Impact of anesthesia on pathophysiology and mortality following subarachnoid hemorrhage in rats

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Abstract
Background: Anesthesia is indispensable for in vivo research but has the intrinsic potential to alter study results. The aim of the current study was to investigate the impact of three common anesthesia protocols on physiological parameters and outcome following the most common experimental model for subarachnoid hemorrhage (SAH), endovascular perforation.

Methods: Sprague-Dawley rats (n = 38) were randomly assigned to (1) chloral hydrate, (2) isoflurane or (3) midazolam/medetomidine/fentanyl (MMF) anesthesia. Arterial blood gases, intracranial pressure (ICP), mean arterial blood pressure (MAP), cerebral perfusion pressure (CPP), and regional cerebral blood flow (rCBF) were monitored before and for 3 hours after SAH. Brain water content, mortality and rate of secondary bleeding were also evaluated.

Results: Under baseline conditions isoflurane anesthesia resulted in deterioration of respiratory parameters (arterial pCO2 and pO2) and increased brain water content. After SAH, isoflurane and chloral hydrate were associated with reduced MAP, incomplete recovery of post-hemorrhagic rCBF (23 ± 13% and 87 ± 18% of baseline, respectively) and a high anesthesia-related mortality (17 and 50%, respectively). Anesthesia with MMF provided stable hemodynamics (MAP between 100-110 mmHg), high post-hemorrhagic rCBF values, and a high rate of re-bleedings (> 50%), a phenomenon often observed after SAH in humans.

Conclusion: Based on these findings we recommend anesthesia with MMF for the endovascular perforation model of SAH.

Keywords: Subarachnoid hemorrhage, Rat, Experimental, Anesthesia, Isoflurane, Chloral hydrate, Medetomidine, Brain edema, Cerebral blood flow, Re-bleeding

Background
Experimental animal models are widely used to study the pathophysiology of subarachnoid hemorrhage (SAH) [1-4]. The induction of experimental SAH is performed by invasive surgical techniques which require continuous multimodal monitoring and adequate sedation and analgesia. Ventilation and, hence, blood gases can be controlled by intubation and mechanical ventilation [5], however, other parameters which are known to critically determine the outcome following experimental SAH such as intracranial pressure (ICP), mean arterial blood pressure (MAP) and regional cerebral blood flow (rCBF) [1,2,6-9] may well be influenced by the applied anesthetic protocol as already demonstrated for experimental models of cerebral ischemia [5] and traumatic brain injury [10,11].

Various inhaled or injectable anesthetics have been used in experimental SAH, e.g. halothane, isoflurane [alone or in combination with nitrous oxide (N2O)] [1,3,4,12,13], chloral hydrate, barbiturates [14-16], ketamine/xylazine or midazolam/medetomidine/fentanyl (MMF) [17-19]. Although experimental SAH models are known to suffer from some variability [2,3,9], little is known to which degree the used anesthetic protocol contributes to this phenomenon.
Therefore, we investigated the effect of three well-established and frequently used standard anesthesia protocols, namely chloral hydrate, isoflurane, and MMF on animal physiology, mortality and brain water content before and after experimental SAH in rats. The ultimate aim of the study was to determine how anesthesia influences the outcome of experimental SAH and, if significant differences are found between different anesthesia protocols, which protocol has the least influence on the pathophysiology induced by the endovascular perforation model, the most frequently used and the presumably most clinically relevant model of subarachnoid hemorrhage.

Methods
In this study we used 38 male Sprague-Dawley rats (250-300 g body weight), purchased from Charles River Laboratories, Sulzfeld, Germany. Eight animals died during experiments (see below), i.e. 30 animals were included in the final analysis. Animals had free access to pellet food until 12 hours prior to surgery. Water was accessible at all time. All experiments were approved by the Ethics Committee of the District Government of Upper Bavaria, Germany.

Anesthesia
Anesthesia protocols were chosen based on the frequency of their use with the endovascular perforation model of SAH (e.g. isoflurane), on their availability (e.g. halothane was not included in the analysis since its production for human use was discontinued resulting in a reduced availability for many laboratories), on their known small influence of cerebral blood flow (chloral hydrate and midazolam / medetomidine / fentanyl), on the ease of their termination (isoflurane and midazolam / medetomidine / fentanyl), and on their level of standardization in experimental and veterinary medicine (all three protocols).

