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MOLECULAR HYDROGEN AFFORDS NEUROPROTECTION IN A TRANSLATIONAL PIGLET MODEL OF HYPOXIC-ISCHEMIC ENCEPHALOPATHY

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Hypoxic-ischemic encephalopathy (HIE) is the major consequence of perinatal asphyxia (PA) in term neonates. Although the newborn piglet is an accepted large animal PA/HIE model, there is no consensus on PA-induction methodology to produce clinically relevant HIE. We aimed to create and to characterize a novel PA model faithfully reproducing all features of asphyxiation including severe hypercapnia resulting in HIE, and to test whether H_2 is neuroprotective in this model. Piglets were anaesthetised, artificially ventilated, and intensively monitored (electroencephalography, core temperature, O_2 saturation, arterial blood pressure and blood gases). Asphyxia (20 min) was induced by ventilation with a hypoxic-hypercapnic ($6\%O_2 – 20\%CO_2$) gas mixture. Asphyxia-induced changes in the cortical microcirculation were assessed with laser-speckle contrast imaging and analysis. Asphyxia was followed by reventilation with air or air containing hydrogen ($2.1\%H_2$, 4 hours). After 24 hours survival, the brains were harvested for neuropathology. Our PA model was characterized by the development of severe hypoxia ($pO_2 = 27 \pm 4$ mmHg), and combined acidosis (pH = 6.76 ± 0.04; $pCO_2 = 114 \pm 11$ mmHg; lactate = 12.12 ± 0.83 mmol/L), however, cortical ischemia did not develop during the stress. Severely depressed electroencephalography (EEG), and marked neuronal injury indicated the development of HIE. H_2 was neuroprotective shown both by the enhanced recovery of EEG and by the significant preservation of neurons in the cerebral cortex, hippocampus, basal ganglia, and the thalamus. H_2 appeared to reduce oxidative stress shown by attenuation of 8-hydroxy-2'-deoxyguanosine immunostaining. In summary, this new PA piglet model is able to induce moderate/severe HIE, and the efficacy of hydrogen post-treatment to preserve neuronal activity/function in this PA/HIE model suggests the feasibility of this safe and inexpensive approach in the treatment of asphyxiated babies.

Key words: *hydrogen, neuroprotection, hypoxic-ischemic encephalopathy, laser speckle contrast imaging, perinatal asphyxia, electroencephalography*

INTRODUCTION

Infections and intrapartum-related complications are the leading causes of child mortality under the age of five (responsible for ~6.3 million deaths annually worldwide), and approximately 40% of these children die during the neonatal period (1, 2). Birth asphyxia is one of the major contributor to early neonatal loss, affecting 3 – 4 newborns out of 1000 live births in developing countries, accounting for around 1 million neonatal deaths annually (3). Prolonged deprivation of oxygen around the time of delivery (4) is defined as perinatal asphyxia (PA) and it is presented with hypoxemia, hypercapnia and acidosis (5) eliciting an immediate redistribution of blood flow to the vital organs thus compromising the nutrition of other tissues. If the respiratory distress-induced compensatory mechanisms are exhausted, tissue hypoperfusion may occur, risking permanent hypoxic/ischemic damage. Hypoxic-ischemic encephalopathy (HIE) is one of the most severe consequence of PA with a wide clinical spectrum among survivors: cerebral palsy (motor skill abnormalities), seizure activity, feeding disorders as well as mental- and somatic retardation, vision-, hearing- and speech disorders that constitute a life-long social and financial burden for the health care system and affected families (6, 7). Critical care of asphyxiated newborns requires immediate and adequate resuscitation techniques (8) along with effective neuroprotective interventions to prevent the progressive deterioration of the neonatal brain function resulting in HIE. Currently mild systemic hypothermia is the gold standard neuroprotective therapy to alleviate asphyxia-induced brain damage (9-11), but clearly further efforts are needed to establish additional therapeutic approaches that can compliment the existing treatment using animal models that have already been an invaluable aid to study the pathophysiology of PA/HIE (12).

