In-depth characterization of a long-term, resuscitated model of acute subdural hematoma–induced brain injury

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OBJECTIVE Acute subdural hematoma (ASDH) is a leading entity in brain injury. Rodent models mostly lack standard intensive care, while large animal models frequently are only short term. Therefore, the authors developed a long-term, resuscitated porcine model of ASDH-induced brain injury and report their findings.

METHODS Anesthetized, mechanically ventilated, and instrumented pigs with human-like coagulation underwent subdural injection of 20 mL of autologous blood and subsequent observation for 54 hours. Continuous bilateral multimodal brain monitoring (intracranial pressure [ICP], cerebral perfusion pressure [CPP], partial pressure of oxygen in brain tissue [PbtO₂], and brain temperature) was combined with intermittent neurological assessment (veterinary modified Glasgow Coma Scale [MGCS]), microdialysis, and measurement of plasma protein S100β, GFAP, neuron-specific enolase [NSE], nitrite+nitrate, and isoprostanes. Fluid resuscitation and continuous intravenous norepinephrine were targeted to maintain CPP at pre-ASDH levels. Immediately postmortem, the brains were taken for macroscopic and histological evaluation, immunohistochemical analysis for nitrotyrosine formation, albumin extravasation, NADPH oxidase 2 (NOX2) and GFAP expression, and quantification of tissue mitochondrial respiration.

RESULTS Nine of 11 pigs survived the complete observation period. While ICP significantly increased after ASDH induction, CPP, PbtO₂, and the MGCS score remained unaffected. Blood S100β levels significantly fell over time, whereas GFAP, NSE, nitrite+nitrate, and isoprostane concentrations were unaltered. Immunohistochemistry showed nitrotyrosine formation, albumin extravasation, NOX2 expression, fibrillary astrogliosis, and microglial activation.

CONCLUSIONS The authors describe a clinically relevant, long-term, resuscitated porcine model of ASDH-induced brain injury. Despite the morphological injury, maintaining CPP and PbtO₂ prevented serious neurological dysfunction. This model is suitable for studying therapeutic interventions during hemorrhage-induced acute brain injury with standard brain-targeted intensive care.

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KEYWORDS GFAP; MAP-2; mitochondrial respiration; oxidative stress; multimodal brain monitoring; trauma

ACUTE subdural hematoma (ASDH) is frequent in traumatic brain injury,¹⁸ particularly in elderly patients²³ and those with comorbidities, with mortality reaching 60%.⁴⁴ Animal models of brain injury mostly use young and healthy rodents, which have a lissencephalic brain,^{10,45} and commonly lack standard intensive care.¹⁰

Large animals with gyrencephalic, human-like brains may improve clinical translation due to similar neuroanatomical structure.³⁹ Standard clinical parameters including neurological assessment need to be integrated into translational research experiments.⁹ However, in addition to cost and necessary infrastructure, large animal models mostly

ABBREVIATIONS ASDH = acute subdural hematoma; CPP = cerebral perfusion pressure; ICP = intracranial pressure; I/E = inspiratory/expiratory; MGCS = modified Glasgow Coma Scale; NO = nitric oxide; NOX2 = NADPH oxidase 2; NSE = neuron-specific enolase; PbtO₂ = partial pressure of oxygen in brain tissue; PEEP = positive end-expiratory pressure; ROS = reactive oxygen species.

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comprise short observation periods (2–10 hours only), and often lack intensive care^{1–3,12,28,30,41,43} and/or a neurological assessment.¹⁰ Studying pathophysiological mechanisms and treatment approaches requires longer-term, large-animal models with a well-reproducible type of injury and integration of current intensive care standards. Since ASDH is a well-reproducible model of acute brain injury,¹¹ we developed a long-term, resuscitated model of ASDH-induced brain injury in human-sized pigs comprising bilateral, multimodal brain monitoring integrating standard intensive care, intermittent neurological assessment, measurement of blood biomarkers of brain injury as well as postmortem morphological and immunohistochemical analyses.⁵

Methods

After obtaining approval from the local Animal Care Committee and the Federal Authorities for Animal Research, we performed the experiments in adherence with the National Institutes of Health Guidelines on the Use of Laboratory Animals and the European Union “Directive 2010/63/EU on the protection of animals used for scientific purposes.” Eleven pigs were used for this study; 2 animals had to be dropped from the study due to cardiovascular failure unrelated to the neurosurgical instrumentation or the ASDH induction, with high-dose norepinephrine needs > 4 hours after ASDH induction, without any necropsy findings of herniation or neurosurgical instrumentation-related injuries. Hence, the data presented refer to 9 Bretoncelles-Meishan-Willebrand pigs of both sexes (5 females and 4 males; median age 11 months [IQR 9–12 months] and weight 65 kg [IQR 57–71 kg]). Coagulation properties in this pig strain closely mimic those in humans,³¹ in contrast to the otherwise species-specific hypercoagulability,²¹ thereby mimicking the time needed for clot formation within the subdural space in humans.

During the last 12 hours preceding the experiments, the pigs received a nutritional solution (Fresubin, Fresenius Kabi) and had free access to water. Prior to induction of anesthesia, premedication consisted of intramuscular 5 mg/kg azaperone and 1–2 mg/kg midazolam. After establishment of intravenous access via an ear vein, anesthesia was induced with propofol (1–2 mg/kg) and ketamine (1 mg/kg).^{18,20} The pigs were endotracheally intubated and mechanically ventilated (ventilator settings: tidal volume 8 mL/kg, respiratory rate 8–12 breaths/minute adapted to achieve a PaCO₂ of 35–40 mm Hg, inspiratory/expiratory [I/E] ratio of 1:1.5, fraction of inspiratory oxygen [FiO₂] of 0.3, positive end-expiratory pressure [PEEP] 10 cm H₂O to prevent formation of atelectasis).^{18,20} Anesthesia was maintained with continuous intravenous propofol (10 mg/kg/hr) and fentanyl (10 µg/kg initial bolus followed by 2.5 µg/kg/hr). A balanced electrolyte solution (10 mL/kg/hr, Jonosteril 1/1, Fresenius Kabi) was infused as maintenance fluid.