Dose regiments were based on the scientific and veterinary literature and our own experience during the past 5 (midazolam / medetomidine / fentanyl) to 20 (chloral hydrate and isoflurane) years. A similar level of sedation between the three protocols was achieved by using the lowest dose necessary to reach surgical anesthesia (as verified by the tail pinch test and concomitant observation of withdrawal reflexes and systemic blood pressure).

Anesthesia was induced by placing animals in a chamber with 4% isoflurane for 2-3 minutes. Thereafter rats were randomized to one of the three standard anesthesia protocols: (1) Chloral hydrate (1 ml/100 g body weight of a 3.6% solution) was injected intraperitoneally (i.p.). Animals were intubated and ventilated with 70% air and 30% O₂ under control of ventilation pressure and rate (Small animal ventilator KTR-4, Hugo Sachs Elektronik, Germany). A third of the initial dosage was applied every 60 minutes in order to maintain anesthesia. (2) Animals were ventilated with 2% isoflurane in 70% N₂O and 30% O₂. (3) Midazolam (2 mg/kg), medetomidine (0.15 mg/kg) and fentanyl (0.0075 mg/kg) were given by intraperitoneal injection. Animals were ventilated with 70% air and 30% O₂. A third of the initial dosage was applied for maintenance of anesthesia every 40 minutes. Atropine (150 mg/kg bodyweight) was injected subcutaneously in all experimental groups to inhibit salivary secretion and induce bronchodilation.

Monitoring
The tail artery was canulated for continuous measurement of mean arterial blood pressure (MAP) and for blood sampling throughout the experiment. Arterial blood gases, pH, serum glucose and lactate were analyzed every 30 minutes. Ventilation rate (in ventilations per minute = vpm) and pressure were adapted in order to maintain physiological arterial pCO₂ values (35-45 mmHg). Temporal muscle and rectal probes were used to monitor brain and body temperature, respectively. A thermostatically regulated, feedback-controlled heating pad and lamp were used to maintain rectal and brain temperature at 37°C.

Intracranial pressure (ICP) was continuously measured using a Codman ICP microsensor (Johnson & Johnson Medical Limited, Berkshire, UK). After drilling a burr hole over the right parietal cortex under constant cooling, the probe was advanced 2 mm into the brain and fixed with dental cement. Cerebral perfusion pressure (CPP) was calculated using the following formula: CPP = MAP - ICP.

A 2-channel laser-Doppler flowmeter (LDF; MBF3D, Moor Instruments Ltd.) was used for continuous monitoring of regional cerebral blood flow (rCBF) in the territory of the middle cerebral artery (MCA) of both hemispheres as previously described [4,13].

Induction of SAH
SAH was induced by endovascular puncture, one of the most widely used models for experimental SAH [1-3], as previously described [4]. Briefly, a 3-0 monofilament was advanced via the external carotid artery (ECA) into the internal carotid artery (ICA) until increase of ICP and bilateral decrease of rCBF indicated SAH. Subsequently, the suture was withdrawn and the ECA ligated.

Experimental groups
In a first series of experiments blood gases, MAP, ICP, CPP and rCBF were continuously monitored for 3 hours under physiological conditions in all three experimental groups (n = 5 each). Afterwards animals were sacrificed for quantification of brain water content. In a second series animals were randomly assigned to one of the three anesthesia groups (n = 5 each) and SAH was induced. All
physiologic parameters were recorded under baseline conditions and continuously over 3 hours after SAH. At the end of observation time anesthesia was terminated and animals were tested for signs of awakening, i.e. adequate spontaneous respiration. Animals that died during surgery or exhibited excessive re-bleedings were replaced until the final group size was n = 5 in all groups. Subsequently, animals were re-anesthetized and sacrificed for quantification of brain water content.

Quantification of brain water content
Brains were removed and the hemispheres were separated and weighed to assess their wet weight (WW). Thereafter, the hemispheres were dried for 24 h at 110°C and their dry weight (DW) was determined. Hemispheric water content (%) was calculated using the following formula: ((WW-DW)/WW) × 100.

