

Molecular hydrogen affords neuroprotection in a new translational piglet model of hypoxic-ischemic encephalopathy

PhD. thesis

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INTRODUCTION

Hypoxic-ischemic encephalopathy (HIE) is regarded as an acute/subacute neonatal cerebral injury related to perinatal asphyxia (PA) around the time of delivery. PA is characterized by the inability of gas exchange resulting in progressive hypoxemia, hypercapnia and mixed acidosis, favouring cerebral ischemia due to haemodynamic and mechanical complications. Although the prenatal diagnosis of PA is difficult as moderate asphyxiation may occur at almost every vaginal delivery, the diagnosis of severe PA, leading to HIE, is based on the following criteria: 1) severe acidosis in umbilical arterial blood ($\text{pH} < 7.0$ and base deficit > 12 mmol/l), 2) the persistence of low (0-3) Apgar scores after 5 min, 3) neonatal neurologic disorders (i.e. coma, seizure activity or muscle hypotonia) and 4) multi organ failure. According to global estimates, ~2 million newborns are victims of severe PA annually. The outcome of PA/HIE shows great variability as PA/HIE are responsible for the majority of neonatal mortality (~6-800.000 newborns/year, ~23%). On the other hand, survivors of PA may manifest a wide spectrum of neurological sequelae, including complete recovery without neurological impairments or with mild consequences (i.e. with learning and attention deficit disorders). Nevertheless, in more severe cases of HIE, permanent symptoms may develop leading subsequently to long-term neurodevelopmental sequelae including seizures, mental and somatic retardation as well as cerebral palsy (CP), characterized by severe sensorimotor deficits, or death. In addition, ~20-50% of HIE survivors (~500.000 neonates/year) remain severely handicapped by the end of childhood. Decreasing the mortality rate of HIE along with the development of severe neurological impairments are the greatest challenges of neonatology.

Nearly until a decade ago, only supportive intensive care existed for HIE patients, but since therapeutic hypothermia (TH) has been established and put in widespread use by today. TH is the only proven neuroprotective HIE therapy decreasing both mortality and the number of survivors with severe disabilities. Unfortunately, the effectiveness of TH is limited, moreover, severe side effects related to the controlled body cooling may develop in newborns (i.e. bradycardia and hypotension, hyperkalemia, errors of haemostasis...), consequently, further neuroprotective interventions are required that could compliment the effects of TH or replace it.

In biomedical research, *in vivo* animal models offer an invaluable aid to reveal unknown molecular mechanisms of pathophysiological processes, moreover, they open the door to study the pathophysiology of PA/HIE and to test putative neuroprotective therapies. In order to reproduce translationally all of the metabolic (i.e. O_2 saturation, plasma glucose and lactate) and functional (i.e. heart rate, blood pressure, EEG activity) changes of PA/HIE observed in the human neonate, the choice of the animal model is critical. During the past few years, a large number of PA/HIE animal models were introduced in the literature partly due to the heterogeneous etiology of human PA, on the other hand, to mirror the hypotheses of the research groups. Small animal models (i.e. guinea pig, rat, rabbit, mouse) contribute significantly to exploring the molecular mechanisms of PA/HIE, whilst the application of large animal models (i.e. sheep, dog, rhesus monkey and pig)

allows multiple instrumentation and increased translational potential to clinical practice. Among these animal models, the newborn pig became the deputy of the term neonate. The advantages of the species include its easy availability and more importantly, that the developmental stage of the central nervous system along with the cerebral metabolism at birth share similarities with the term human newborn's brain. During the past few decades numerous PA/HIE swine models were introduced, i.e. selective global cerebral ischemia induced by raised intracranial pressure, asphyxiation by suspended artificial ventilation, by bilaterally induced pneumothorax or by cardiac arrest. Nowadays, hypoxic ventilation with or without reversible bilateral carotid artery occlusion (BCAO) has become the most popular way to induce HIE, however, hypercapnia, also a major hallmark of PA, is not always developed by the end of the insult. Moreover, the clinical relevance of mechanical vessel occlusion is questionable.

