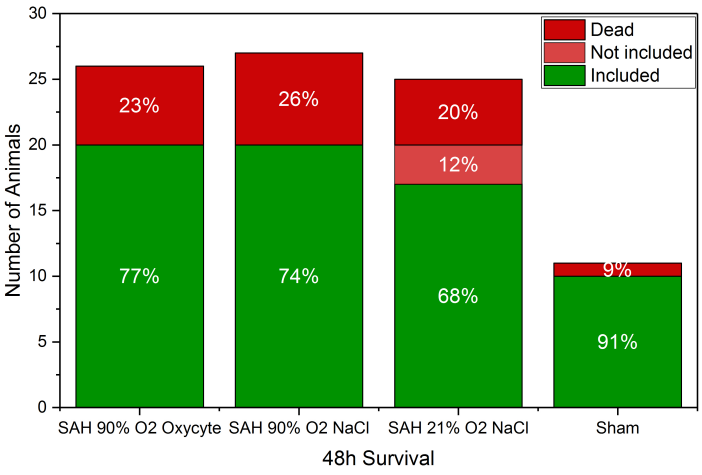


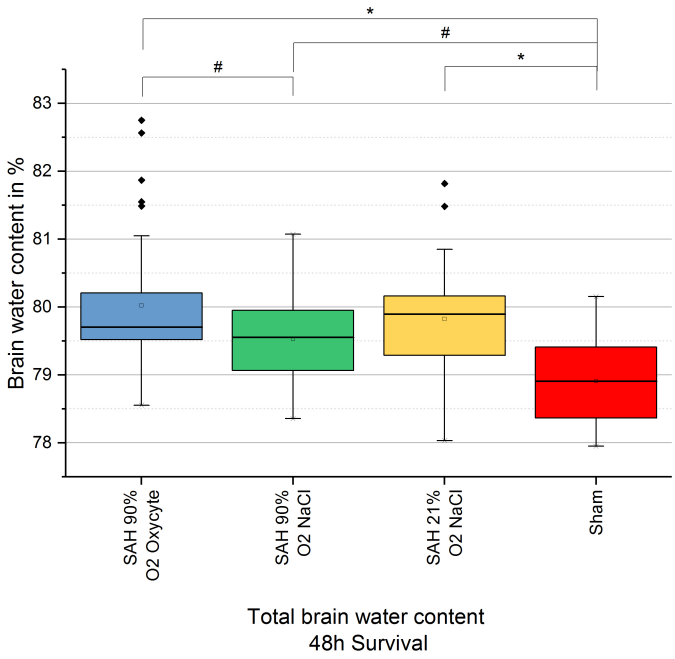
Figure 34:
Survival after 48h of observation; SAH 90%O2 Oxycyte: 26 animals operated, 20 animals (77%) included, 6 animals (23%) died before completing 48h of observation; SAH 90%O2 NaCl: 27 animals operated, 20 animals (74%) included, 7 animals died before completing 48h of observation; SAH 21%O2 NaCl: 25 animals operated, 17 animals (68%) included, 3 animals (12%) excluded due to an unsuccessful intravenous injection, 5 animals (20%) died before completing 48h of observation; Sham: 11 animals operated, 10 animals (91%) included, 1 animal died before completing the 48h of observation.

Despite a considerably longer observation period, the mortality was not elevated in the groups with 48h of observation compared to the groups with shorter survival periods. With the exception of the sham-group, which should not show any mortality anyway, rates averaged between 20% and 26%. Further analysis with a chi-square test (1 expected cell frequency below 5; Monte Carlo method with 99% confidence coefficient) revealed no significant correlation between the mortality and the different groups ($\chi^2(3) = 1,30$, $p = 0,707$). The total operation time (no outliners excluded, data normally distributed, homogeneity of variances given) and the time to SAH (no outliners excluded, data normally distributed, homogeneity of variances given) was analysed with a one-way ANOVA. For the total operation time no significant differences were found ($F(3, 63) = 1,78$; $p = 0,160$). There was a significant difference in the time to SAH between the four groups ($F(3, 63) = 3,51$, $p = 0,020$). A post-hoc Tukey's test revealed a significant difference ($p = 0,012$) between the total operation time of the SAH 90%O2 Oxycyte group and the SAH 21%O2 NaCl group (mean time difference -8 min, 95%-CI[-14, -1]). There were no significant differences between the treatment time on day 1 (no outliners excluded, data not normally distributed; $\chi^2(3) = 3,48$, $p = 0,323$), the observation time between treatment 1 and treatment 2 (no outliners excluded, data not normally distributed; $\chi^2(3) = 5,13$, $p = 0,162$), the treatment time on day 2 (no outliners excluded, data not normally distributed; $\chi^2(3) = 6,99$, $p = 0,072$) and the total treatment time (no outliners excluded, data not normally distributed; $\chi^2(3) = 7,18$, $p = 0,067$) between the different groups, as analyses by Kruskal-Wallis tests showed. The ICP baseline (no outliners excluded, data not normally distributed), the ICP raise (no outliners excluded, data normally distributed, homogeneity of variances not given) and the ICP maximum (no outliners excluded, data not normally distributed) were also analysed by Kruskal-Wallis tests and showed significant differences for the ICP increase ($\chi^2(3) = 25,28$, $p < 0,001$) and the ICP maximum ($\chi^2(3) = 25,24$, $p < 0,001$), however no significant differences between the groups for the ICP baseline ($\chi^2(3) = 1,49$, $p = 0,685$). As expected, further analysis by post-hoc Dunn's tests, including a Bonferroni-correction, resulted in significant differences only between the SAH groups and the sham group for the ICP raise (SAH 90% O_2 Oxycte vs. sham: $p_{corr} < 0,001$; SAH 90% O2 NaCl vs. sham: $p_{corr} < 0,001$; SAH 21% O_2 NaCl vs. sham: $p_{corr} < 0,001$) and the ICP maximum (SAH 90% O_2 Oxycte

vs. sham: $p_{corr} < 0{,}001$; SAH 90% O2 NaCl vs. sham: $p_{corr} < 0{,}001$; SAH 21% O_2 NaCl vs. sham: $p_{corr} < 0{,}001$).

The analysis of the brain water content of the right hemisphere (no outliners excluded, data normally distributed, homogeneity of variances given) by a one-way ANOVA found significant differences between the groups ($F(3, 63) = 5{,}82$, $p = 0{,}001$). A post-hoc Tukey's test revealed significant differences between the SAH 90% O2 Oxycyte group and the sham group, as well as between the SAH 21% O2 NaCl group and the sham group (SAH 90% O2 Oxycyte vs. sham: mean difference 1,07%, 95%-CI[0,37, 1,78], $p = 0{,}001$; SAH 21% O2 NaCl vs. sham: mean difference 0,88%, 95%-CI[0,15, 1,61], $p = 0{,}011$). For the left hemisphere (no outliners excluded, data normally distributed, homogeneity of variances given) a one-way ANOVA also showed significant differences ($F(3, 63) = 5{,}16$, $p = 0{,}003$). A post-hoc Tukey's test again showed significant differences between the SAH 90% O2 Oxycyte group and the sham group, as well as between the SAH 21% O2 NaCl group and the sham group (SAH 90% O2 Oxycyte vs. sham: mean difference 1,16%, 95%-CI[0,34, 1,97], $p = 0{,}002$; SAH 21% O2 NaCl vs. sham: mean difference 0,95%, 95%-CI[0,11, 1,79], $p = 0{,}020$).