After surgical exposure, a 9-F catheter sheath was inserted into the right femoral vein for placement of a 3-lumen venous catheter (Arrow International) into the iliac vein. The right carotid artery was surgically exposed for placement of a precalibrated ultrasonic flow probe around

the vessel. Both femoral arteries were exposed for placement of a 4-F PiCCO catheter (PULSION Medical Systems SE) for continuous cardiac output, pulse pressure variation measurement, and blood sampling. A catheter was placed in the urinary bladder via midline minilaparotomy.

The skull was exposed, and a craniotomy (approximately 20 mm) was drilled over the left and right parietal cortices (Fig. 1). After exposing the dura on the left side, a small incision was made, and a catheter was inserted approximately 5 mm into the subdural space for later initiation of the ASDH. On the contralateral (right) side, an additional burr hole was similarly placed. Afterward, microdialysis catheters and multimodal brain monitoring probes (Neurovent-PTO, Raumedic AG) were inserted about 10–15 mm into the brain parenchyma in both hemispheres. The multimodal probes were placed under visual control for intracranial pressure (ICP), partial pressure of oxygen in brain tissue (PbtO₂), and temperature measurements. The probes were calibrated before insertion according to the manufacturer’s specifications. After placement, all catheters were allowed to equilibrate for about 1 hour, and recording was started when PbtO₂ values were stable. At the end of the neurosurgical procedure, the burr holes were closed using bone wax, which also served for fixation of the catheter as well as the microdialysis and ICP probes (Fig. 1). During surgery, hydroxyethyl starch 6% 130/0.42 (Vitafusal, Serumwerk) was used to maintain pulse pressure variation, with a maximum dose of 30 mL/kg according to the manufacturer’s specifications. The bilateral neurosurgical procedure was performed to avoid sham experiments, i.e., the hemisphere without ASDH served as the control for the hemisphere with ASDH, and complies with the 3R principle (replacement, reduction, and refinement of animal research).²⁵

Figure 1 shows the experimental protocol. After neurosurgical instrumentation, in order to mimic a patient’s situation immediately before the occurrence of ASDH, at least 1 hour before induction of ASDH, FiO₂ was reduced to 0.21 and the PEEP to 0 cm H₂O, and the I/E ratio was set to 1:2 for baseline measurements. Thereafter, 20 mL of autologous blood (corresponding to 12.5% of brain volume) was injected using an automated syringe pump (rate 90 mL/hr to avoid clotting in the syringe; Perfusor Space, B. Braun Melsungen AG) via the subdural catheter above the left parietal cortex. This amount of blood was injected based on that in previous studies on porcine ASDH^{30,38,41} that had also investigated the effects of blood volumes corresponding to 10%–15% of the brain volume. Figure 2 shows a typical time course of ICP during and until the first 30 minutes after ASDH induction. Two hours after subdural blood injection, resuscitation was initiated: cerebral perfusion pressure (CPP = mean arterial pressure – ICP) was titrated to baseline levels; if CPP remained below baseline values despite volume resuscitation, norepinephrine was used to restore CPP. Fluid was administered (10 mL/kg/hr Jonosteril), and the respirator settings for mechanical ventilation were as follows: PEEP of 10 cm H₂O, FiO₂ of 0.3, and I/E ratio of 1:1.5. Temperature management aimed to achieve brain normothermia. Every 12 hours, the anesthesia depth was reduced until ad-

FIG. 1. A: Neurosurgical instrumentation. Via craniotomy above the left and right parietal cortices, microdialysis and parenchymal multimodal probes were inserted on the ipsilateral/left and contralateral/right sides. After exposing the dura on the left side, a small incision was made, and a catheter was inserted 5 mm into the subdural space for later initiation of the ASDH. The probes were placed under visual control for parenchymal ICP monitoring and $P_{bt}O_2$ and brain temperature measurements. **B:** Experimental protocol. At least 1 hour before induction of the ASDH, F_{iO_2} was reduced to 0.21 and PEEP to 0 cm H₂O, and the I/E ratio was set to 1:2 for subsequent baseline measurements. After the ASDH-induction phase, the following standard resuscitation protocol was used: CPP was titrated to levels before the induction of the ASDH (if CPP remained below baseline values despite volume resuscitation, norepinephrine was used to restore CPP), while fluid administration and lung-protective mechanical ventilation were used (respirator settings: PEEP 10 cm H₂O, F_{iO_2} 0.3, and I/E ratio 1:1.5). F_{iO_2} was stepwise adjusted to maintain an arterial hemoglobin oxygen saturation (S_aO_2) $\geq 95\%$; temperature management was aimed to achieve normothermia in the brain. Every 12 hours, after reduction of the anesthesia depth, a porcine-adapted MGCS score (adapted from that of Platt et al.³⁵) was used for neurological assessment. Figure is available in color online only.

equate spontaneous breathing resumed, and neurological function was assessed using a swine-adapted modified Glasgow Coma Scale (MGCS; Table 1).³⁵ At the end of the experiments, i.e., after 54 hours of intensive care, after further deepening of anesthesia, the pigs were euthanized via injection of KCl. We strove to make the observation period as long as possible, with termination after 54 hours being imposed by practical limitations, in particular, personnel available for providing ICU care and sample processing.