An ideal neuroprotectant alleviating the symptoms of HIE should fulfill the following criteria: 1. promising results in reliable PA/HIE translational animal models then later in clinical trials, 2. easy and inexpensive administration at bedside, 3. no/minimal side effects, 4. ability to pass the biological membranes and barriers, and finally 5. good clearance. Beside the proven neuroprotective effects of TH, numerous interventions have already showed promising results in various PA/HIE animal models, but surprisingly, not all of them (i.e. nicardipine; magnesium) could pass the clinical trials, due to probably the observed side effects. Thus, new promising candidates are currently under active scope of research. To name a few candidates, xenon (Xe) inhalation combined with TH was proven to be neuroprotective in a newborn piglet model of PA/HIE, although recent clinical trials testing TH combined with Xe inhalation could not report augmented neuroprotection in HIE survivors against controlled body cooling only. In addition, Xe is expensive, limiting/excluding the routine application of the noble gas in developing countries. Furthermore, allopurinol, an inhibitor of xantin oxidase, could offer neuroprotection only in milder cases of HIE in clinical trials. Currently, the most attractive candidates for future clinical trials are N-acetylcysteine, the antidote of paracetamol intoxication along with melatonin and erythropoietin, affording neuroprotection via their anti-inflammatory and anti-apoptotic effects.

Exploring the possible neuroprotective effects of molecular hydrogen (H₂) in a translationally relevant PA/HIE piglet model has become the focus of our research group. In 2007, Ohsawa *et al.* introduced molecular H₂ and they demonstrated that 2-4% H₂ inhalation dosedependently decreased the infarct volume in a focal ischemia adult rat model. According to our previous results, postasphyxial H₂ inhalation alleviated neurovascular dysfunctions in our newborn piglet HIE model, manifested by preserved vascular responsiveness of the pial arteries for different vasoactive stimuli. However, in our previously reported PA piglet models, the applied PA elicited only moderate neuronal injury, making more difficult to explore the putative neuroprotective effects of molecular H₂, hence significant neuroprotection was not found in all examined cerebral areas.

To further assess the neuroprotective potency of H₂, our major aims were the followings:

- 1.) We investigated the effects of BCAA on cerebrocortical microcirculation in newborn piglets both under normoxic and hypoxic conditions to study the necessity and efficacy of mechanical vessel occlusion on the severity of HI insult.
- 2.) We sought to establish and profoundly characterize (i.e. metabolic-, haemodynamic-, cerebrocortical perfusion- along with brain electrical activity changes) a new PA/HIE piglet model aiming to approach the human clinical conditions. In addition, we obtained unique data on the brain interstitial pH (pH_{brain}) changes during asphyxia and the subacute phase of HIE.
- 3.) Using our new PA/HIE model we sought to test the potential neuroprotective effects of molecular H₂ administration, based on electroencephalography (EEG) and neuropathology analysis.
- 4.) Finally, we assessed the effect of H₂ on cortical oxidative neuronal damage to test whether the effects of H₂ are in accordance with its proposed antioxidant mechanism of action.

MATERIALS AND METHODS

1. Animals

Our experiments were performed in newborn (~1-day-old) male Large-White piglets (body weight: 1.5-2.5 kg, n=48). Anaesthesia was induced by intraperitoneal injection of sodium thiopental then piglets were intubated via tracheotomy and artificially ventilated (fraction of inspired O₂ [FiO₂]: 0.21, respiratory rate [RR]: 30-35 l/min, peak inspiratory pressure: 120-135 mmH₂O) with warmed, humidified synthetic medical air. The right femoral vein was catheterized for continuous infusion of morphine, midazolam and fluids throughout the experiments. A second catheter was placed either into the right femoral artery in piglets, subjected to BCAA, or into the right carotid artery in piglets, subjected to PA/HIE research, for continuous monitoring of mean arterial blood pressure (MABP) and heart rate (HR). To elicit BCAA, remotely controlled vascular occlusion cuffs were secured around both exposed common carotid arteries.

During the entire experiment, the electrocardiography, MABP, HR and O₂ saturation were continuously monitored and recorded from all experimental groups. Core temperature was maintained in the physiologic range (38.5±0.5°C) by servo-controlled heating lamp. Regular arterial blood samples served for blood gas and metabolite analysis.

After suturing the surgical incisions, animals were divided into 6 groups:

- 1) in group 1 (n=7) we evaluated the effects of BCAA (2-2 min) on cerebrocortical microcirculation by laser speckle contrast imaging (LSCI) under various ventilation conditions.
- 2) in group 2 (n=7) time control animals after the operation survived 24 hours.
- 3) in group 3 (n=8) animals were asphyxiated (20 min, 6%O₂-20%CO₂) and reventilated with room air for 24 hour survival.
- 4) in group 4 (n=8) animals after PA were reventilated with a gas mixture containing 2.1% molecular H₂ during the first 4 hour of the observation period, then with room air for the remaining 20 hours.
- 5) group 5 (n=5) served for evaluating the effects of our experimental asphyxia on cerebrocortical microcirculation by LSCI.
- 6) Finally, in group 6 (n=13), pHbrain changes were determined in our new PA/HIE model.