Figure 35:
The total brain water content after 48 hours survival time; SAH 90%O2 Oxycyte: 80,03±0,94%, SAH 90%O2 NaCl: 79,53±0,58%, SAH 21%02 NaCl: 79,83±0,73%, Sham: 78,91±0,65%; Significance: SAH 90%O2 Oxycyte vs. SAH 90%O2 NaCl: p=0,02 (#), SAH 90%O2 Oxycyte vs Sham: p<0,001 (*), SAH 90%O2 NaCl vs. Sham: p=0,017 (#), SAH 21%O2 NaCl vs. Sham: p<0, 001 (*).



The analysis of the whole brain water content (no outliners excluded, data normally distributed, homogeneity of variances given) by a one-way ANOVA also resulted in significant differences between the groups ($F(3, 130) = 10{,}74$, $p < 0{,}001$). This time the post-hoc Tukey's test revealed significant differences between the SAH 90% O2 Oxycyte and the SAH 90% O2 NaCl group and between the three SAH groups and the sham group (SAH 90% O2 Oxycyte vs. SAH 90% O2 NaCl: mean difference 0,50%, 95%-CI[0,06, 0,93], $p = 0{,}020$; SAH 90% O2 Oxycyte vs. sham: mean difference 1,11%, 95%-CI[0,58, 1,65], $p < 0{,}001$; SAH 90% O2 NaCl vs. sham: mean difference 0,62%, 95%-CI[0,08, 1,15], $p = 0{,}017$; SAH 21% O2 NaCl vs. sham: mean difference 0,92%, 95%-CI[0,34, 1,47], $p < 0{,}001$).

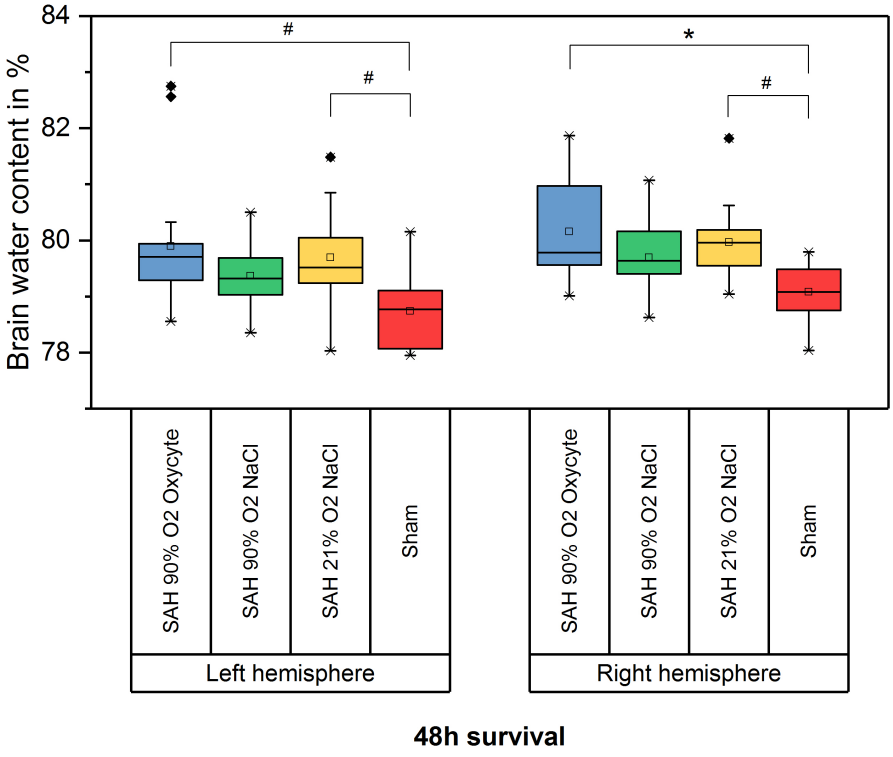


Figure 36:
The brain water content in the hemispheres after 48 hours survival time; Left hemisphere: SAH 90%O2 Oxycyte 79,89±1,03%, SAH 90%O2 NaCl 79,37±0,54%, SAH 21%O2 NaCl 79,69±0,80%, Sham 78,74±0,70%; Right hemisphere: SAH 90%O2 Oxycyte 80,16±0,86%, SAH 90%O2 NaCl 79,69±0,58%, SAH 21%O2 79,97±0,65%, Sham 79,08±0,57%; Significance left hemisphere: SAH 90%O2 Oxycyte vs. Sham: p=0,002 (#), SAH 21%O2 NaCl vs. Sham: p=0,02 (#); Significance right hemisphere: SAH 90%O2 Oxycyte vs. Sham: p<0,001 (*), SAH 21%O2 NaCl vs. Sham: p=0,011 (#).

Since the data of the neuroscore after 24h and 48h was interval-scaled and analyses showed no normal distribution and no homogeneity of variances, the results could not be analysed by a normal mixed ANOVA. Therefore the data was analysed by a procedure developed by Winer and Shirley, consisting of a data transformation into ranks, followed by a mixed ANOVA with Bonferroni corrected post-hoc tests. (Shirley, 1981; Winer et al., 1991). As a result, the assessment by a Levene's test continued to show no homogeneity of variances after the data transformation (NS 24h: $p < 0,001$; NS 48h: $p < 0,001$), but homogeneity of covariances, as assessed by Box's test ($p = 0,707$), could be achieved. Calculation of chi square for the two main effects and their interaction resulted in a significant difference for the treatment ($\chi^2(3) = 20,66$, $p < 0,001$) and the neuroscore ($\chi^2(1) = 11,73$, $p < 0,001$), but no significant difference for their interaction ($\chi^2(3) = 4,52$, $p > 0,05$). Results from the mixed ANOVA with post-hoc analyses showed a significant difference between the results of the neuroscore of day 1 and day 2 (NS 24h vs. NS 48h: $p < 0,001$) and between the SAH groups and the sham group (SAH 90% O2 Oxycyte vs. sham: $p < 0,001$; SAH 90% O2 NaCl vs. sham: $p < 0,001$; SAH 21% O2 NaCl vs. sham: $p < 0,001$) in general. The mNSS results for the SAH 90% O2 Oxycyte group ($p = 0,039$) and the SAH 21% O2 NaCl group ($p = 0,001$) changed significantly from the testing at 24h to the testing at 48h, whereas the results of the neuroscore between day 1 and day 2 showed no significant differences for the SAH 90% O2 NaCl group ($p = 0,085$) and the sham group ($p = 1,000$). If the results of the neuroscore were analysed separately for the testing after 24h and after 48h, the tests showed a significant difference between the SAH-groups and the sham group (NS 24h: SAH 90% O2 Oxycyte vs. sham: $p < 0,001$, SAH 90% O2 NaCl vs. sham: $p < 0,001$, SAH 21% O2 NaCl vs.

sham: p < 0,001; NS 48h: SAH 90% O2 Oxycyte vs. sham: p < 0,001, SAH 90% O2 NaCl vs. sham: p = 0,001, SAH 21% O2 NaCl vs. sham: p < 0,001).