Intracerebral tissue metabolites were determined using an automated microdialysis system (CMA 600 Microdialysis Analyzer, CMA/Microdialysis AB) equipped for glutamate, lactate, pyruvate, and glucose. Microdialysis catheters (70 microdialysis bolt catheter, M Dialysis AB) were implanted bilaterally after perforation of the dura, and lowered to a depth of 10–15 mm and perfused (perfusion fluid, CMA/Microdialysis AB) by a microdialysis pump (CMA/102 microdialysis pump, CMA/Microdialysis AB). After calibration according to the manufacturer's specifications, microdialysate samples were collected in

microvials (MDialysis AB) over 3 hours (i.e., from 1 hour before until 2 hours after each measurement time point) and analyzed immediately after collection. Due to technical failure of the automated analyzing system, there were numerous dropouts and/or implausible values for the glutamate and pyruvate measurements, so that only lactate and glucose data are presented.

Serum blood levels of S100 β , MAP-2, GFAP, and neuron-specific enolase (NSE) were determined using commercially available porcine specific kits (S100 β , MAP-2, GFAP, and NSE, all BlueGene BioTech).

As a marker of lipid peroxidation, 8-isoprostane plasma concentrations were measured using a commercially available immunoassay kit (ELISA kit, Cayman Chemicals). Plasma nitrate+nitrite levels, the stable metabolites of nitric oxide (NO), were analyzed with the chemiluminescence method after reduction of nitrate+nitrite to NO with vanadium chloride (Sievers NOA 280i analyzer, GE Analytical Instruments). Blood samples for these measurements were taken before as well as 2, 14, 26, 38, 50, and 54 hours after induction of the ASDH (see Fig. 1).

FIG. 2. Graph showing an exemplary time course of the ICP during and immediately after ASDH induction over the first 30 minutes (recording frequency 1 Hz). Twenty milliliters of autologous nonheparinized blood was injected using an automated syringe pump via the subdural catheter above the left parietal cortex. Red arrows mark the beginning (left arrow) and the end (right arrow) of the injection time. The temporary drop immediately after termination (right arrow) of the ASDH induction is due to the turning of the 3-way stopcock and the closure of the catheter. Figure is available in color online only.

Homogenized brain tissue specimens from regions adjacent to the surgical instrumentation sites of both sides (brain area next to the ASDH ipsilateral and corresponding right area) were analyzed immediately postmortem for mitochondrial respiratory activity using “high-resolution respirometry” (Oxygraph-2K, Oroboros Instruments). After supplementation of substrates for complexes I and II and ADP, the respiratory capacity in the state of oxidative phosphorylation (coupled state, OXPHOS) was assessed. Maximal respiratory capacity of the electron transfer system (ETS) in the uncoupled state was measured after the addition of 4-(trifluoromethoxy) phenylhydrazone (FCCP). LEAK respiration compensating for proton leakage or slipping was reported as a percentage of ETS capacity. Data are normalized for tissue wet weight.

Immediately postmortem, animals were decapitated, and the brain was removed. The fresh brain was cut sagittally to separate the left and right hemispheres; for protein biochemistry, we sampled an entire slice (5–6 mm thick) from the right hemisphere of the parietal cortex, and for mitochondrial measurements, we took small tissue samples (300 mg) of both the ipsi- and contralateral prefrontal cortex. The remaining brain was fixed for 6 days in 4% formalin. Then, the brain was sectioned in 4-mm sections from frontal to occipital, which were then embedded in paraffin. Standard 3- to 5- μ m, and, using a modified protocol, 70- μ m, paraffin thick sections were cut and mounted on Superfrost plus (VWR International) slides. General neuropathological evaluation was performed on H & E-stained sections.

Immunohistochemical evaluation of the brain slices around the surgical instrumentation sites (brain area next to the ASDH ipsilateral and corresponding right area) was performed for formation of 3-nitrotyrosine, which mirrors the reaction of the superoxide radical with NO to peroxyni-

TABLE 1. MGCS adapted to swine

Level of consciousness	
Responsive to environment	6
Depression or delirium; limited addressable, pronounced sleep tendency	5
Semicomatose, responsive to visual stimuli	4
Semicomatose, responsive to auditory stimuli	3
Semicomatose, responsive only to repeated noxious stimuli	2
Comatose, unresponsive to repeated noxious stimuli	1
Motor activity	
Normal posture, normal spinal reflexes	6
Hemiparesis, tetraparesis, or decerebrate rigidity	5
Recumbent, intermittent extensor rigidity	4
Recumbent, persistent extensor rigidity	3
Recumbent, permanent extensor rigidity with opisthotonus	2
Recumbent, hypotonia of muscles, depressed or absent spinal reflexes	1
Brainstem reflexes	
Normal pupillary light reflexes and oculocephalic reflexes	6
Slow pupillary light reflexes and normal to reduced oculocephalic reflexes	5
Bilateral unresponsive miosis with normal to reduced oculocephalic reflexes	4
Pinpoint pupils with reduced to absent oculocephalic reflexes	3
Unilateral, unresponsive mydriasis with reduced to absent oculocephalic reflexes	2
Bilateral, unresponsive mydriasis with reduced to absent oculocephalic reflexes	1

This scale was used for the intermittent neurological assessment. Modified from Platt SR, Radaelli ST, McDonnell JJ: The prognostic value of the modified Glasgow Coma Scale in head trauma in dogs. *J Vet Intern Med* 15:581–584, 2001, John R. Wiley & Sons, Inc., ©American College of Veterinary Internal Medicine, with permission.