2. Laser speckle contrast imaging (LSCI) and analysis (LASCA)

Animals of groups 1 and 5 were placed in prone position with the head fixed in a stereotactic frame then a closed cranial window with 3 injectible ports was inserted over the left parietal region. The subarachnoidal space under the cranial window was filled with warmed, pH equilibrated, artificial cerebrospinal fluid through the ports. Our custom-designed speckle imager and the LASCA software allow to monitor rapid changes in tissue perfusion by determining the autocorrelation decay time (τ) of interference patterns produced by laser light (laser speckle) scattered from moving particles (red blood cells). The average velocity of the moving particles is directly proportional to $1/\tau$. Accordingly, the cranial window was illuminated with a polarized light

($\lambda=808$ nm, 200 mW) and speckle images were recorded (1 Hz, 2 ms) through an operating microscope by a monochrome camera, visualised online with a custom-made software in LabVIEW and stored on a personal computer. LASCA was performed offline; regions of interests (5x5 pixels [$\sim 100 \mu\text{m}^2$], 4 for each LSI image in each animal) were selected over the pial arteries (in group 1) and over the cortical parenchyma (in groups 1 and 5) not obstructed by surface pial vessels. First, the determined $1/\tau$ values were averaged/animal, next, normalized for the baseline, and then the data were expressed as relative changes from baseline. Pial arteriolar diameter changes were determined offline from speckle images using our custom designed software and were expressed as relative changes from baseline.

3. BCAO

Cerebrocortical perfusion was measured in animals of group 1 first, at baseline condition, then during hypoxia ($\text{FiO}_2=0.1$), finally under asphyxic condition (halted artificial ventilation). 2 mins BCAO-s were performed at baseline and in the 5th and 30th minute of hypoxia.

4. PA/HIE experimental groups

Animals for PA/HIE (groups 2., 3. and 4.) research were placed into a neonatal incubator. Vital parameters were continuously monitored and the urinary bladder was tapped by suprapubic puncture at 12 hour of survival. To avoid infections during the experiments, iv. antibiotics (Penicillin+Gentamicin) were given at the beginning and at 12 hour of survival. Seizures were treated with 1-2 bolus injections of midazolam.

After the surgical procedure, an hour recovery period allowed stabilization of the monitored physiological parameters prior obtaining baseline values. After obtaining the baseline physiologic parameters, animals were divided into 3 groups: time control group (n=7, group 2), asphyxia group (n=8, group 3) and asphyxia + H₂ treated group (n=8, group 4). PA in groups 3 and 4 was induced by switching ventilation from medical air to a hypoxic-hypercapnic gas mixture (6% O₂-20%CO₂, balance N₂) for 20 minutes, reducing the RR to 15 l/min and stopping the fluid/glucose administration. Piglets were reventilated (RR: 30 l/min) in group 3 with medical air for 24 hours, whereas in group 4 with a gas mixture containing H₂ gas (2.1% H₂, 21% O₂, balance N₂). In group 4, H₂ administration was stopped after 4 hours and ventilation with medical air was resumed till the end of the observation period.

5. EEG recordings

EEG activity in groups 2., 3. and 4. was recorded (impedance: <5kOhm, bandpass filters: 1-70 Hz, sampling rate: 250 Hz) via subcutaneously inserted silver pin electrodes above the fronto-parietal and occipital regions. Data were stored on a hard disc of a personal computer. EEG activity was analysed offline with two approaches. First, 10-minute-long EEG epochs recorded at the beginning of each hour after PA were scored using an amplitude-based, incremental scoring system where continuous and high amplitude background activities (>10 μV) were given lower scores while severely depressed and isoelectric activities (<10 μV)

received higher ones. Second, EEG power spectrum analysis of the same EEG epochs was performed and absolute band powers were calculated. Total EEG power (μV^2) (summation of bands) values were selected to quantitatively characterize the recovery of brain electrical activity following PA and to complement the semiquantitative and the more observer-dependent scoring system.

6. pHbrain measurements

pHbrain measurements were performed in animals of group 6. After fixing the head of the animals in a stereotaxis, two small circular craniotomies were made bilaterally over the fronto-parietal cortex, and the dura mater was gently removed. Proton selective microelectrode (PSM) and the self-made Ag/AgCl reference electrodes (RE) were installed ~2-2 mm deep into the exposed cortex. The PSM was calibrated before each measurement in 3 different warmed buffer solutions (pH: 6.10, 7.10, and 8.10, respectively). The potential difference between the PSM and the RE was amplified, converted to direct current then visualized and recorded online with WinEDR freeware. Evaluation of the recordings was performed offline: by applying linear regression analysis, the signals from the calibration solutions were fitted with a curve and finally, the potential difference was converted and expressed on a pH scale. As the technique allows stable continuous pHbrain measurements reliably only for 3-4 hours, different time windows were chosen to be assessed in the animals (baseline, PA and the first 4 hours of survival, 8th-14th hours and 20th-24th hours of survival)