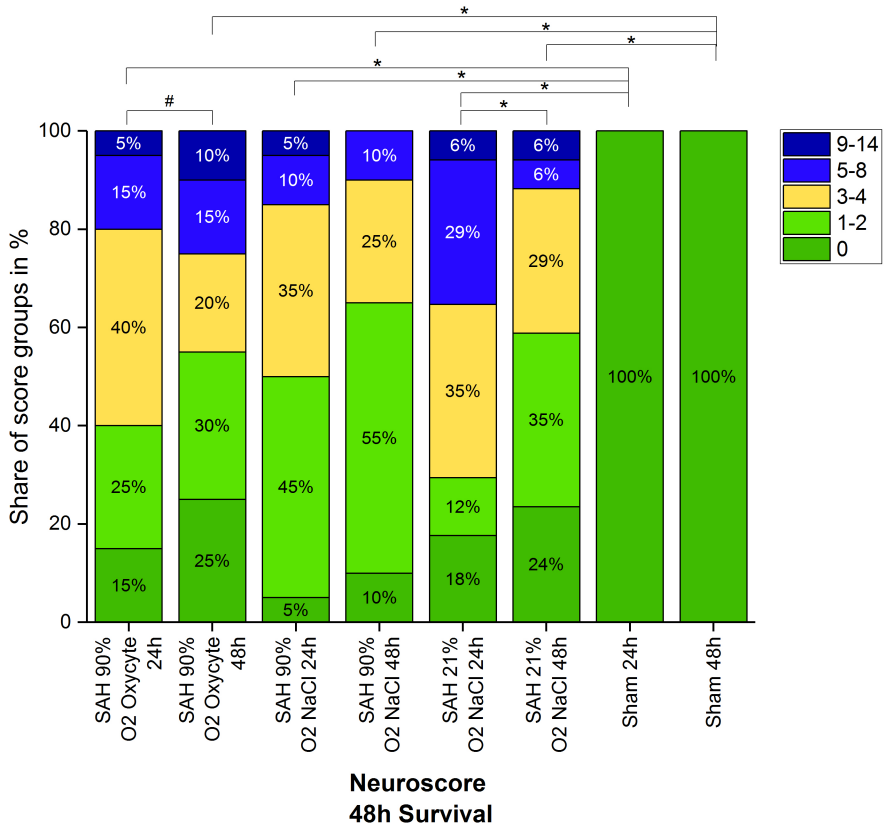


Figure 37:
The neuroscore after 48 hours survival; Results for the first testing after 24 hours of observation: SAH 90%O2 Oxycyte: 0 points three animals (15%), 1-2 points five animals (25%), 3-4 points eight animals (40%), 5-8 points three animals (15%), 9-14 points one animal (5%). SAH 90%O2 NaCl: 0 points one animal (5%), 1-2 points nine animals (45%), 3-4 points seven animals (35%), 5-8 points two animals (10%), 9-14 points one animal (5%). SAH 21%O2 NaCl: 0 points three animals (18%), 1-2 points two animals (12%), 3-4 points six animals (35%), 5-8 points five animals (29%), 9-14 points one animal (6%). Sham: all animals scored 0 (100%); Results for the second testing after 48 hours of observation: 0 points five animals (25%), 1-2 points six animals (30%), 3-4 points four animals (20%), 5-8 points three animals (15%), 9-14 points two animals (10%). SAH 90%O2 NaCl: 0 points two animals (10%), 1-2 points eleven animals (55%), 3-4 points five animals (25%), 5-8 points two animals (10%), 9-14 points no animal (0%). SAH 21%O2 NaCl: 0 points four animals (24%), 1-2 points six animals (35%), 3-4 points five animals (29%), 5-8 points one animal (6%), 9-14 points one animal (6%). Sham: all animals scored 0 (100%);
Significance after 24 hours of observation: SAH 90%O2 Oxycyte vs. Sham: p<0,001 (*), SAH 90%O2 NaCl vs. Sham: p<0,001 (*), SAH 21%O2 NaCl vs. Sham: p<0,001 (*); Significance after 48 hours of observation: SAH 90%O2 Oxycyte vs. Sham: p<0,001 (*), SAH 90%O2 NaCl vs. Sham: p=0,001 (*), SAH 21%O2 NaCl vs. Sham: p<0,001 (*); Significance between 24 and 48 hours of observation: SAH 90%O2 Oxycyte: p=0,039, SAH 21%O2 NaCl: p=0,001)
Trends: In general, an improvement of the score at 24 hours to the score at 48 hours can be seen. The SAH 90%O2 Oxycyte-group is the exception: the animals split into two groups, one getting better and one getting worse.

In summary, a higher total brain water content in the Oxycyte group compared with the control group of oxygen treatment alone could be observed (figure 35). The results from the neuroscore showed an interesting trend: After 48h 25% of the animals treated with Oxycyte scored 0 in the mNSS, which is double the quantity of the animals in the 90vol% oxygen-control in the same score category (10%), but identical to the portion of animals in the 21% oxygen-control group

without oxycyte. On the other end of the score range, in both control groups without Oxycyte (90vol% oxygen or 21vol% oxygen), the portion of animals showing worst outcome at least remained stable or even decreased at 48h compared with 24h, whereas the portion of animals in the oxycyte-treated group increased from 5 to 10% (figure 37).
Thus, animals treated with Oxycyte appear to be divided into two factions – one part recovered during the second 24 hours of the treatment, thereby ameliorating their score, and the other part further deteriorated during this period. Therefore, Oxycyte might have caused positive effects in a subgroup of animals and may have induced harm to another subgroup, the reason for which is not known so far. In contrast, both SAH-controls without Oxycyte application displayed an overall improvement of the results from the testing at 24h to the testing at 48h post-SAH. This difference was almost exclusive due to a recovery of the animals with moderate to severe damage.
In conclusion, the results from this series affirm the suspicion that the treatment with Oxycyte worsens the neurologic outcome in this setting. However, the question remains if this applies to all animals or just a fraction. Regarding the effects of the treatment with highly-concentrated oxygen, the results from this series indicate no noxiousness but rather positive effects on the outcome.