Statistical analysis
Statistical analysis was performed with SigmaStat 3.1 (SPSS Science Inc., Chicago, IL, USA). Blood gas parameters were analyzed with Kruskal-Wallis - ANOVA on ranks followed by Student-Newman-Keuls as post hoc test, and for physiologic parameters ANOVA on ranks/ Dunn’s Method was applied. Two points in time of the same group were compared with Friedman - repeated measures ANOVA on ranks. Statistical significance of results was assumed at p < 0.05 and a power of > 0.8. Data are presented as means ± SEM if not otherwise indicated.

Results
All three anesthesia protocols provided sufficient sedation and analgesia for surgical intervention - the animals showed no signs of untimely awakening or discomfort at any time. Pinching of the hind paw did not trigger a withdrawal reflex or an increase in blood pressure.

Ventilation rate and blood gases
Under physiological conditions anesthesia with isoflurane required a significant increase of ventilation rate during the 180 minutes of observation to keep PaCO₂ within normal limits (58 ± 2 vpm vs. 27 ± 1 vpm in rat anesthetized with MMF; p < 0.05; Table 1).

After SAH induction, anesthesia with isoflurane and chloral hydrate required an increase of the ventilation rate that reached statistical significance (p < 0.05) compared to MMF after 180 minutes (47 ± 3, 42 ± 4, and 34 ± 3 vpm, respectively) (Table 1). Ventilation pressure was not different between groups (12.6 ± 0.2, 12.7 ± 0.2 and 12.6 ± 0.4 cmH₂O for chloral hydrate, isoflurane and MMF, respectively).

Arterial pCO₂ and pH were not significantly different between the three anesthesia protocols under physiological conditions or after SAH induction. Arterial pO₂ in the isoflurane group, however, decreased significantly during the course of the experiment (p < 0.05) and was significantly lower compared to chloral hydrate and MMF (p < 0.05) after 60 and 180 minutes under physiological conditions (116.0 ± 6.3, 89.6 ± 2.8, and 135.6 ± 8.8 mmHg for chloral hydrate, isoflurane and MMF, respectively). Accordingly, in SAH groups a decline in arterial pO₂ was observed for isoflurane (p < 0.05), with values significantly different from the chloral hydrate and MMF group at 60 minutes after SAH (Table 1).

ICP, MAP, CPP, and rCBF
There was no significant difference in ICP (values around 5 mmHg) between the three groups under physiological conditions. SAH resulted in an immediate increase in ICP to a peak of 42 ± 8, 28 ± 4 and 61 ± 17 mmHg in the chloral hydrate, isoflurane and MMF group, respectively (Figure 1A). This peak was followed by a plateau of 15-20 mmHg that was reached after 15 minutes in all groups.

Under physiological conditions animals subjected to MMF anesthesia exhibited a significantly (p < 0.05) higher MAP (~100 mmHg) compared to the groups anesthetized with chloral hydrate (~80 mmHg) or isoflurane (~70 mmHg). After SAH MAP remained significantly (p < 0.05) elevated in the MMF group (Figure 1B). Of note, the sudden rise in ICP after SAH triggered a Cushing reflex in MMF anesthetized animals: MAP acutely increased by ~20 mmHg (from 98 ± 7 to 118 ± 12 mmHg) and returned to baseline values 15 minutes later. In animals anesthetized with chloral hydrate or isoflurane, however, this expected physiological increase in blood pressure was not observed. On the contrary, MAP actually dropped by 20 mmHg and recovered only slowly thereafter (Figure 1B) indicating that chloral hydrate and isoflurane interfere with physiological blood pressure regulation.

Due to the higher MAP under physiological conditions, CPP was significantly (p < 0.05) higher in MMF anesthetized rats (~75 mmHg) compared to those animals which received chloral hydrate (~75 mmHg) or isoflurane (~65 mmHg). Following SAH, CPP decreased by ~30-45 mmHg in all experimental groups. In the MMF group, CPP returned to near baseline values of 88 ± 7 mmHg within 5 minutes after SAH. For chloral hydrate and isoflurane a constantly reduced CPP was observed after SAH (51 ± 7 and 41 ± 3 mmHg, respectively; p < 0.05 vs. MMF).