7. Neuropathology

24 hours after the end of asphyxia, the brains were perfused with cold (4°C) physiologic saline solution through the catheterized common carotid arteries in groups 2., 3. and 4. Brains were collected and were immersion fixed in 4°C , 4% paraformaldehyde solution and further processed after two weeks. Paraffin embedded, $4\ \mu\text{m}$ sections were made from numerous brain areas. The degree of cerebrocortical neuronal damage was determined adapting a previously published scoring system: the pattern of neuronal injury (none < scattered < grouped < panlaminar) was determined in 20-20 non-overlapping fields of vision (200x magnification) in each assessed cortical region. Then, scores (0-9) were given to each region based on the frequency (% of 20 examined fields) of the most severe pattern of injury observed. The neuronal damage in the ganglionic cell layer of the cerebellum, basal ganglia, thalamus and the hippocampal CA1 and CA3 regions was assessed with cell counting (in 10, 5, 5, 3, 3 fields of vision respectively; 200x magnification). The impact of asphyxia on cerebellum, hippocampus and subcortical brain regions was expressed as the percentage of damaged neurons. In order to be able to compare the severity of asphyxia-induced cortical neuronal damage at 24 hour of survival between the present new and our previously published asphyxia method (20 min ventilation with 6% O_2 -20% CO_2 versus 8 min suspension of artificial ventilation) we re-analyzed the cortical samples from the ASPH group (n=9) of the previous study using the scoring system as well.

8. 8-hydroxy-2'-deoxyguanosine (8-OHdG) immunohistochemistry

Parietal cortex and CA1 tissue microarrays from groups 2., 3. and 4. were produced from the paraffin tissue blocks, sectioned at 4 μm and processed for 8-OHdG immunohistochemistry using an automated immunostainer. The slides were scanned and homogenous, strong nuclear 8-OHdG immunoreactivity was considered as a sign of oxidative damage. The ratio of such nuclei to the total number of cell nuclei was determined.

9. Neuron specific enolase (NSE) ELISA

1 ml arterial blood samples were taken from groups 2., 3. and 4. at regular intervals (at baseline, in the 18th minute of asphyxia and in the 4th and 20th hour of survival) into EDTA coated centrifuge tubes supplemented with 40 μl protease inhibitor cocktail procured following the manufacturer's directions. The blood samples were centrifuged at 2200 g and 4°C for 5 minutes and the plasma was transferred to fresh microcentrifuge tubes and stored at -80°C. Blood plasma NSE levels were determined using a commercially available, porcine-specific sandwich ELISA kit and a microplate reader ($\lambda= 450 \text{ nm}$)

10. Statistical analysis

Data were analysed with one-way- or two-way repeated measure of analysis of variance (RM ANOVA) followed by the Student-Newman-Keuls *post hoc* test. For non-parametric data RM ANOVA on ranks and for pairwise comparisons the Student-Newman-Keuls *post hoc* test was applied. Results were expressed as mean \pm S.E.M. Level of significance (p) was set at 0.05.

RESULTS

1. The effects of BCAA on cortical blood flow (CoBF) under different ventilation conditions.

Prior the onset of hypoxic ventilation, vital parameters of the animals (group 1.) were in the respected physiological ranges and did not differ significantly from the other experimental groups at baseline (core temperature: $38.5\pm 0.5^{\circ}\text{C}$, O_2 saturation: $94\pm 1\%$, MABP: 79 ± 3 mmHg and HR: 163 ± 11 1/min).

Hypoxia ($9\%\text{O}_2$, N_2 , 35 min) resulted in acidosis (pH_a : from 7.42 ± 0.03 to 7.29 ± 0.04), hypoxemia (P_aO_2 : from 79 ± 8 to 22 ± 2 mmHg) but normocapnia (P_aCO_2 : from 37 ± 7 to 37 ± 5 mmHg) was retained. Then, room air ventilation restored blood gas values. The subsequently induced asphyxia (suspended ventilation with clamped endotracheal tube for 7 min) elicited more severe acidosis (pH_a : 6.73 ± 0.02), hypoxemia (P_aO_2 : 14 ± 3 mmHg) and hypercapnia (P_aCO_2 : 87 ± 6 mmHg).

The 1st BCAA performed at baseline did not influence CoBF. Hypoxic ventilation elicited cortical hyperemia and vasodilation, nevertheless, the BCAA-s performed in the 5th and 30th minute of hypoxia did not affect the hyperemic response.