trite; for NADPH oxidase (NOX) expression, the subtype NOX2 of which is referred to be mainly responsible for reactive oxygen species (ROS) production in brain tissue;²⁶ for extravasation of albumin as a marker of blood-brain barrier dysfunction; and for brain tissue, GFAP, a marker of reactive gliosis after brain injury.⁴⁰ The sections were deparaffinized and microwaved for 90 seconds in citrate buffer (pH 6.0) for heat-induced antigen retrieval, blocked for 30 minutes in 10% goat serum, and incubated with the following antibodies: rabbit anti-nitrotyrosine (Merck Millipore), rabbit anti-GFAP (Abcam), rabbit anti-IBA1 (Abcam), mouse anti-NOX2 (gp91phox, Becton Dickinson Bioscience), and rabbit anti-albumin (Abcam). Primary antibody detection was performed using the Dako REAL detection system (anti-mouse, anti-rabbit, Dako) and visualized with red chromogen (DakoREAL) followed by counterstaining with Mayer's hematoxylin (Sigma-Aldrich). Histomorphology was visualized using an Axio Imager.A1 microscope (Zeiss) with a $\times 10$ objective. Two random 800,000- μ m² regions were quantified for intensity using the AxioVision 4.8 software (Zeiss). Results are presented as mean densitometric sum red.

Results

Table 2 summarizes systemic hemodynamics, acid-base state, gas exchange, lung mechanics, and metabolism. The pigs' temperatures dropped during the surgery and were restored to normal levels after 14 hours of resuscitation. Except for a statistically significant, but clinically irrelevant, decrease over time of glycemia, lactatemia levels, and the right carotid artery flow, all parameters remained stable throughout the experiment. In all animals, macroscopic evaluation at necropsy showed a solid subdural clot of about 2 cm in diameter on the left cortical surface (Fig. 3). In addition, in all animals hemorrhage had broken through the subarachnoid into the intracerebral space, with bleeding reaching the lateral ventricles in 3 animals.

Figure 2 demonstrates that ICP sharply rose on ASDH induction and remained increased in both hemispheres until the end of the experiment. Table 2 also summarizes the results of the multimodal brain monitoring. At 36 hours of intensive care, i.e., 38 hours after ASDH induction, the ICP in the left hemisphere was significantly higher than that in the right hemisphere ($p = 0.024$). Despite the increased ICP, fluid resuscitation and continuous intravenous norepinephrine needed by all animals (median/IQR infusion rate median $0.10 \mu\text{g/kg/min}$ [IQR $0.04\text{--}0.39 \mu\text{g/kg/min}$]) allowed the CPP to be maintained at baseline levels. Strikingly, PbtO_2 values significantly differed between the two hemispheres at baseline ($p = 0.048$), but this difference disappeared throughout the experiment; overall, PbtO_2 was not significantly affected over time. Despite the macroscopically visible damage, the well-maintained CPP and unchanged PbtO_2 were associated with a stable MGCS score throughout the entire experiment.

Figure 4 shows the brain microdialysis glucose and lactate concentrations. At baseline the median lactate levels were 2.2 mmol/L (IQR $1.0\text{--}4.0 \text{ mmol/L}$) in the ipsilateral/left hemisphere and 1.7 mmol/L (IQR $0.2\text{--}2.6 \text{ mmol/L}$) in the contralateral/right hemisphere. After ASDH induction, left-sided lactate levels increased up to a median of 2.9 mmol/L (IQR $1.4\text{--}4.4 \text{ mmol/L}$), however, with pronounced interindividual variation, but remained stable in the right hemisphere. Tissue glucose levels at baseline were 0.4 mmol/L (IQR $0.3\text{--}1.0 \text{ mmol/L}$) in the ipsilateral and 0.4 mmol/L ($0.1\text{--}0.6 \text{ mmol/L}$) in the contralateral hemispheres. The ipsilateral glucose concentrations significantly decreased to values of 0.01 mmol/L (IQR $0.01\text{--}0.3 \text{ mmol/L}$) over time, whereas they remained unchanged in the right hemisphere. Again, there were considerable interindividual variations.

The systemic markers of NO and ROS formation as well as brain injury are summarized in Table 2. Except for the $\text{S100}\beta$, which significantly decreased over time, none of these parameters (8-isoprostane, nitrite+nitrate, GFAP, NSE, or MAP-2) were significantly affected. Figure 4 shows that mitochondrial respiratory activity of immediate postmortem brain specimens taken from regions adjacent to the surgical instrumentation did not differ between the two hemispheres.

Figure 5 shows typical examples of the histological findings: all swine showed periventricular lymphocyte infiltration, the degree of which was moderate and differed among individual animals. Three animals also showed

minor granulocyte infiltration, possibly documenting secondary infection associated with the surgical instrumentation. There were no significant histological signs of tissue hypoxia, although dark eosinophilic neurons were found in the injured hemisphere near the ASDH site.

Figure 6 shows typical examples as well as the quantitative analysis of the immunohistochemical evaluation. ASDH induction was associated with marked 3-nitrotyrosine formation, extravascular albumin accumulation, and NOX2 expression, which coincided with a significantly higher GFAP expression in the cortex next to the ASDH compared with the corresponding region in the contralateral hemisphere. Moreover, analysis of the dorsomedial prefrontal cortex with a distant location to the ASDH site revealed GFAP-positive astrogliosis and more complex morphologies of IBA-1 immunoreactive cells, indicating microglial activation on the left hemisphere with ASDH, whereas these changes were not present in the contralateral dorsomedial prefrontal cortex.