5. Discussion

5.1. Experimental design

5.1.1. Mice SAH-model

Experimental research into SAH so far basically uses two different models of haemorrhage induction in a variety of animals. (Lee et al., 2009; Prunell et al., 2003) Traditionally, animals were injected with blood into a CSF-cistern, which allows an easy control of haemorrhage volume, control-groups with saline injection and therefore an independent analysis of effects due to the presence of blood or a raised ICP. The endovascular perforation model was introduced in 1995 as an alternative procedure. (Bederson et al., 1995; Veelken et al., 1995) It is considered to better mimic the pathophysiological processes taking place after a SAH, especially the mechanisms responsible for the EBI. There is no need for a craniotomy in this model, so the skull persists as a closed system. But the haemorrhage can still be induced at a location where normally many aneurysms rupture spontaneously. Provoking a vessel perforation while the normal arterial blood pressure persists allows to simulate the direct mechanical impact to the tissue and induces the pathological mechanisms that evolve due to the rupture of the vessel and the damage to the endothelium. Therefore, the same clinical symptomatology as in humans after a SAH can be observed, ranging from delayed vasospasms to neurological dysfunction, pronounced brain oedema and similar mortality and morbidity. However, contrasted with the blood injection model, this method also brings disadvantages. Its mechanism of induction implicates a difficult control of the haemorrhage volume, thereby causing a wider variation of results and a need for bigger sample sizes. In addition, the degree of the haemorrhage depends on the filament size and the force applied to perforate the vessel. To reduce the problem of variety, in this work, all interventions were carried out only by the author. Nonetheless, significant differences between different groups regarding the operation time unfortunately could not completely be avoided. However, there were no significant differences in ICP raise and ICP maximum between the SAH-groups. Therefore, we can assume that the mice in the different groups experienced a comparable impact by the SAH. On the other side, in contrast to the SAH-groups there was no relevant ICP increase in the sham-groups, resulting in significant differences in ICP raise and ICP maximum between both groups.

Furthermore, prior to the induction of the SAH, the vessels must be clipped and the insertion of the filament may reduce the blood flow, both possibly leading to short periods of moderate hypoperfusion. In consequence, a skilled surgeon is needed in order to reduce the hypoperfusion time and results must be controlled by sham-groups undergoing the same procedure. Finally, this model does not allow to distinguish between effects caused by the blood release and the increased ICP, which, however, neither was necessary in this study. In conclusion, the endovascular perforation model became the method used in most studies, since the focus of the research into SAH changed to a better understanding of the processes responsible for EBI. (Sehba et al., 2012) Consequently, it was also chosen for the experiments in this work.

Originally, SAH-models were adapted mv≥ for mice because genetic alterations are easier to achieve. (Feiler et al., 2010) Even though this was not necessary in this case, mice were chosen for this study to allow a good comparability with other publications, since nowadays this model is widely used in the investigation into SAH. Additionally, mice offer the advantage of being relatively reasonable priced, they are easy to manage in a laboratory and they are well studied as a species. However, because of their size the intervention must be carried out microsurgically,

which again requires a skilled surgeon. Furthermore, the physiology, the neuroanatomy and the metabolism of rodents are known to be different from humans. (Sehba et al., 2012) Most importantly, there exist differences in the composition and functionality of the microcirculation. (Faraci et al., 1990) In consequence, results obtained by the study of rodents can differ from results in humans. (Carmichael, 2005)

5.1.2. Anaesthesia and Analgesia

The combination of Fentanyl, Midazolam and Medetomidine is widely used in experimental settings in rodents. Besides being secure, its advantages are a good control of the depth of anaesthesia, a quick effectiveness after injection, as well as the possibility of a prompt antagonisation with a fast recovery and resumption of food and water intake. The drawbacks of this combination, hypothermia and hypoxia, are also known for other common ways of anaesthesia and can easily dealt with by intubation and body temperature control. Especially the possibility of an antagonisation, combined with an easy and safe control of the anaesthesia and analgesia during the surgery, explains its popularity, which is why it was used in this work. (Fleischmann et al., 2016) However, there are reports of neuroprotective properties for both, the combination of the three substances (Ozden et al., 2004) as well as for each substance individually. (Chi et al., 2010; Ito et al., 1999; Jolkkonen et al., 1999) Still, the only established form of anaesthesia that is not linked to neuroprotection so far is chloral hydrate. (Ozden et al., 2004) Besides being more difficult to control, there is no efficient way of antagonisation available and only recently Huske et al. emphasised its severe systemic toxicity and therefore generally questioned the use of chloral hydrate in animal experiments. (Huske et al., 2016) Furthermore, it is not known if the combination of Fentanyl, Midazolam and Medetomidine still exerts a neuroprotective effect on the outcome after being antagonised.
The fast-acting antagonisation of Fentanyl and Medetomidine however leaves the animal rapidly without sufficient analgetic protection after recovery from anaesthesia. Therefore, in respect to animal protection, a sufficient analgesia must be guaranteed during the observation period. Buprenorphine is often used in many experimental settings due to its reliable analgetic effects, moderated by its binding to the opioid μ-receptor. However, Buprenorphine was also proven to be neuroprotective, probably by the same way of action. (Ozden et al., 2004) Thus, the analgesia was changed to Carprofen during the course of the experiments, after the consistency of the results from brain water analyses was secured. Carprofen is a nonsteroidal anti-inflammatory drug, more precisely a COX-2-inhibitor, and did not show any neuroprotection in a publication on optical nerve axotomy. (Ozden et al., 2004) Furthermore, it was shown to be as effective as Buprenorphine in preventing postoperative pain in rats. (Liles et al., 1994) Nonetheless, by the nature of its action, Carprofen has anti-inflammatory properties and, as described in the introduction, inflammation plays an important role in the pathophysiology after SAH. So, Carprofen could theoretically have neuroprotective effects in this setting, too. This is even more plausible, as Carprofen was shown to reduce cerebral damage and improve the functional recovery after traumatic brain injury in a more recent work, which has a similar pathophysiology to SAH. (Thau-Zuchman et al., 2012).
Since all substances used for anaesthesia and analgesia during the experiments can show neuroprotective effects, it cannot be excluded that the results were influenced by this. Consequently, a potential neuroprotective effect of Oxycyte might have been covered hereby. A possibility to investigate this would be to use chloral hydrate as anaesthetic, despite its consequences for animal health. However, the problem of a sufficient analgesia during the

observation period would still remain. On the other hand, patients experiencing a SAH also receive a treatment with analgetics and, in case of interventional clipping or coiling of the aneurysm, anaesthetics. Hence, the evaluation of the neuroprotective potential of PFCs together with analgetics and anaesthetics simulates a realistic handling in a clinical environment.

5.1.3. Methods

5.1.3.1. ICP

The monitoring of the ICP is crucial for a controlled SAH-induction in the endovascular perforation model. Since the vessel perforation occurs without visual control, the sudden rise in ICP is the only possibility to notice the initiation of the haemorrhage. Furthermore, the steepness and the range of increase give more information about the quality and quantity of the haemorrhage, thereby making the degree of the SAH comparable between different animals. But, to measure the ICP reliably the probe must be placed subdural, making its insertion the only moment during the intervention the brain is directly exposed to the surroundings. Hereby, brain parenchyma can be damaged, which can falsify the quantification of the brain oedema in the end. Therefore, any damage to the brain tissue caused by the probe must be avoided as far as possible. This was the intention behind the change of the measuring system towards a smaller probe in between animal series. Most importantly, consistency of results could be ensured by comparing ICP values measured with both systems.