Asphyxia resulted in significant hypoperfusion, however this ischemia was also reversible upon reventilation.

2. Characterization of the PA stress

20 min asphyxia induced a biphasic response in HR and MABP values: after the onset of asphyxia, O_2 saturation fell rapidly while HR and MABP increased simultaneously, remaining elevated for minutes during the insult. Then HR along with MABP decreased uniformly in all asphyxiated animals till the end of asphyxia. Interestingly, severe hypotension (MABP < 50 mmHg) did not develop in the asphyxiated animals.

Our PA stress was characterized by similar acidosis (pH_a : 6.78 ± 0.02), hypercapnia (P_aCO_2 : 119 ± 6 Hgmm) and hypoxemia (P_aO_2 : 26 ± 2 Hgmm) in groups 3., 4., 5. and 6. (data from group 3.) In addition, arterial blood samples at the end of the stress verified severely reduced base excess (by 17.4 ± 5.0 mmol/l), central desaturation (from $94\pm 5\%$ to $13\pm 4\%$), hyperglycemia (from 4.2 ± 0.4 mmol/l to 9.3 ± 0.9 mmol/l) and lactic acidosis (from 1.6 ± 1.0 mmol/l to 10.3 ± 2.5 mmol/l) (data from group 5.)

The EEG became isoelectric within 1-2 minutes after the onset of asphyxia and it is noteworthy that CoBF increased and remained elevated during the asphyxic insult (group 5.). Finally, pH_{brain} decreased progressively and robustly during the hypoxic-hypercapnic insult from 7.08 ± 0.02 to 5.75 ± 0.27 that largely exceeded the pH drop in the arterial blood (pH_a : from 7.53 ± 0.27 to 6.79 ± 0.07) in this group (6.).

3. Characterization of the HIE development

Cardiovascular instability in 1-1 piglets (at 8 and 12 hours, respectively) in groups 3. and 4. resulted in a 12.5% (2 out of 16 animals) mortality rate, consequently, data of these animals were excluded from the results.

Reventilation from asphyxia, irrespective of the composition of the gas mixture, elicited a quick restoration of O_2 saturation followed by uniform elevation in HR and MABP in all experimental groups with an immediate reactive hyperaemic response in CoBF changes revealed in group 5. Vital parameters remained

in the respective physiologic ranges during the entire experiment and did not show statistical difference between groups. Interestingly, only HR was markedly elevated in group 3 and statistically different from the time control animals.

Although blood lactate levels were still elevated 1 hour after the end of asphyxia (6.42 ± 2.39 mmol/l), they returned to baseline by the 4th hour of survival. Brain electric activity was gradually restored in piglets reventilated with room air containing 2.1% H₂ (group 4), resulting in lower EEG scores and statistically higher total EEG power, whilst EEG remained flat (high EEG scores with low power) in group 3. It is noteworthy, that 2 animals in this group manifested electro-clinical convulsions at the 9th and 13th hour of survival.

PA did not induce elevations in plasma NSE levels that were 96.2 ± 6.7 % and 106.1 ± 36 % of baseline levels at 4 and 20 hour of survival, respectively (group 3, n=6).

Finally, pH_{brain} (group 6.) was gradually restored within 1-2 hours under room air reventilation and marked pH_{brain} alterations were not observed during the subsequent survival period (between 8-14 and 20-24 hours).

4. Neuropathology results

Histology analysis of group 2. revealed minimal brain injury displaying low-frequency occurrence of scattered damaged neurons. Asphyxia induced extensive brain damage by 24 hour of survival, indicated by laminar or even confluent panlaminar necrotic areas in the cortical areas and by the high incidence of damaged neurons in other brain regions (group 3).

Molecular H₂, administered during the first 4 hour of reventilation (group 4.), alleviated the cortical damage and preserved neurons also in other examined areas.

For comparison, we applied the neuropathology scoring system on the asphyxia group from our previously reported 8 min PA model. We found that the summated cortical histopathology scores of that group were significantly smaller than the values of our current new PA/HIE model (17;10;21 vs. 32;20;36; median; 25th – 75th percentiles, respectively). We conclude that our new model could elicit more robust cortical damage compared to the previously employed PA method.

In addition, 8-OHdG immunohistochemistry analysis revealed intensive nuclear staining in the parietal cortex (73 ± 7 %) and the hippocampal CA1 (46 ± 14 %) subfield in the asphyxiated animals (group 3.). 2.1% H₂ reventilation (group 4.) reduced oxidative stress indicated by the lower incidence of immunopositive nuclei (34 ± 9 % and 7 ± 6 %, respectively).