Discussion

The purpose of this study was to describe a clinically relevant, long-term, resuscitated porcine model of ASDH-induced acute brain injury. The main results were as follows: 1) ASDH caused nitrosative and oxidative stress, while the neurosurgical instrumentation alone had no effect. 2) Local brain injury was not reflected by systemic markers of oxidative or nitrosative stress or markers of brain injury. 3) Despite the morphological damage, maintenance of CPP and PbtO_2 prevented severe impairment of tissue energy metabolism and, hence, serious neurological dysfunction.

We chose ASDH as model of acute brain injury because of its reproducibility and clinical relevance,^{18,23,44} which resulted in different manifestations of brain injury beyond the subdural hemorrhage, possibly due to secondary bleeding and/or pressure-related rupture of the arachnoid and pia maters. Since the results of all investigated parameters were similar, we suspect that the bleeding in the lateral ventricles mirrored different manifestations due to anatomical variations. Porcine models of ASDH using multimodal brain monitoring have been described for up to 12 hours only using injected blood volumes of $2\text{--}9 \text{ mL}$.^{30,41} We injected 20 mL of blood into the subdural space, corresponding to 12.5% of the brain volume, because previous studies on porcine ASDH had used comparable ratios ($10\%\text{--}15\%$) of the volume of subdural blood injected and brain volume.^{30,38,41} In contrast to the aforementioned studies,^{30,41} we used human-sized animals with a median body weight of 65 kg and, consequently, weight-adapted the amount of blood injected into the subdural space. The ICP increased in both sides with higher ipsilateral values throughout the experiment, with the contralateral ICP increase most likely being mass effect due to intracranial pressure spreading. The present model focused on the effects of an ASDH-induced brain injury in human-sized adult swine together with the integration of guideline-based intensive care therapy. Hence, it is likely that the long-term anesthesia and the surgical instrumentation also affected our findings, in particular with respect

TABLE 2. Hemodynamics, gas exchange, acid-base status, metabolism, bilateral neuromonitoring, MGCS, blood markers of brain injury as well as oxidative and nitrosative stress

Parameter	At Baseline	2 Hrs After ASDH Induction	14 Hrs After ASDH Induction	26 Hrs After ASDH Induction	38 Hrs After ASDH Induction	50 Hrs After ASDH Induction	54 Hrs After ASDH Induction
Heart rate, beats/min	91 (82–101)	82 (75–104)	104 (93–124)	82 (70–107)	75 (70–97)	83 (73–98)	92 (67–120)
Mean arterial pressure, mm Hg	121 (114–132)	122 (113–132)	124 (113–134)	123 (107–133)	110 (104–128)	124 (112–133)	121 (98–130)
Cardiac output, mL/min/kg	147 (124–161)	132 (113–152)	119 (103–149)	120 (93–146)	116 (99–163)	133 (88–163)	166 (94–199)
Rt carotid artery flow, mL/min/kg	9.1 (8.4–18.5)	7.9 (5.8–13.3)	4.4 (3.3–8.1)	4.7 (2.4–7.2)	4.2 (2.9–9.6)	4.1 (3.1–6.2)*	4.8 (2.4–5.8)*
Arterial pH	7.54 (7.52–7.57)	7.55 (7.52–7.59)	7.54 (7.51–7.57)	7.52 (7.51–7.55)	7.50 (7.48–7.55)	7.52 (7.49–7.55)	7.52 (7.48–7.53)
PaO ₂ , mm Hg	109 (98–137)	101 (93–104)	138 (127–144)	134 (131–139)	132 (127–138)	133 (122–137)	131 (124–137)
PaO ₂ , mm Hg	37 (34–40)	35 (34–39)	36 (34–38)	38 (35–39)	37 (35–41)	37 (34–39)	37 (35–40)
Arterial base excess, mmol/L	9.2 (6.9–10.1)	8.8 (6.7–9.8)	6.9 (5.0–10.4)	7.9 (5.7–9.2)	6.6 (5.3–7.9)	6.4 (5.0–7.7)	6.7 (5.3–8.0)
Arterial lactate, mmol/L	1.2 (1.0–1.3)	1.0 (0.9–1.4)	0.5 (0.5–0.9)*	0.5 (0.5–0.6)*	0.7 (0.5–0.8)*	0.6 (0.5–0.9)*	0.8 (0.5–0.9)
Arterial glucose, mg/dL	108 (103–114)	117 (114–124)*	88 (85–95)*	78 (70–84)*	86 (75–92)*	74 (65–101)*	87 (62–107)
ICP, mm Hg							
Lt hemisphere	7 (2–17)	18 (17–21)*	19 (16–22)*	15 (14–20)	15 (14–20)†	15 (14–20)	19 (16–25)
Rt hemisphere	5 (3–9)	14 (11–18)*	14 (11–21)*	13 (10–14)*	11 (11–14)*,†	12 (11–18)*	14 (13–18)*
CPP, mm Hg							
Lt hemisphere	116 (109–125)	111 (97–115)	109 (96–118)	105 (96–126)	92 (80–119)	115 (109–119)	114 (86–121)
Rt hemisphere	117 (108–135)	108 (93–120)	106 (96–122)	110 (87–126)	101 (92–120)	110 (85–118)	101 (76–117)
PbtO ₂ , mm Hg							
Lt hemisphere	19 (5–33)†	16 (5–36)	30 (5–39)	13 (7–36)	27 (15–39)	24 (18–25)	20 (15–25)
Rt hemisphere	72 (47–92)†	33 (4–43)	21 (8–46)	25 (17–59)	32 (9–48)	26 (11–45)	25 (10–37)
Brain temperature, °C							
Lt hemisphere	35.8 (34.9–37.1)	36.4 (35.7–37.4)	38.0 (37.6–38.3)*	38.4 (38.0–38.5)*	38.1 (38.0–38.6)*	38.3 (38.1–38.3)*	38.2 (38.1–38.3)*
Rt hemisphere	34.9 (34.7–35.0)	35.9 (35.3–36.5)*	37.9 (37.0–38.4)*	38.4 (37.6–38.4)*	38.5 (37.9–38.6)*	38.3 (38.0–38.3)*	38.2 (38.0–38.6)*
MGCS score	14 (12–17)	13 (11–14)	13 (13–14)	13 (12–14)	13 (12–14)	14 (13–14)	13 (12–14)
8-isoprostane, pg/mL	81 (72–91)	81 (57–94)	78 (69–91)	82 (77–110)	99 (67–250)	78 (66–163)	89 (72–107)
nitrate+nitrite, µmol/L	11 (2–16)	10 (2–13)	5 (0–7)	5 (0–7)	5 (1–7)	6 (0–7)	6 (1–7)
GFAP, pg/mL	421 (319–1377)	379 (249–1299)	321 (236–995)	334 (187–1089)	450 (183–1031)	402 (180–912)	418 (256–879)
MAP-2, ng/mL	3 (2–12)	3 (2–11)	3 (2–9)	3 (1–9)	3 (1–8)	2 (1–7)	3 (1–6)
NSE, ng/mL	53 (44–133)	50 (37–108)	39 (28–94)	29 (23–88)	26 (20–79)	23 (19–72)	34 (19–72)
S100β, ng/mL	3.7 (3.0–10.2)	4.0 (3.0–9.6)	2.5 (2.0–8.3)	1.4 (1.1–6.6)	1.0 (0.8–5.4)*	0.8 (0.6–4.8)*	0.8 (0.6–5.0)*