Anesthesia with chloral hydrate or MMF did not increase rCBF during the 3 hour observation time (7 ± 13% and 5 ± 6% rCBF increase vs. 30 min after initiation of anesthesia, respectively), while in isoflurane anesthetized rats rCBF increased by over 1/3 (35 ± 22% rCBF increase vs. 30 min after initiation of anesthesia).
In all animals SAH resulted in a sharp decline of ipsilateral rCBF ($p < 0.05$) to ~10-30% of baseline (Figure 2). No recovery was observed in the isoflurane group while in MMF and chloral hydrate anesthetized rats rCBF recovered to almost baseline values (92 ± 13% and 87 ± 18%, respectively). Contralateral rCBF recovered within the 3 hours observation period in all groups.

Brain water content (BWC)
Following 4.5 hours of anesthesia, brain water content was in the physiological range in the MMF group (78.8 ± 0.1%) but increased in animals anesthetized with chloral hydrate or isoflurane ($p < 0.05$; 79.5 ± 0.1% and 79.6 ± 0.1%, respectively; Figure 3) suggesting either opening of the blood brain barrier or, more likely, increasing cerebral blood volume due to anesthesia-induced vasodilation.

Three hours after induction of SAH brain water content was 79.5 ± 0.2%, 80.1 ± 0.4%, and 78.9 ± 0.1% in the chloral hydrate, isoflurane and MMF groups, respectively. SAH did not result in an increase in brain water content in chloral hydrate and MMF anesthetized animals ($p < 0.05$ vs. MMF; Figure 3).

Mortality and re-bleeding after SAH
There was no mortality during surgical preparation and sham operated animals did not show any mortality.

After SAH, animals anesthetized with chloral hydrate had the highest rate of intraoperative mortality (50%) followed by animals anesthetized with MMF (29%) and isoflurane (17%, Figure 4A).

Significant re-bleedings, which were identified by a recurrent ICP increase of > 20 mmHg, were detected in chloral hydrate (1/10, 10%) and MMF (4/7, 57%) anesthetized animals, in the latter contributing to the mortality in this group (Figure 4B). In total 8 out of the 38 animals used for the current study died prematurely resulting in n = 30 animals being included in the final analysis.

Discussion
In the present study we evaluated the effect of three widely used and well standardized anesthesia protocols on pathophysiology and outcome following subarachnoid hemorrhage (SAH) in rats. Surprisingly, different anesthesia protocols resulted in completely different pathophysiologies following SAH. Isoflurane and chloral hydrate anesthesia resulted in low blood pressure, lack of the expected physiological post-ictal Cushing response, impaired recovery of post-hemorrhagic ischemia and no re-bleedings, while a combination of midazolam, medetomidine and fentanyl (MMF) maintained physiological blood pressure, allowed the rising ICP to trigger the expected increase in systemic blood pressure (Cushing reflex), resulted in complete recovery of post-hemorrhagic cerebral blood flow and caused a significant number of re-bleedings, an important characteristic of SAH in humans.

Ventilation and arterial blood gases
Most agents used for small animal anesthesia cause a dose dependent depression of central respiratory activity.
leading to deterioration in arterial blood gases [20,21]. Therefore, particularly with longer duration experiments, intubation and mechanical ventilation is used to keep blood gases within physiological limits [5]. Animals in the isoflurane group experienced a gradual decrease in arterial pO2 (blood oxygenation) and, similarly to chloral hydrate, an increase in arterial pCO2 that required continuous adjustments of ventilation parameters. Airway irritation and impaired mucociliary clearance have previously been described for inhalative agents like isoflurane and halothane [22,23] and most probably account for the reduced gas exchange and deterioration in blood gases. Keeping arterial pCO2 within physiological limits is particularly critical following pathological conditions, as carbon dioxide is not

![Figure 1](image1.png)

**Figure 1** (A) Time course of ICP until 180 minutes after SAH. No significant differences were observed between groups. (B) Time course of dynamic MAP changes until 180 minutes after SAH. MAP was significantly higher before and after SAH when animals were anesthetized with MMF as compared to chloral hydrate or isoflurane. SAH induced a Cushing response (transient increase of MAP due to increased ICP) in animals anesthetized with MMF while no such response was observed in the isoflurane or chloral hydrate groups. ICP and MAP were recorded continuously, averaged over 3 minutes, and displayed as means ± SEM (n = 5 in each group; *p < 0.05 vs. isoflurane, #p < 0.05 vs. chloral hydrate; Kruskal-Wallis ANOVA on ranks followed by Dunn’s post-hoc test).