5.1.3.2. Brain oedema

In neurologic studies the extent of cerebral oedema is often measured as it is easy to quantify and generally correlates well with the magnitude of the neuronal damage and with the severity of the haemorrhage after a SAH. (Claassen et al., 2002; Katzman et al., 1977) In this work, the extent of brain oedema was determined by measuring the brain water content. This was done by using the wet/dry-weight method, which is widely used in research for the assessment of cerebral oedema. Yet, this method is sensitive to some influences and, hence, the accuracy of the results depends on the measuring conditions. Most importantly, findings can be falsified by changing temperatures, altered humidity and air flow. (Chen et al., 2014) Consequently, these parameters must be kept constant to avoid misrepresentation. These presuppositions were considered while measuring the brain water content. The continuity of the environment was guaranteed and the brain tissue was protected from external influences by closed glass containers. However, it has to be considered that, while calculating the brain water content by the wet/dry-weight method, the influence of damage dealt by brain swelling is neglected. Since the skull is a closed system, already small changes in brain water content can entail important changes in pressure, tissue damage and swelling. (Keep et al., 2012) This might explain why results from measuring the brain water content and the neuroscore can differ between groups.

5.1.3.3. Neuroscore

The Neurologic Severity Score used in this work is an adaption for SAH of the score employed by Pfeilschifter et al. (Pfeilschifter et al., 2012) The modification was necessary as, so far, the score had been used for the evaluation of animals with ischaemic strokes. (Chen et al., 2001) The test procedure was not changed, but pathologic symptoms were valued differently. Due to the neuroanatomy, a bleeding in the subarachnoid space surrounds the whole brain parenchyma whereby damage can develop at any point. In consequence, a strictly unilateral clinical

syndrome that is typically seen after an ischaemic stroke is not characteristic for a SAH. That is why any impairment on any site was counted as pathological during the test procedure.
This version of the mNSS has some clear advantages. While it is not very time-consuming, it is not only easy to perform but does neither require elaborated instruments. At the same time, it allows to evaluate the most important motor, sensory and balancing functions, as well as reflexes and their integration among each other. However, on the other side, the scores are as well very dependent on the operator. Therefore, it is decisive that the animals are always evaluated by the same person to guarantee consistency of the results. Another drawback during the evaluation of the mice were a lack of fine discrimination in the lower scores. Slight impairments, as often present after 48h, are not sensitively differentiated by the scoring system. On the other hand, to attain a higher score animals must be severely handicapped. An important handicap for the survivors of an SAH are also cognitive deficiencies. Cognitive functions were not tested either but are difficult to assess in animals in general.

5.1.4. Experimental groups

5.1.4.1. Randomisation and blinding

Since randomisation of the experiments and blinding of the investigator are important quality criteria in research, it was tried to implement them in this study as much as the experimental design and the capacity of the laboratory made it possible.
A randomisation was granted by assigning the animals to the different treatment groups by drawing lots. By waiting with this process till the last moment possible an influence on the preceding operational procedure was excluded. However, the randomisation was limited because the assignment to different treatment groups included only groups with the same observation period at a time. Planning and coordinating a complete randomisation of all groups and observation periods would have overstressed the capacity of the author and could therefore unfortunately not be performed.
Since the randomisation took place only directly before the SAH-induction, the author was blinded during most of the operation, as the intervention was performed uniformly. However, for the procedure of inducing the haemorrhage a blinding was not possible as the surgeon realises how far the filament is advanced into the ICA and has to surveil the development of the ICP. The same applies for the procedure of intravenous drug administration. Since PFCs have to be emulsified for intravenous application (see chapter 1.2.1), Oxycyte is a lacteal, cloudy liquid that differs clearly from a saline solution. Even if the syringes would be covered externally the difference would be noticeable during the injection. Therefore, the surgeon could not be blinded during the drug administration. After the intervention, the mice were labelled to be able to distinguish them during the observation time. Even though the label gave no hint about the treatment group, a recognition of the treatment group could have been possible during the subsequent evaluation of the neuroscore which was performed by the author without further blinding measures. This could have been avoided if the neuroscore would have been evaluated by another person. Alternatively, a blinding would have been possible if the mice were brought to the author by another person for the evaluation. However, due to the small team size in the laboratory, the presence of an extra person could not be ensured at all time during the very time-consuming experiments.
In summary, due to the circumstances some minor limitations in randomisation and blinding had to be accepted in the realisation of the experiments.

5.1.4.2. Oxygen treatment

As already stated, the capacity of PFCs to carry oxygen linearly depends on the prevailing partial pressure. In consequence, to reach the maximum PFC oxygen uptake the emulsion must be ventilated with pure oxygen. For most PFCs it is known that without the ventilation of highly concentrated oxygen they fail to deliver a sufficient supply to maintain the cell homeostasis, since the resulting oxygen delivery is inferior to the one of haemoglobin. (Kim et al., 2004; Spiess, 2009) Therefore, to exploit the maximum capacity of Oxycyte, in this study mice were treated with highly concentrated oxygen. Furthermore, neuroprotective proof of Oxycyte, so far, was shown accompanied by additional oxygenation. (Woitzik et al., 2005; Zhou et al., 2008)
However, like explained earlier, beneficial effects of PFCs are not only caused by an increased oxygen delivery. For example, the facilitation of diffusion is also regarded as an important advantage of these substances. By these properties, PFCs could possibly improve tissue oxygenation even without further oxygenation. There is one study supporting this theory by showing beneficial effects of Oxycyte after spinal cord injury while animals were ventilated only with 30vol% oxygen. (Yacoub et al., 2014) For SAH, this was not tested in this study but would be interesting to explore in the future.
Regarding this issue, further investigation into Dodecafluoropentane, a PFC with unique properties, might be interesting. Due to a low boiling point, it persists in the human body in a state between liquid and gaseous thereby offering the highest oxygen carrying properties known for PFCs, even to the point of making further oxygenation unnecessary. Besides, it also offers additional advantages. It can be stored at room temperature, lower dosages are needed for sufficient oxygen supply and it does not accumulate in organs to the same degree as other PFCs. (Johnson et al., 2009) However, what makes it especially interesting for testing in SAH is its proven neuroprotective properties in ischaemic stroke without additional oxygenation. (Culp et al., 2012; Mullah et al., 2016)

5.1.4.3. Observation time

As the first series of experiments should compare the effect of an oxygen treatment alone with the data of an Oxycyte treatment from Hambauer (Hambauer, 2017), the duration of the observation period was predetermined to be 24h. From research looking into EBI it is known that most pathomechanisms are initiated during this time. Therefore, it can be expected that ischaemia already resulted in neuronal cell loss. In consequence, differences in brain tissue damage and impaired functionality should be assessable by this time.
In the following series, a 4h-interval was chosen to evaluate very early effects of the treatment. Since some mechanisms occurring after SAH were shown to evolve already minutes to hours after the haemorrhage, it seemed interesting to investigate if Oxycyte might change the outcome already in this early period. However, it has to be considered that ischaemic tissue might still be hibernating after this period and consequently cell loss might not be as pronounced. Nonetheless, differences in neuronal functionality should be assessable.
To investigate the long-term outcome after application of a low-dose treatment with Oxycyte, the time span of 48h was chosen. Thereby, most of the 72 hours in which EBI is considered to take place had passed. In contrast, animal mortality is not expected to be as elevated as after 72h, which would lead to a selection of animals with better outcome. Besides, the number of animals that needed to be operated to attain a sufficient amount of animals that could be included in the evaluation would be significantly higher. During this observation time neuronal loss and brain

oedema get sufficient time to evolve, allowing to investigate the consequences of treatment better. Furthermore, by choosing this observation period, neurologic functionality could be evaluated twice, after 24h and 48h.