Values are median (IQR). Values at baseline represent data after completion of the surgical instrumentation, i.e., immediately before induction of the ASDH.

* $p < 0.05$ versus baseline within each hemisphere.

† $p < 0.05$ ipsilateral/left versus contralateral/right side.

FIG. 3. Photograph of a porcine brain immediately after termination of the experiment (**A**), showing a solid clot above the left hemisphere that has just released from its still-visible original position. Macroscopic visible damage of the formalin-fixed brain slices on the ipsilateral/left hemisphere (**B1 and C1**) is contrasted with its contralateral/right hemisphere (**B2 and C2**). Figure is available in color online only.

to the systemic biomarkers. However, investigation of pathophysiological challenges and/or possible therapeutic interventions during acute hemorrhage or trauma-induced brain injury using current resuscitation standards would also require long-term anesthesia and surgical instrumentation, and, therefore, control animals without anesthesia, surgery, and/or baseline injury would have provided data from completely different conditions. We performed identical, bilateral neurosurgical instrumentation procedures on the left (ipsilateral) and on the right (contralateral) hemispheres so that the “sham-instrumented” hemisphere could be used as a control for the ASDH hemisphere. Due to the significant macroscopic (Fig. 3) and immunohistochemical (Fig. 6) differences (in particular with respect to 3-nitrotyrosine formation, albumin extravasation, and NOX2 expression) between both sides, we strongly suggest that the ipsilateral findings were related to the ASDH rather than the neurosurgery. Moreover, this approach also allowed for complying with the 3R principle to reduce the number of animals.²⁵

Experiments lasted for 54 hours under intensive care conditions, including neurological assessment using an adapted MGCS.³⁵ To our knowledge, no large-animal model of ASDH has yet been described that includes multimodal brain monitoring and full-scale intensive care over a resuscitation period of 54 hours. A study on ASDH in 3-week-old piglets described neurological deficits at days 3 and 7,³⁸ but these animals did not receive intensive care.

Other porcine studies on ASDH^{30,41} did not describe any detailed neurological assessment. Clearly, MGCS scores in our study were lower than normal values. However, baseline assessment was taken after the end of the neurosurgical instrumentation, and, therefore, the depth of anesthesia was only reduced to levels allowing for spontaneous breathing rather than complete awake status. Nevertheless, neither ASDH induction nor prolonged anesthesia had any further effect on the neurological function.

In contrast to earlier large-animal studies of ASDH-induced and/or traumatic brain injury,^{1,12,28,38,41} we measured ICP bilaterally. Since systemic hemodynamics remained stable due to fluid resuscitation and norepinephrine infusion, CPP was well maintained throughout the experiment, which was ultimately associated with an unaffected MGCS score. These observations are in line with a short-term (6 hours) study of acute traumatic brain injury in piglets, which showed attenuated metabolic disturbances and brain injury when a CPP of 70 mm Hg rather than 40 mm Hg was targeted.¹²

Monitoring of PbtO₂ to detect cerebral ischemia has been advocated for the management of patients after severe head injury.⁴² In that study, the duration of periods with PbtO₂ ≤ 15 mm Hg were directly related to poor neurological outcome and mortality.⁴² In our study, PbtO₂ values remained unchanged over time and did not show statistically significant differences between the two hemispheres. However, data recorded showed large variations, probe malfunctions with implausible values and, consequently, various data could not be used. Moreover, individual values were below the above-mentioned threshold, indicating regional cerebral hypoxia and/or ischemia. At first glance, this finding is in contrast to the unaffected MGCS scores. However, comparably broad variations of PbtO₂ values with standard deviations of up to 50% of the recorded mean values were observed in a pig study on brain death due to a gradual increase in ICP resulting from inflation of an epidural balloon.³⁶ A more recent study characterizing a multiparameter brain probe in juvenile pigs (maximum duration 5–6 hours) undergoing various physiological challenges (hypoxia, hypercapnia, norepinephrine infusion) with or without cortical impact also reported individual PbtO₂ < 15 mm Hg with neurosurgical instrumentation alone as well as standard deviations of the recorded mean PbtO₂ levels of up to 50%. In that study, approximately 20% of the recorded PbtO₂ values had to be discarded completely.²⁷