![Figure 2](image2.png)

**Figure 2** Regional cerebral blood flow of the hemisphere ipsilateral to hemorrhage measured by laser Doppler fluxmetry until 180 minutes after SAH. SAH resulted in an immediate reduction of rCBF in all groups with a significantly better recovery in the MMF group (*p < 0.05 vs. isoflurane). rCBF was recorded continuously, averaged over 3 minutes, and displayed as percentage (%) of pre-hemorrhagic baseline (means ± SEM; n = 5 in each group; Kruskal-Wallis ANOVA on ranks followed by Dunn’s post-hoc test).

![Figure 3](image3.png)

**Figure 3** Brain water content (BWC) 3 hours following SAH or in respective control animals. Streaked gray area indicates the range of normal brain water content in animals not subjected to long-term anesthesia. BWC is within the normal range under MMF anesthesia while it is elevated in rats anesthetized with isoflurane (p < 0.05) or chloral hydrate (Control). Following SAH BWC increased only in animals anesthetized with isoflurane while it remained unchanged in animals receiving chloral hydrate or MMF. Values are presented as mean ± SEM for n = 5 in each group, *p < 0.05 vs. isoflurane (Kruskal-Wallis ANOVA on ranks followed by Dunn’s Method).
only a potent regulator of cerebrovascular tone [24] but hypercapnia and subsequent acidosis are known to increase ischemic brain injury [25,26]. Also, hypoxemia, a symptom of respiratory distress which is frequently observed after SAH [27,28], has been shown to increase the likelihood of a poor outcome [29-31]. In contrast to isoflurane and chloral hydrate the MMF regimen did not affect ventilation; pO₂ and pCO₂ remained in the physiological range without necessitating constant adjustment of ventilator settings. Therefore, in our hands, MMF anesthesia provided the most stable physiological conditions (especially in combination with mechanical ventilation), allowing prolonged, hassle-free surgery.

MAP, ICP, CPP, and rCBF
Anesthesia with chloral hydrate or isoflurane caused a reduction of MAP, most likely due to their well known negative effect on cardiac function and vascular tone [20,32]. In contrast, MAP remained closest to physiological values of conscious rats (100-110 mmHg) [33] in the MMF group. The difference in MAP amongst the groups correlated with the ICP increase during SAH induction, which implies that the amount and severity of hemorrhage in this model is directly influenced by pre-hemorrhagic MAP. MAP monitoring is, therefore, an absolute prerequisite for the proper and reproducible induction of experimental SAH.

The acute stage of SAH is frequently accompanied by derangements in arterial blood pressure [3,31,34]. A hypertensive reaction, as exhibited in MMF animals, is described as central activation of vasomotor centers to restore cerebral perfusion in the presence of raised ICP, i.e. the well known Cushing reflex [35,36]. The lack of such a robust physiological reflex in the chloral hydrate and isoflurane group suggests that these agents severely affect the cardiovascular system, thereby altering post-hemorrhagic pathophysiology in an uncontrolled manner. Our data, therefore, indicate that studies on the pathophysiology of SAH under chloral hydrate or isoflurane anesthesia may need to be re-interpreted carefully, since there is a high probability that they may have been performed under a disturbed cardiovascular baseline.

Even more divergent effects of the three anesthesia protocols were observed when post-hemorrhagic rCBF was measured. Whilst rats anesthetized with MMF showed an almost complete recovery from SAH-induced ischemia due to high MAP and CCP, rCBF did not recover at all in animals receiving isoflurane anesthesia. Since isoflurane and chloral hydrate have a very similar suppressive effect on systemic blood pressure these findings suggest that isoflurane may have an additional vasodilatory effect on cerebral vessels, as also suggested by others [37]. The mechanisms responsible for this effect seem to be a selective upregulation of nitric oxide synthesis in the brain by volatile anesthetics [38]. These findings suggest that the use of isoflurane and other volatile anesthetics should be discouraged when studying SAH.