DISCUSSION

The major findings of the studies were the followings:

- 1) BCAA *per se* does not influence cerebrocortical perfusion and pia arteriolar blood flow velocity during different ventilation (normoxic and hypoxic) conditions in newborn piglets.
- 2) We introduced a novel PA/HIE newborn piglet model. By characterizing our model, we demonstrated all the major metabolic hallmarks of PA (hypoxemia, hypercapnia with mixed acidosis) to be present in a translationally relevant degree. Although the reventilation restored blood gases along with the metabolic (i.e. glucose, lactate) parameters within hours, HIE development was clearly demonstrated on one hand, by the severely depressed brain electrical activity throughout the observation period, and on the other hand, by the detection of extensive neuronal damage revealed by neuropathology analysis.
- 3) We successfully employed ion-selective electrodes to study pHbrain changes over the subacute phase of HIE development in a large animal model. We found that (1) pHbrain dropped to extremely low values during asphyxia exceeding the blood acidosis by more than 1 pH unit, (2) after the gradual restoration of pHbrain, there were no secondary pHbrain alterations despite the development of HIE at least during the 24 hour survival period.
- 4) Administration of molecular H₂ starting immediately after PA afforded neuroprotection assessed in the subacute phase (1st day) of HIE, indicated by the restoration of brain electric activity and the better preservation of neuronal viability in various cortical and subcortical brain areas. Moreover, H₂ exerted its neuroprotection likely through alleviation of the asphyxia-induced oxidative stress shown by the reduction of 8-OHdG immunopositive neuronal nuclei in the assessed regions: the parietal cortex and the hippocampal CA1 subfield.

PA/HIE is under the active scope of research in the field of neonatology. In order to decrease PA/HIE related mortality and disability, advanced resuscitation protocols, neuroprotective interventions and innovative diagnostic tools are required. Preclinical animal experiments offer the chance to study the pathomechanisms of HIE and to test putative neuroprotective approaches.

The newborn piglet, as a deputy of the term neonate, is one of the most accepted large animal models in paediatric research since the 1980-s. Historically, first quite unsophisticated swine PA models were introduced, for instance by covering the head of the animals with a face mask. Today, ventilation with reduced FiO₂ gas mixtures often combined with BCAA is the most popular method to induce experimental PA. We are aware of only one comparative study that found no difference in the severity of neuronal damage induced by hypoxia alone or hypoxia+BCAA. Our results showed that BCAA did not affect cortical blood flow in accordance with these results. This result might be surprising at first, but may be explained by the unique cerebrovascular architecture of the pig profoundly different from the human anatomy. In the pig, the circle of Willis arises from a complex vascular network, the so-called *rete mirabile*. The intracranial part of the *rete* is

embedded in the cavernous sinus around the oval foramen of the skull from which the reconstituted internal carotid artery joins the circle. This *rete* represents a dense network of small arterial arborisation receiving afferent branches from the ascending pharyngeal artery, and from other extra- and intracranial arteries, offering rich vascular communication along both sides of the skull. Haaland *et al.* along with Burbridge *et al.* using angiography, described in great detail the well-developed anastomosis system between the intra- and extracranial arteries of the pig. They concluded that extracerebral artery ligation would likely not induce focal cerebral ischemia in pigs. To the best of our knowledge, our current results reported the effects of different ventilation conditions with BCAA on the cortical microcirculation for the first time in newborn pigs. Our LSCI/LASCA measurements revealed unchanged cerebrocortical blood flow in response to BCAA under normoxic or hypoxic ventilation. Consequently, BCAA likely does not affect HIE severity in a significant matter in this species.

Based on our previous experience and pilot studies, we established a new PA/HIE piglet model focusing on the critical metabolic hallmarks of PA (mixed acidosis, hypoxia and hypercapnia) characterizing both the human as well as the naturally occurring porcine PA conditions. Development of HIE in our model was confirmed by the severely depressed EEG activity. It was reported that the ability of the Apgar scores with the umbilical blood gas values is weak to predict safely the severity and outcome of HIE, thus, EEG is thought to be an important method to identify early and accurately sick babies who could benefit from neuroprotective interventions. The prognostic value of EEG is well-known since 1972 when Monod *et al.* reported a retrospective EEG study on 270 asphyxiated children, proclaiming that normal neonatal EEG has favourable prognostic significance for good outcome in childhood. Since then, numerous clinical studies could confirm the good predictive value of early (during the first 24-72 hours of life) EEG recordings, therefore, assessing brain electric activity has become a routinely applied method in the field of neonatology. Nevertheless, the interpretation of neonatal electric patterns requires trained neurophysicians because of many reasons. First, cortical electric activity varies progressively with postnatal development, second, there is no clear consensus how to grade EEG patterns in neonates with HIE, and in addition, some anticonvulsants interfere with the EEG waves and suppress brain electric activity sometimes entirely. Thus, to quantify EEG patterns, numerous scoring systems were introduced, principally based on the amplitude of the waves, the continuity, the hemispheric asymmetry, the presence or absence of sleep-wake cycles, seizures along with special EEG features. In the currently presented PA/HIE piglet model, a subjective and an objective analytical method both could confirm the presence of HIE, moreover, the beneficial effects of molecular H₂.