5.2. Results

In the first series of experiments with an observation time of 24 hours we see significant differences in the brain water content and in the neuroscore between the SAH group that received an oxygen treatment and the sham group. We can see the same differences between the SAH group without oxygen treatment and the sham group, although not always that clearly and significantly. This effect would probably become more clearly with a bigger sample size. In the third series of experiments with an observation time of 48 hours we see the same significant differences between the SAH group treated with Oxycyte and the sham group, as well as between the SAH control group treated at ambient air and the sham group. With regard to the SAH control group receiving an oxygen treatment we see a significant difference in comparison to the sham group only for the total brain water content and the neuroscore but not for the individual hemispheres. Hence, there are significant differences as well but not as pronounced as for the other two SAH groups. In conclusion, effects of the operation procedure on the results, besides the SAH-induction, can be excluded as far as possible.
Although not significant, the evaluation of the early period after the SAH pointed in the direction of a slightly better outcome in animals treated with Oxycyte during the first 4h. This might be the case because the tissue oxygen tension was shown to be most seriously decreased in the first hours after a SAH. (Westermaier et al., 2009b) So, during this time the positive effects of an increased free oxygen delivery could outweigh the detrimental ones. In contrast, in the long run the results from the 24 hours and 48 hours observation periods indicate that in a part of the animals the treatment with Oxycyte has worsened the outcome compared to a sole treatment with highly concentrated oxygen. However, the outcome of the other part of mice who received the Oxycyte treatment tended to better. Interestingly, the animals who improved their neuroscore from the first to the second rating were those with a neuroscore of less or equal to 4 after 24 hours. Consequently, in animals with only slight brain damage Oxycyte seemed to be able to better the outcome, while in those severely hit by the SAH its impact is detrimental. The question is, why did the treatment with Oxycyte not only fail to improve the outcome in more severely hit animals but worsen it?

5.2.1. Neuroprotection of PFCs

By looking through literature some findings evoke doubts regarding the neuroprotective effects of PFCs.
For example, in a study investigating the effects of PFCs on brain damage after transient artery occlusion neuroprotective qualities could only be found after short-time observation but were not verifiable on longer terms. (Kolluri et al., 1986) However, these experiments were undertaken with Fluosol, a first-generation PFC. In contrast, the neuroprotective effects of Oxycyte still were persistent after two weeks after traumatic brain injury. (Zhou et al., 2008)
Between studies there exist also different ways of drug application. While in this work Oxycyte was applied by a single shot injection, other investigators have used a slow infusion for the application of a PFC. In a direct comparison of these two ways of application a rapid infusion of

the substance initially decreased the cerebral concentration of oxygenated haemoglobin, whereas during and after a slow infusion this effect was not observed. (Davies et al., 2013) Still, the decline in oxygenated haemoglobin was only present for a few minutes and thereafter led to an increase in brain oxygenation, like in the animals who were infused slowly. But, during the first minutes this effect could be responsible for additional hypoxia.
Nonetheless, these observations are not very likely to be responsible for the unfavourable outcome found in this work. First of all, as stated above, long-lasting neuroprotective effects for Oxycyte were proven. Secondly, Oxycyte did also show neuroprotection in publications where the substance was applied by a single injection. (Woitzik et al., 2005) And finally, if these mechanisms were the reason for the adverse outcome, one would expect their influence to be present continuously, though maybe not affecting groups with different observation time equally but at least the subjects within one series of experiments.

5.2.2. Adverse effects of PFCs

As already mentioned in chapter 1.2.1., even if PFCs are generally considered to be safe they can influence physiological processes in a way that could lead to detrimental side effects.
Firstly, there have been reports of systemical adverse effects for PFCs constantly, also including newer substances. Amongst others, this includes impairment of different mechanisms of the coagulation (Cronin et al., 2016; Spahn et al., 1999), the generation of hypertension and ileus disease (Spahn et al., 2002), reports of an increased number of strokes (Palmer et al., 2014) and flu-like symptoms as headache and dizziness (Leese et al., 2000). Even though most of these side effects were not regarded as severe, they could have had a decisive influence on an organism already struggling with the impact of a SAH.
Secondly, the good solubility of PFCs for gases also includes nitric oxide. Therefore, once the substance is present in the damaged brain region, it efficiently absorbs the available nitric oxide and thereby takes it away from vasomotoric mechanisms in the endothelium and smooth muscle cells. (Rafikova et al., 2004) The consequence is a resulting vasoconstriction which was shown to last for about 30 minutes after the infusion of a PFC. (Ortiz et al., 2013) Thus, during this time the perfusion can be insufficient and cause ischaemia. Furthermore, this effect was shown to be dose-dependent, as higher dosages of PFCs produce a more rapid nitric oxide scavenging and a more pronounced vasoconstriction. (Rafikova et al., 2004) Hence, the concentration of Oxycyte could have been too high to let the positive effects prevail.
Thirdly, under physiological conditions PFCs cannot cross the blood-brain barrier. (Ahrens et al., 2011) However, after the haemorrhage the BBB gets rapidly and diffusely destroyed, therefore possibly allowing PFC molecules to penetrate into the brain tissue. Although so far it is not known how the cell homeostasis gets affected by this, the direct contact to the substance could possibly induce a variety of deleterious processes. Potential influences could be an aggravation of the existent inflammation and an impairment of the cell metabolism or the intercellular communication.
And fourthly, PFCs were shown to facilitate the transformation of methaemoglobin to oxyhaemoglobin. (Kozlova et al., 2016) As already mentioned, oxyhaemoglobin plays an important role in a variety of mechanisms involved in the SAH-pathophysiology and contributes to their destructive effects. Hence, an increase in its concentration would probably lead to an enhancement of these mechanisms.
Anyway, similar to the previous chapter, if these mechanisms would be responsible for the worse outcome after the treatment with Oxycyte one would expect all animals to be affected by them.