Brain water content
Our findings demonstrating increased brain water content in rats anesthetized with isoflurane are supported by previous studies where volatile anesthetics increased
brain water content in healthy rats and dogs [11,39]. These findings are in agreement with the local vasodilatory effects of isoflurane on the cerebral vasculature; vasodilation increases cerebral blood volume and hence brain water content [40-42].

Isoflurane anesthesia increases brain water content following brain injury, e.g. after experimental traumatic brain injury [11] and after focal cryogenic lesion [43] as also observed in the current study. This is most likely caused by the effect of isoflurane on cerebral blood volume (see above), however, brain damage due to prolonged post-hemorrhagic ischem/hypoperfusion [44] would also be expected to be involved in the development of brain edema in animals anesthetized with isoflurane.

**Mortality**

The two major determinants of post-hemorrhagic mortality in our study were lack of recovery of cerebral hypoperfusion and re-bleeding. Interestingly, the occurrence of hypoperfusion and re-bleeding depended exclusively on the anesthetic protocol used. Hypoperfusion-/ischemic-induced death only occurred after isoflurane and chloral hydrate anesthesia. While isoflurane seems to have a direct CBF-reducing effect (see above), chloral hydrate-induced death was, in most cases, due to episodes of arterial hypotension followed by cessation of rCBF following intraperitoneal re-application of chloral hydrate, as also reported in experimental stroke [45]. This specific problem may, however, be overcome by continuous application of chloral hydrate using a intraperitoneal catheter [46].

Mortality occurring in animals anesthetized with MMF was, in all cases, related to post-hemorrhagic re-bleedings, which are an integral part of the pathophysiology of SAH in humans. The underlying mechanisms of MMF-induced re-bleedings are the maintained MAP and CPP. High MAP and CPP, although beneficial for cerebral perfusion, increase the risk for re-hemorrhages [47] with a high mortality rate [48], as also suggested by our findings. Hence, using MMF for anesthesia during experimental SAH in rats reproduces an important component of SAH in patient, i.e. delayed re-bleedings, which is usually missing when using when chloral hydrate or isoflurane. Accordingly, only when rats were anesthetized with MMF the pathophysiology of SAH was properly reproduced by the endovascular perforation model.

**Handling for general anesthesia in rats**

All three protocols proved to be suitable for surgical anesthesia, however, only MMF appears to not have any negative effect on the pulmonary system, thereby resulting in a far more stable maintenance of arterial pCO2 and pO2, with fewer interventions by the experimentalist compared to isoflurane and chloral hydrate. When considering that MMF anesthesia can be terminated by injection of respective antagonists and that anesthesia under chloral hydrate is difficult to judge and to terminate [49], it seems fair to conclude that MMF is superior to the other two investigated protocols.

**Conclusion**

We investigated if and how three widely used and well standardized anesthesia protocols [isoflurane, chloral hydrate, and a combination of midazolam, medetomidine, and fentanyl (MMF)] affect pathophysiology and outcome following experimental SAH in rats. Our experiments demonstrate that when rats are anesthetized with MMF key properties (re-bleedings, Cushing reflex, moderate mortality) known to be integral parts of the pathophysiology of SAH in humans [47,48,50] are replicated by the endovascular perforation model of experimental SAH. Our findings demonstrate that the choice of anesthesia can have a significant impact on animal models of disease and suggest that MMF should be used for future studies using the endovascular perforation model of SAH.

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**Authors’ contributions**

KH carried out the SAH experiments, performed data analysis, and drafted the manuscript. RT participated in the design and coordination of the study and supervised the experiments. KS participated in the design of the study and edited the manuscript. ET supported KH in performing the experiments and analyzing the data. NP initiated, designed, and coordinated the study and finalized the manuscript. All authors read and approved the final manuscript.

**Competing interests**

The authors declare that they have no competing interests.

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