Alterations in pH_{brain} can also significantly affect neuronal injury. Regulation of pH in the brain is finely tuned under physiologic conditions, thus, perturbations in extracellular pH would highly influence neuronal excitability. *In vitro* studies reported that acidosis suppresses the activity of the N-methyl-D-aspartate (NMDA) receptor ion channel, meanwhile alkalosis facilitates ion currents through the NMDA receptors in rat hippocampal neurons. Moreover, environmental pH changes similarly modulate the conductance and gating

properties of voltage-gated Na⁺ and Ca⁺⁺ ion channels in rat hippocampal cells. Helmy *et al.* reported that asphyxia, induced by hypoxic-hypercapnic (9% O₂-20% CO₂) gas mixture in spontaneously breathing rat pups, elicited cerebral acidosis and interestingly, during reventilation, the restored pH_{brain} shifted progressively towards alkalosis that was associated with the increased incidence of seizures in the pups. Because of its importance to determine neuronal injury, numerous *in vivo* studies recorded pH_{brain} changes in various HI animal models. However, quantitative data on pH_{brain} changes during/after PA in large animal models are very scarce in the literature, thus, our current observations provide important new information to the pathophysiology of HIE in piglets. We are aware of only one previous study that assessed pH_{brain} with similar technique after PA in piglets. In this study, Bender *et al.* reported pH_{brain}: 6.26±0.14, almost half pH unit higher than in our study, likely at least in part due to the much lower P_aCO₂ values (61±1 vs. 160±23 mmHg, vs. present study, respectively). In addition, those experiments contained numerous components facing the current neonatal resuscitation guidelines, worsening the outcome of PA, i.e. resuscitation was initiated by 100% O₂ and iv. bicarbonate solution was administered to counteract acidosis. More importantly, the study did not follow up on pH_{brain} changes beyond 4-hour survival, in contrast, our current measurements extended the observation period considerably, to 24 hours of survival. Our results suggest that pH_{brain} alterations play an important role in the pathophysiology of HIE development as very severe acidosis during the acute phase of asphyxia, and possibly brain alkalosis after the first 24-hour of reventilation. This suggestion is corroborated by observations in human neonates. Magnetic Resonance Spectroscopy (MRS) is a promising method to measure and to follow mainly intracellular pH changes in the brain. Using MRS, Hope *et al.* reported no significant changes in brain pH (7.13±0.05) from term asphyxiated newborns during the first day of life that essentially match our experimental results in the present study. Importantly, brain pH rose during the following days indicating the belated development of brain alkalosis. Robertson *et al.* reported also brain alkalosis appearing in term neonates in the first 2 weeks of life after PA. The extent of neonatal brain injury showed good correlation with the elevation of cerebral pH that persisted for months afterwards, corresponding to poor neurodevelopmental outcome. Interestingly, Chopp *et al.* reported in adult rats that forebrain ischemia also triggered post-ischemic cerebral alkalosis appearing first only after 24 hour of survival and was still present at 48 hours. Dynamics of pH_{brain} alterations in response to HI stress can thus clearly show species, and age specific variations. The large animal piglet HIE model may follow a more similar course to the human, and may be markedly different from the rat.

H₂ is a colourless, odourless, tasteless and nontoxic gas, that can be abundantly produced from water hydrolysis or chemical reactions and, thus, easily affordable and available in contrast to Xe. Although H₂ is known to be flammable in air, it cannot combust below 4% and more importantly, the optimal neuroprotective concentration around 2% (according to Ohsawa *et al.*) could be safely administered even in a hospital settings. The major advantages of the gas are the small molecular diameter along with the amphiphilic solubility, thus, H₂ can be dissolved in aqueous solutions and be able to penetrate biological membranes as well as the cell nucleus or mitochondria, moreover, it is able to penetrate easily the blood-brain-barrier as well. Ono *et al.*

reported that 30 minute inhalation of a gas mixture containing 2-4% H₂ in air raises blood plasma H₂ levels dose dependently to 10-20 µmol/l. We administered H₂ mixed in medical air as in sedated and artificially ventilated patients this method offers the easiest way to administer the neuroprotectant. Notably, infusion and oral consumption of H₂ rich saline solution were also proved to be safe in other clinical studies, i.e. in patients surviving stroke. In addition, oral administration of lactulose can dramatically induce bacterial fermentation and contribute to endogenous H₂ production as well.