The neuroprotective application of molecular hydrogen was first employed by Ohsawa *et al.* who reported that hydrogen was a selective hydroxyl radical scavenger and was found neuroprotective in an adult rat stroke model (13). Since then, many preclinical and even clinical studies reported widespread cytoprotective, anti-inflammatory and anti-apoptotic effects of this gas (14). Concerning PA/HIE research, some (15, 16) but not all (17) studies found molecular hydrogen to be neuroprotective in rodent PA/HIE models. As far as we know,

our research group was the first to test the effect of molecular hydrogen in a large animal (newborn piglet) model following asphyxia induced by airway obstruction. We found that reventilation after 8 – 10 min asphyxia with a gas mixture containing 2.1% hydrogen gas prevented both acute (1 hour) and delayed (24 hours) neuronal-vascular dysfunction assessed by determination of PA-sensitive cerebrovascular responses (18, 19). Unfortunately, the neuronal damage induced by this stress was relatively mild to be able to fully assess the neuroprotective potential of hydrogen, as electroencephalography (EEG) recovered in 24 hours in virtually all asphyxiated animals and neuropathology revealed also only mild to moderate neuronal injury in the untreated asphyxiated animals too (19). Therefore, we sought to establish a translational moderate/severe PA/HIE piglet model that reproduced all clinical features of PA. Although, the newborn piglet has become an accepted large animal model of the human term neonate, the precise methodology to elicit a clinically feasible experimental PA remains undetermined. Numerous piglet PA models were employed in the past decades, ranging from bilaterally induced pneumothorax (20), suspended ventilation (21) or raised intracranial pressure to produce selective cerebral ischemia (22) offering limited clinical feasibility in terms of human PA pathophysiology. Recently, hypoxic ventilation/reoxygenation followed by airway obstruction (the Johns Hopkins model (23)) or hypoxic ventilation with bilaterally occluded carotid arteries (the University College London model (24)) are extensively applied methods (i.e. 6% O_2 for 30 min. (25)). We have recently shown with laser speckle contrast imaging (LSCI), that bilateral carotid artery occlusion does not reduce cortical blood perfusion in piglets (26) in accordance with the unique anatomy of extracerebral large arteries (27), although, bilateral carotid artery occlusion (28) or 4-vessels occlusion (29) will induce true cerebral ischemia in rats. Furthermore, hypoxic ventilation alone does not induce severe hypercapnia that could critically affect the development of seizures (30) - a common complication in clinical management of PA/HIE.

The purpose of the present study was twofold: 1) to establish and to characterize a clinically relevant and reproducible PA/HIE piglet model that reproduces the human conditions well. To describe the developing asphyxia in detail, we also determined cortical blood flow (CoBF) changes during asphyxia and in the acute reventilation period; 2) to test the putative neuroprotective effect of hydrogen in our severe PA/HIE model. We also performed a meta-analysis on the structural brain damage between the current and our previously reported PA model (19).

MATERIALS AND METHODS

Animals

Newborn (~1 day old) male Large-White piglets (body weight: 1.5 – 2.5 kg, n = 28) were obtained from a local company (Pigmark Ltd., Co., Szeged, Hungary) and delivered to the laboratory on the morning of the experiments. All procedures were approved by the Animal Care and Use Committee of the University of Szeged. Anaesthesia was induced by intraperitoneal injection of sodium thiopental (45 mg/kg; Sandoz, Kundl, Austria). Piglets were intubated *via* tracheotomy and artificially ventilated with warmed, humidified medical air (21% O_2, balance N_2) that could be supplemented with oxygen *via* a pressure-controlled neonatal ventilator. Respiratory settings (fraction of inspired O_2 (FiO_2): 0.21 – 0.25; respiratory rate (RR): 30 – 35 1/min, peak inspiratory pressure: 120 – 135 mmH_2O) were adjusted to maintain blood gas values and oxygen saturation in the physiologic range. The right femoral vein was catheterized under aseptic conditions to maintain anaesthesia/analgesia with a bolus injection of morphine (100 µg/kg; Teva, Petach Tikva, Israel) and midazolam (250 µg/kg; Torrex Pharma, Vienna, Austria), then with continuous infusion of morphine (10 µg/kg/h), midazolam (250 µg/kg/h) and fluids (5% glucose, 0.45% NaCl 3 – 5 ml/kg/h) throughout the whole experiment, as artificial ventilation-induced stress (31) affects the neuro-endocrine system in intubated neonates. A second catheter was placed into the right carotid artery for continuous monitoring of mean arterial blood pressure (MABP) and heart rate (HR). This artery was chosen as ligation of the femoral artery resulted in critical ischemia of the hindlimb over the chosen survival period (personal observations), in contrast, unilateral carotid artery occlusion has been shown to unaffect cerebral blood flow (32). After suturing the surgical incisions, animals were placed into a neonatal incubator (SPC 78-1; Narco Air-Shields, Inc., Hatboro, Pa., USA) in prone position then supplied with electrocardiograph (ECG) and electroencephalograph (Natus Neurology, Middleton, WI, USA) electrodes. Rectal temperature was measured continuously and kept in the physiologic range (38.5 ± 0.5°C) with a servo-controlled heating lamp. Oxygen saturation, MABP, HR and ECG were continuously monitored using a Hewlett-Packard M1094 monitor (Palo Alto, California, USA) and recorded online (MecifView, Arlington, Mass., USA). Prophylactic antibiotics were given intravenously (i.v.): penicillin (50 mg/kg/12 h, Teva, Petah Tikva, Israel) and gentamicin (2.5 mg/kg/12 h, Sanofi, Paris, France). Seizures were treated with 1 – 2 bolus injections of midazolam (250 µg/kg) according to (33). The urinary bladder was tapped by suprapubic puncture at 12 hour of survival. Arterial blood samples (~300 µl/sample) were checked (ABL 5, Radiometer, Denmark or EPOC Blood Analysis, Epocal Inc., Ottawa Canada) at baseline, at the end of asphyxia; then at selected intervals up to 20 hours to keep blood gas values in the physiological range (*Fig. 1*). After the surgical procedure, one hour recovery period allowed stabilization of monitored physiological parameters prior obtaining baseline values. These parameters were recorded then during PA and for 10 minutes at the beginning of each survival hour.