What concerns the depletion of NO, the disruption of the BBB and the increased concentration of oxyhaemoglobin, all of these mechanisms are activated early after a SAH. Therefore, if they were responsible for the negative impact their effect should be explicit after 4 hours and not the opposite. While systemical side effects could well affect animals that are more severely struck by the haemorrhage particularly, one would expect to observe rather an increased mortality than marked differences in the neurologic functionality. Additionally, systemic side effects are not reported consistently and especially not in a sufficient quantity to explain the worse outcome after exposure to Oxycyte in this work.

5.2.3. Oxygen and PFCs

In most publications that showed neuroprotection for a treatment with PFCs, the animals were ventilated with highly concentrated oxygen in addition to the PFC-injection in order to increase the brain oxygenation. As already stated, neuroprotective effects of PFCs were found for neurologic diseases like ischaemic stroke, traumatic brain injury and spinal cord injury. Since the author was not able to reproduce this effect after SAH, the question arises in which detail this disease differs from the others.
The most prominent and apparent difference between SAH and both, ischaemic stroke and traumatic brain injury, is the high amount of fresh blood the brain tissue is exposed to during the haemorrhage. As already stressed in chapter 1.1.3., consequences of the release of fresh blood are an increased formation of free radicals and a greater extent of oxidative stress. Both are important mechanisms participating in EBI. Dependent on the volume of the haemorrhage, this process can even be as pronounced as to rapidly exhaust the intrinsic radical defence systems. This was proven in animals (Marzatico et al., 1993) as well as in humans (Gaetani et al., 1997). Additionally, hyperoxia is also known to potentially promote oxidative stress and, thereby, to induce tissue damage by oxygen toxicity. (Buonocore et al., 2010) Consistently, in an analysis of hyperoxia treatment to high-grade SAH-patients the treatment significantly increased the rate of DCI and resulted in worse outcome. (Jeon et al., 2014) Still, increasing the oxygen supply to ischaemic cells by raising the brain tissue oxygenation was the therapeutic strategy behind the PFC-treatment in this work. However, while an increase in oxygen partial pressure and an augmented amount of free oxygen can be achieved, oxygen toxicity and free radical formation is promoted as a secondary effect. (Daugherty et al., 2004; Demchenko et al., 2012) In conclusion, whether or not the radical defence systems become exhausted after a SAH depends on the quantity of radial production, hence on the severity of the haemorrhage. Once the radical defence systems are exhausted a further promotion of oxidative stress by PFCs has the potential to increase the damage to the brain tissue.
There is further evidence that the damaging effect of PFCs might be due to an increased radical formation. In a study of PFC-treatment on temporary middle cerebral artery occlusion Pereira et al. were able to counteract detrimental effects of the PFC-treatment by the additional application of mannitol (Pereira et al., 1988), a substance which is known for its radical scavenging properties (Larsen et al., 2002). Likewise, Liu et al. could reverse damage from a PFC-application under hyperbaric oxygen conditions by the administration of the radical scavenger Edavarone. (Liu et al., 2012) Furthermore, as displayed in chapter 1.1.3., oxidative stress impairs the regulation of the vascular tone. The endothelium and smooth muscle cells get damaged by the radical species and the vessel's response to vasoconstrictive agents intensifies. By these mechanisms, oxidative stress causes sustained vasoconstriction. Interestingly, a combination of Oxycyte and highly-concentrated oxygen treatment was found to potentially induce a long-lasting decrease of CBF

by around 20% (Demchenko et al., 2012), which could be explained by impaired vascular regulation due to radical formation. Accordingly, hypoperfusion and ischaemia can result.
In conclusion, the differences in the outcome for animals with light and severe damage after the treatment with Oxycyte could be explained by a different degree of exhaustion of the endogenous radical scavenging systems. Consequently, in brains that are only lightly damaged antioxidant systems would not be fully consumed by radicals arising from the pathomechanisms active after a SAH but would still be capable to counteract additional radical creation resulting from increased oxygen levels. In this scenario, the positive consequences of a treatment with Oxycyte would outweigh its negative implications. In contrast, in severely damaged brains initial radical creation would already completely exhaust protective mechanisms, leaving the cells defenceless to further damage from oxidative stress created by hyperoxia.
This hypothesis is further supported by recent publications. As specified in chapters 1.1.3.2.4. and 1.2.3., HIF-1α is an important mediator of cell death due to ischaemia and was found to be down-regulated when PFCs exert neuroprotection. Anyway, the formation of radicals and the creation of oxidative stress were shown to be indispensable for the upregulation of HIF-1α. (Guzy et al., 2005) In 2015, Zhang et al. were able to successfully reduce EBI by a treatment with a PFC in a study with 24 hours of observation. (Zhang et al., 2015) This included decreased levels of brain oedema and BBB disruption, less neuronal cell death and improved neurologic functionality. A year later, the same group showed this improvement was due to a downregulation of HIF-1α and its target genes. (Xu et al., 2016) However, we do not know if this neuroprotection is the effect of less radical production. Still, this might be the most probable explanation, since one important difference in the study design was that animals were not further oxygenated after the injection of the PFC. Instead, the PFC emulsion was loaded with oxygen by aeration prior to the infusion. This could result in a less sustained hyperoxia and consequently less radical formation as well. At the same time possible detrimental effects of a treatment with PFCs without any oxygenation, as, for example, NO-scavenging, can be avoided by this design.
Besides, there are some other interesting details this work and the study from Zhang et al. differ in. Instead of perfluoro-tert-butylcyclohexane, their PFC emulsion was based on perfluorooctyl-bromide, which can be formed into nanoparticles half in size and has the property of an increased oxygen carrying capacity. As already stated above, the emulsion was oxygenated prior to the injection without further application of oxygen afterwards. If the degree of radical formation is responsible for the antipodal outcomes, this seems to be an important difference. Moreover, rather than injecting the substance directly after finishing the induction of the SAH, the substance was applied only 1 hour later. However, If the time point of the PFC-application results in a difference of the neuroprotective capacity and by which mechanisms are not known and might be interesting to be investigated in the future. And finally, Zhang et al. used chloral hydrate as anaesthetic, without further application of an analgetic drug during the observation time. As discussed in 4.1.2., this solves the trouble of a possible neuroprotection by anaesthetics and analgetics. However, leaving the animal without sufficient analgesia during the observation time is ethically doubtable and would not be approved by most ethical committees. Nevertheless, under certain circumstances PFCs seem to have neuroprotective effects in SAH.

5.3. Future prospects

The research on the treatment with PFCs in SAH is still in an early development and there is a lot more to explore. From what we know so far possible modifications to already existing treatments can be deduced that would be interesting to investigate.

To begin with the setup of the experiments in this work, there is theoretically the option of adding a treatment against the oxygen toxicity. The simplest way to do so would be the application of a radical scavenger together with the PFC. Thereby, the formation of further radicals would be blocked while increased oxygen levels could still counteract the ischaemia. Radical scavengers have been tested as unique treatments in ischaemic stroke (Isahaya et al., 2012) and SAH (Munakata et al., 2009) and showed beneficial effects regarding the outcome. However, to the authors knowledge, radical scavengers have not been investigated in the combination with PFCs as a treatment after SAH so far. With regard to the newest findings mentioned above, PFCs might not cause damaging levels of oxygen radicals if applied without the additional creation of a sustained hyperoxia. But radical scavengers could still add a further protective effect.