Probably, the most important result of the present thesis is administering molecular H₂ after experimental PA can afford neuroprotection in our large animal HIE translational model. Unfortunately, the exact molecular target of the H₂ is still not revealed. In the seminal work of Ohsawa *et al.*, cell free experiments showed that H₂ selectively decreases the levels of hydroxyl and peroxynitrite radicals, whilst the levels of other reactive species (i.e. hydrogen peroxide, superoxide anion or nitric oxide) remained unchanged. The selective ability of the gas to neutralize principally the hydroxyl radical is supposed by the strongest oxidative strength of the radical. Due to these advantageous physicochemical properties of H₂, numerous research groups tested molecular H₂ in various HI/reperfusion animal models, focusing on its anti-oxidant, anti-inflammatory and anti-apoptotic effects. By today, H₂ was introduced to almost every field of research where oxidative stress played an important role in cellular damage. For instance, in a myocardial ischemia rat model, 2% H₂ inhalation significantly improved left ventricular contraction meanwhile reduced the infarct size and the level of oxidative injury, assessed by 8-OHdG immunohistochemistry staining. In addition, H₂ rich saline markedly ameliorated the renal functions after experimental ischemia in a rat model, moreover, it decreased the level of the pro-inflammatory cytokines and alleviated oxidative stress by decreasing the tissue levels of malondialdehyde (MDA) and 8-OHdG. In an other model of retinal ischemia/reperfusion injury, daily H₂ inhalation for a week significantly preserved the functions of retinal ganglion cells in rats by inhibiting the overexpression of inflammatory cytokines. Furthermore, molecular H₂ was found to be neuroprotective in various middle cerebral artery occlusion rat models as well as H₂ administration markedly decreased the infarct size, attenuated oxidative injury, indicated by the decreased levels of MDA immunostaining, caspase-3 staining along with the number of 8-OHdG positive cells in the infarct area. Finally, H₂ was reported to increase the activity of anti-oxidant enzymes. Our results correlate well to other published observations as the immunohistochemistry analysis of 8-OHdG staining verified alleviated oxidative damage in the H₂ treated group.

Our research effort focused on assessing the putative neuroprotective effect of molecular H₂ in a translational model of moderate/severe HIE. To reach this goal, we first critically examined the methodology and the translational potential of previously published piglet PA/HIE models. During this process, we demonstrated that BCAO in the newborn piglet does not affect the cerebrocortical microcirculation i.e. it does not induce significant ischemia under various ventilation conditions. Thus, the usefulness of mechanical vessel occlusion to augment the severity of PA insult has been questioned. We then successfully designed a subacute

HIE model, where anaesthesia/analgesia and the methodology to induce PA have been chosen to maximize the translational potential of the model. Our new PA/HIE model has been painstakingly characterized: changes in metabolism, hemodynamic parameters, brain electrical activity and uniquely also pH_{brain} were determined, and demonstrated to comply with the data obtained from neonates. Our pH_{brain} measurements yielded that acidosis during PA may be 10 times (1 pH unit) higher compared to the arterial blood. Neuropathology revealed that our new model was capable inducing severe and reproducible structural brain damage in accordance with the suppression of brain electric activity. Using this established PA/HIE model we demonstrated that reventilation with 2.1% molecular H₂ facilitated the recovery of EEG amplitudes and ameliorated neuronal damage virtually in all examined areas. The mechanism of molecular H₂ action moreover appears to include alleviated oxidative stress, indicated by the lower number of 8-OHdG immunopositive nuclei in the assessed regions. Our data suggest a marked neuroprotective potential of post-insult administered molecular H₂, therefore we aim to assess the neuroprotective effects of H₂ combined with TH in the future.

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PUBLICATIONS RELATED TO THE SUBJECT OF THE THESIS

Domoki F, Zolei-Szenasi D, Olah O, Toth-Szuki V, Nemeth J, Hopp B, Bari F, Smausz T (2014) Comparison of cerebrocortical microvascular effects of different hypoxic-ischemic insults in piglets: a laser-speckle imaging study. *J. Physiol. Pharmacol.* 65(4): 551-8.

Nemeth J, Toth-Szuki V, Varga V, Kovacs V, Remzso G, Domoki F (2016) Molecular hydrogen affords neuroprotection in a translational piglet model of hypoxic-ischemic encephalopathy. *J. Physiol. Pharmacol.* 67(5): 677-689.