Electroencephalography (EEG)

EEG activity was recorded via subcutaneously inserted silver pin electrodes above the fronto-parietal and occipital regions. The impedance of all electrodes was checked to be below 5 kΩ. Bandpass filters were applied between 1 – 70 Hz, and an automatic noise filter (notch filter) rejected the 50 Hz component. EEG signal was amplified (Nicolet EEG v32, Natus Medical Inc, San Carlos, California, USA), recorded (sampling rate: 250 Hz) and visualized online during the entire experiment with the manufacturer's software (Nicolet One). Data were stored on a hard disc of a personal computer. EEG activity was analysed offline with two approaches. First, 10 minute EEG epochs recorded at the beginning of each hour after PA were scored by a researcher unaware of the experimental group using an amplitude-based, incremental scoring system (*Table 1*), according to (34). Briefly, continuous and high amplitude background activities (> 10 µV) were given lower scores while severely depressed and isoelectric activities (< 10 µV) received higher ones. In addition, if seizures appeared in the evaluated hour, 2 extra points were added. Second, EEG power spectrum analysis of the same EEG epochs was performed by the NicoletOne Review software using Fast Fourier Transformation (FFT, linear detrending, Hamming window; δ 1 – 4 Hz, θ 4 – 8 Hz, α 8 – 12 Hz, β 12 – 30 Hz) 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.

Experimental perinatal asphyxia (PA)

After obtaining the baseline physiologic parameters, animals were divided into 3 groups (*Fig. 1*): time control group (CTR, n = 7), asphyxia group (ASPH, n = 8) and asphyxia + hydrogen treated group (ASPH + H_2, n = 8). Animals were randomized between groups ASPH and ASPH + H_2 by coin flip. PA in groups ASPH and ASPH + H_2 was induced by switching ventilation from medical air to a hypoxic-hypercapnic gas mixture (6% O_2, 20% CO_2, balance N_2) for 20 minutes, reducing the RR to 15 1/min and stopping the fluid/glucose administration. Piglets were reventilated (RR: 30 1/min) in group ASPH with medical air, whereas in group ASPH + H_2 with a gas mixture containing hydrogen gas (2.1% H_2, 21% O_2, balance N_2). In group ASPH + H_2, after 4 hours hydrogen treatment was stopped and ventilation with medical air was resumed. An additional group of animals (group LSCI, n = 5) was added to assess CoBF changes during asphyxic conditions. Animals received similar anaesthesia and instrumentation regimen as the other groups then 20 minute asphyxia was induced as in groups ASPH and ASPH + H_2 followed by room-air reventilation and short-term survival. Details of LSCI methodology are given in the respective section below.

Histology

Twenty-four hours after the end of asphyxia, the brains were perfused with cold (4°C) physiologic saline through the catheterized common carotid arteries. Brains were gently collected and were immersion-fixed in 4°C, 4% paraformaldehyde solution and further processed after two weeks. Paraffin embedded, 4 µm sections were made from the frontal, temporal, parietal, occipital lobes. The haematoxylin-eosin stained slides were evaluated by a researcher blinded to the experimental groups with light microscopy (Leica Microsystems, Wetzlar, Germany). Damaged neurons were identified using the major hallmarks of dark eosinophilic cytosol, as well as pyknotic or disrupted nuclei. The degree of cerebrocortical neuronal damage was determined adapting a previously published scoring system (*Table 2*) (35) that allowed more comprehensive study of these large brain regions compared to the cell counting used in our previous studies (18, 19). Briefly, the pattern of neuronal injury (none < scattered < grouped < panlaminar) was determined in 20 – 20 non-overlapping fields of vision under 20× magnification in each assessed cortical region. Then, scores (0 – 9) were given to each region based on the