Another option of expanding the treatment with PFCs involves NO. In chapters 1.1.3. and 1.2.1. the central role of NO in the pathophysiology of SAH was emphasized and PFCs were identified to be able to dissolve and carry it. The possibility of pre-loading PFC-particles with NO and the influence on the organism was studied by Ortiz et al. (Ortiz et al., 2013) In their experiments an injection of a PFC without preloading with nitric oxygen produced a vasoconstriction lasting 30 minutes. During this time, the tissue is at risk of hypoperfusion and ischaemia. In contrast, a PFC-injection pre-loaded with NO resulted in vasodilation. In addition, they found that PFC-micelles created an environment for oxygen and NO to efficiently react to dinitrogen trioxide and other molecules that lead to vasorelaxation. Furthermore, if PFCs are pre-loaded with NO, they no longer scavenge the NO released from the endothelium and smooth muscle cells but can rather supply them in order to assure physiological vasoregulation. Since due to its short living a pre-load with NO would only offer a temporary supply, Ortiz et al. also proposed the idea to combine this treatment with the continuous application of other NO-releasing substances. (Ortiz et al., 2013)

Avoiding radical formation seems to be the key for neuroprotection after a PFC-treatment in SAH. As already mentioned in 4.1.4.1., by now, newer substances are available that can increase brain tissue oxygenation without further oxygen application, just by breathing air. (Mullah et al., 2016) By using these substances, the risk of radical formation can be reduced and resulting oxygen toxicity and vasoconstriction possibly avoided. Therefore, these newer PFCs offer an interesting perspective for the treatment of EBI and the prevention of DCI. Nonetheless, it might still be interesting to investigate the effects of Oxycyte without additional oxygenation as well, since this setting reduced cell death in a model of spinal cord injury when it was combined with an inspirational oxygen fraction of only 30vol%. (Yacoub et al., 2014)

Finally, there are also interesting possibilities arising regarding the way of application of the PFC. Only recently, Yao et al. successfully applied a PFC through the lung. (Yao et al., 2015) This was achieved by encapsulating the PFC into a nanocarrier, a substance class that was already used for the application of other substances via the lung. Most importantly, in an in-vivo experiment in rats this way of application resulted in a systemic availability of the PFC. Besides being less complicated than the application via intravenous injection, this route of drug delivery offers important advantages. Firstly, it is non-invasive. Secondly, the lung offers a large surface for absorption, which eases the uptake. Thirdly, Yao et al. also found a high systemic bioavailability with a rapid onset of the therapeutic action. Fourthly, the dosage can probably be reduced, since the PFC gets distributed directly systemically by avoiding the first-pass metabolism in the liver. (Yao et al., 2015) These properties turn the application via the lung into a reliable but most importantly safe and painless way of application.

The possible fields of investigation seem to grant PFCs a promising future in the search for a treatment to reduce morbidity after a SAH.

6. Conclusion

Taking everything into account, there can be drawn three important conclusions from this work. Firstly, combining a PFC-injection with the ventilation with highly-concentrated oxygen as a treatment after a SAH shows the tendency to worsen the outcome of the animals that are already more severely affected by the consequences of the haemorrhage in comparison to the treatment with highly-concentrated oxygen or no treatment. In contrast, the animals with less severe damage from the SAH seem to benefit from the PFC-treatment. The most probable explanation is a difference in the functionality of radical scavenging systems caused by a different degree of radical production after the haemorrhage which correlates with the quantity of the blood liberated. Other authors have found a neuroprotective effect for PFCs if injected without subsequent oxygenation. Therefore, it can be concluded that a continuous hyperoxia after a SAH probably leads to more radical production and worsens the outcome.
Secondly, while Oxycyte might already be neuroprotective without additional oxygenation, there are newer substances that possess improved gas transporting properties that make additional oxygenation no longer necessary to increase the brain oxygenation. The additional treatment with NO or radical scavengers might result in further advantages. Furthermore, the absorption through the lung is a possible future route of administration with many advantages.
And thirdly, currently available, sufficient and easy controllable anaesthetic and analgesic regimes imply neuroprotection. Consequently, in order to differentiate the neuroprotective effect dealt by every substance applied, control groups must be chosen wisely and adequately.

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Danksagung:

Mein Dank gilt meiner Doktormutter Frau Prof. Dr. Ute Lindauer für die Betreuung, Beratung und Unterstützung bei der Planung, Durchführung und Auswertung meiner Experimente und der Verfassung meiner Dissertation. Dank ihrer freundlichen und nahbaren Art hat sie stets ein familiäres Arbeitsklima geschaffen und war auch kurzfristig bei Fragen und Problemen zu erreichen. Gleichzeitig habe ich durch ihre konstruktive Kritik in dieser Zeit viel über theoretische und praktische Wissenschaft gelernt.

Weiterhin möchte ich Svetlana Lachmann danken. Als gute Seele des Labors hatte sie bei allen Höhen und Tiefen ein offenes Ohr und hat mich und die anderen Mitarbeiter nach Kräften unterstützt und dabei niemals die Geduld verloren. Ebenso möchte ich mich bei Irina Eskina bedanken, die ebenfalls jederzeit bei Fragen und Problemen tatkräftig zur Seite stand.

An viele schöne Momente im Labor kann ich mich aber auch Dank meiner Mitdoktoranden erinnern. Für die freundliche, kollegiale und gelegentlich auch sehr lustige Atmosphäre möchte ich ihnen sehr danken. Ein spezieller Dank geht dabei an Sebastian Hambauer für die geduldige Einarbeitung in die doch komplexe Operationstechnik, aber auch an Andreas Bauer und Fritz Schneider für die zahlreichen Stunden die wir zusammen verbracht haben.

Eine wichtige Unterstützung waren auch die zahlreichen wichtigen Freunde die mich über die Jahre begleitet haben. Ob durch Anregungen zur Methodik, gemeinsame Stunden in der Bibliothek, konstruktive Kritik und Korrekturen von Text und Abbildungen oder einfach zur Ablenkung und Entspannung – ihnen allen bin ich außerordentlich dankbar, dass sie mich auf dem Weg begleitet und bei den verschiedenen Etappen unterstützt haben.

Nicht zuletzt bin ich auch meiner Familie zu großem Dank verpflichtet. Meinen Eltern, dass sie mich zu dem Menschen erzogen haben der ich bin, mir kritisches Denken gelehrt haben und mich gleichzeitig in schwierigen Phasen aufgefangen und unterstützt haben, und meinen Brüdern, die ebenfalls immer für mich da waren, wenn ich sie gebraucht habe. Ich weiß ihre Unterstützung sehr zu schätzen und bin froh über unseren gemeinsamen Zusammenhalt.