Microdialysis lactate concentrations in the ASDH-challenged hemisphere increased up to 2.9 mmol/L without significant differences from baseline or between the hemispheres. Microdialysis glucose levels significantly decreased in the ipsilateral, hematoma-affected hemisphere to values < 0.1 mmol/L, whereas the contralateral hemisphere did not show any significant effect. Our findings are in line with a porcine study of controlled cortical impact reporting microdialysis lactate values up to 2.16 mmol/L and a drop of glucose values to 0.1 mmol/L.¹ Comparable microdialysis data were shown in a brain-death study in pigs with lactate values of up to 3.0 mmol/L and glucose levels of 0.1 mmol/L.²⁷ The nonsignificant decrease of microdialysis lactate during the experiment was most likely

FIG. 4. A: Microdialysis lactate and glucose levels are presented as boxplots (median, IQR, maximum/minimum) over time at baseline (pre) and after (2–54 hours) the induction of the ASDH of the ipsilateral (left, with the ASDH) and the contralateral (right) hemisphere. *Significantly different from baseline in the ipsilateral glucose concentrations. **B:** Mitochondrial respiratory capacity of brain samples. Mitochondrial function was analyzed using high-resolution respirometry (Oroboros Oxygraph-2K, Oroboros Instruments) in homogenized tissue specimens collected immediately postmortem from brain regions directly adjacent to the surgical instrumentation site. ETS = maximal respiratory capacity of the electron transfer system in the uncoupled state; LEAK = respiration compensating for proton leakage or slipping and is reported as percentage of ETS; OXPHOS = mitochondrial respiratory capacity in the state of oxidative phosphorylation (coupled state). Data are normalized for tissue wet weight and represent 4 animals. Figure is available in color online only.

observed because of the CPP maintenance and the volume substitution during the intensive care therapy. Due to the missing data of microdialysis glutamate and pyruvate in the present study, the validity of the microdialysis measurements may be regarded as questionable. However, previous studies on porcine ASDH by Alessandri et al.¹ and Purins et al.³⁶ showed similarly huge variations in microdialysis glutamate and pyruvate, the overall data being comparable to our findings. Clearly, the metabolic disturbances were only present in the hematoma-carrying hemisphere, and less pronounced than in these authors' studies. Again, this observation is most likely due to the strict adherence to a brain-targeted resuscitation protocol in our study.

The S100 β plasma concentrations significantly decreased throughout the experiment. Increased S100 β plasma levels have been reported in porcine traumatic brain injury.¹³ However, a study in piglets undergoing cortical impact did not show any increase of S100 β ,⁶ and another study on hypothermic cardiac arrest in juvenile pigs only described an initial, transitory rise of S100 β , which then decreased even below baseline values in the further exper-

iment.³⁷ In the present study, high levels of S100 β were already present at baseline (median 3.7 ng/mL [IQR 3.0–10.2 ng/mL]), and decreased below baseline levels until the end of the experiments. Different time courses of S100 β might be related to various potential sources of S100 β .¹⁹ Hence, our study confirms the limits of this parameter to mirror severity of brain injury.^{6,37} The interpretation of blood-brain biomarkers in general is critically discussed,^{6,13} and findings of the present study differ from those of previous studies due to the use of guideline-directed intensive care.

GFAP is described as a marker of the severity of head injury.^{24,32} In the present study, GFAP serum levels did not change significantly, whereas immunohistochemical analysis showed significantly higher GFAP expression in the ipsilateral, hematoma-carrying hemisphere. Increased serum GFAP levels have been reported after cortical impact in swine; however, these animals underwent brain injury together with hemorrhagic shock, i.e., markedly reduced CPP over 2 hours.²² Nevertheless, our immunohistochemical findings of markedly higher GFAP expression are in line with those in studies demonstrating hyperintensity of

FIG. 5. Histological findings of the brain. Exemplary H & E–stained sections. All swine showed periventricular lymphocyte infiltration, the degree of which was moderate and differed between individual animals. In addition, 3 animals also showed minor granulocyte infiltration, most likely documenting secondary infection associated with the surgical instrumentation. There were no significant histological signs of tissue hypoxia, although dark eosinophilic neurons were found in the injured hemisphere near the ASDH site. **A and B:** Typical perivascular mixed cell (granulocyte and lymphocyte) inflammation. **C–E:** Reactive inflammatory cells (lymphocytes and granulocytes), which migrate from the ventricle into the brain parenchyma. Bar = 1 mm (A and C), 0.2 µm (D), and 100 µm (B and E). Figure is available in color online only.

GFAP as an indicator of reactive gliosis after brain injury in rats⁴⁰ and swine.¹⁷ Moreover, GFAP-positive astrogliosis was accompanied by activation of IBA-1-positive microglial cells, which was not only seen next the ASDH site but also at distant locations.

Plasma NSE remained unaffected in our study. A doubling of NSE serum levels has been reported in piglets on day 1 after cortical impact, regardless of the degree of histological damage.⁶ However, animals in that study did not receive intensive care.

Plasma levels of MAP-2 are suggested as a parameter for ischemic brain injury in humans,³⁴ and, in fact, increased MAP-2 levels were found in the CSF in patients with severe traumatic brain injury.³³ Rats⁸ and swine²⁹ showed decreased immunohistochemical MAP-2 expressions from injured brain regions, while increased plasma MAP-2 levels could be detected shortly after ischemia in the rat.³⁴ In contrast, MAP-2 levels did not show any significant changes over time in our present study. This discrepancy is most likely due to unaffected CPP and PbtO₂ values resulting from strict adherence to therapeutic guidelines.

Primary and secondary brain injury are associated with impaired bioenergetics and mitochondrial dysfunction and/or damage.¹⁶ Significant alterations in cerebral mitochondrial bioenergetics were described in juvenile swine with traumatic brain injury, surprisingly most pronounced in the contralateral, unaffected hemisphere.²⁰ In contrast, in our study, mitochondrial respiration did not differ between both hemispheres. However, we investigated adult animals with mature brains, and, again, strictly adhered to brain-targeted intensive care guidelines.

Brain tissue nitrotyrosine expression, a marker of peroxynitrite formation⁴ has been reported in traumatic brain injury in mice¹⁴ and rats.¹⁵ In patients with severe brain injury, nitrotyrosine levels in the CSF were indicative of poor neurological outcome.⁷ In the present study, nitrotyrosine and NOX2 as indicators of increased oxidative and nitrosative stress were significantly increased in the ipsilateral, blood-injected hemisphere, when compared with the contralateral hemisphere, whereas systemic markers of NO and ROS formation, 8-isoprostane and nitrite+nitrate plasma levels, remained unchanged. The ipsilateral nitro-

FIG. 6. Immunohistochemical analysis of brain specimens and changes in IBA-1 and GFAP immunoreactive cells distant from the ASDH site. **A–D:** Immunohistochemical analysis of the brain specimens. Examples and quantitative results of the densitometric analysis of animals and representative histological images (original magnification $\times 10$ objective) of the brain immunohistochemistry for 3-nitrotyrosine (**A**; $n = 7$ ipsilateral, $n = 7$ contralateral), extravascular albumin (**B**; $n = 7$ ipsilateral, $n = 7$ contralateral), NOX2 (**C**; $n = 6$ ipsilateral, $n = 6$ contralateral), and GFAP (**D**; $n = 6$ ipsilateral, $n = 6$ contralateral). Distinct hemisphere cross-sections were dissected, fixed in formalin, dehydrated, and embedded in paraffin. **FIG. 6. (continued)**→

FIG. 6. Slices of 3-mm thickness were cut and mounted on slides. Primary antibody detection was performed by an alkaline phosphatase–conjugated secondary antibody and visualized with an alkaline phosphatase substrate red chromogen followed by counterstaining with hematoxylin. Two representative 800,000-mm² sections per slide were graded. Quantification of the intensity of the red chromogen was performed. Results are presented as median densitometric sum red. Boxplots display median, IQR, and range. **E–H:** Changes in IBA-1 and GFAP immunoreactive cells distant from the ASDH site. The dorsomedial prefrontal cortex displays IBA-1 immunoreactive activated microglial cells and GFAP-positive astrogliosis on the ipsilateral hemisphere (E and G) subjected to ASDH as seen in 70- μ m-thick brain sections. In the dorsomedial prefrontal cortex of the contralateral hemisphere (F and H), resting IBA-1-positive microglia are found, and astrogliosis is mild. Bar = 100 μ m (E–H). Figure is available in color online only.

tyrosine formation is in line with those in these previous studies,^{14,15} while the unchanged serum markers indicate that maintenance of CPP and PbtO₂ might prevent signs of systemic oxidative and nitrosative stress.

Conclusions

We describe a clinically relevant, long-term, resuscitated porcine model of ASDH-induced acute brain injury. ASDH was associated with pronounced morphological damage, which was only related to the hematoma, whereas the neurosurgical instrumentation alone had no effect. Local brain injury was not reflected by systemic markers, most likely as a result of maintenance of CPP and PbtO₂, which in turn prevented impairment of tissue energy metabolism and, subsequently, major neurological dysfunction. This model is suitable for studying pathophysiological challenges and/or therapeutic interventions during acute hemorrhage- and/or trauma-induced brain injury, under current resuscitation standards.

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Disclosures

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

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Conception and design: Kapapa, Radermacher. Acquisition of data: Datzmann, Kapapa, Merz, Unmuth, Hoffmann, Mathieu, Mayer, Mauer, Röhrer, Möller, Nussbaum, Calzia, Gröger, Radermacher, Wepler. Analysis and interpretation of data: Datzmann, Kapapa, Scheuerle, McCook, Merz, Unmuth, Mathieu, Mauer, Yilmazer-Hanke, Möller, Nussbaum, Calzia, Gröger, Hartmann, Radermacher, Wepler. Drafting the article: Datzmann, Radermacher. Critically revising the article: Datzmann, Kapapa, Scheuerle, McCook, Unmuth, Hoffmann, Mayer, Mauer, Röhrer, Möller, Calzia, Radermacher, Wepler. Reviewed submitted version of manuscript: Datzmann, Kapapa, Yilmazer-Hanke, Möller, Calzia, Radermacher, Wepler. Approved the final version of the manuscript on behalf of all authors: Datzmann. Statistical analysis: Datzmann, Radermacher, Wepler. Administrative/technical/material support: Datzmann, Kapapa, Scheuerle, McCook, Merz, Hoffmann, Mathieu, Mayer, Mauer, Röhrer, Yilmazer-Hanke, Nussbaum, Calzia, Gröger, Hartmann. Study supervision: Radermacher.

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