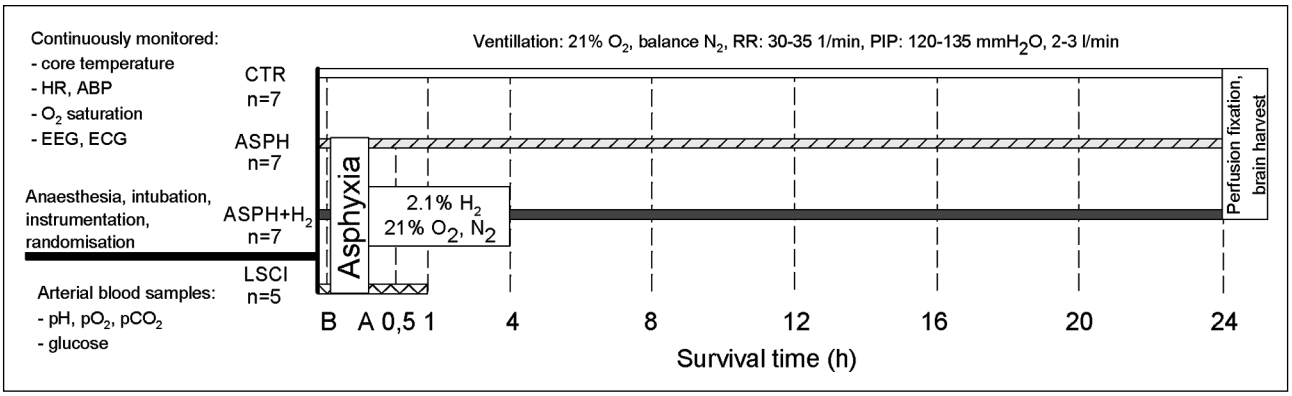

Fig. 1. Experimental protocol and groups. The putative neuroprotective effect of molecular hydrogen (H_2) against asphyxia-induced hypoxic-ischemic encephalopathy was assessed in 3 groups. After instrumentation and recording of baseline (B) physiological parameters (core temperature, heart rate (HR), arterial blood pressure (ABP) and oxygen saturation as well as ECG and EEG), animals were divided into the following experimental groups: piglets in group CTR (n = 7, white) served as normoxic time controls. Piglets in groups ASPH (n = 7, light grey, hatched) and ASPH + H_2 (n = 7, dark grey) were subjected to 20 min asphyxia (ventilation with 6% O_2, 20% CO_2, balance N_2; with a respiratory rate (RR) reduced from 30-35 to 15 1/min). Animals in group ASPH were reventilated with medical air whereas in group ASPH + H_2 by a H_2-containing gas mixture (2.1% H_2, 21% O_2, balance N_2). Ventilation in group ASPH + H_2 was switched back to medical air at 4 hour of survival. The brains were collected for neuropathology analysis at 24 hour of survival. Piglets in group LSCI (n = 5, white, crosshatched) were equipped with a closed cranial window to determine the acute effect of asphyxia on the cortical microcirculation using laser speckle contrast imaging (LSCI).

Table 1. Amplitude-based scoring system was applied to visually assess brain electric activity during experimental asphyxia and the evolution of HIE. High amplitude patterns (> 10 µV) during the initial 10 minute of given time points received lower (1 – 3) scores, while severely depressed (< 10 µV) brain electric activity was given higher (4 – 7) ones. The presence of seizure activity during the evaluated period was indicated by adding 2 extra points to the assessed background activity.

Score	**Amplitude based EEG pattern**
1	> 25 µV dominating pattern
2	> 25 µV with short durations (1 – 5 s) below
3	25 – 10 µV
4	10 – 15 µV
5	Low voltage (< 5µV) with bursts (> 25 µV, 1 – 5 s)
6	< 5µV
7	practically isoelectric
+2	electro/clinical convulsion

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 non-overlapping areas (in 10, 5, 5, 3, 3 fields of vision respectively; under 20× magnification) as in (19). The impact of asphyxia on cerebellum 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 (19) asphyxia method (20 min ventilation with 6%O_2/20%CO_2 versus 8 min suspension of ventilation), we re-analyzed the cortical samples from the ASPH group (n = 9) of the previous study using the scoring system as well, and we present the combined neuropathology scores in the Results for this group as well.

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

Parietal cortex tissue microarrays were produced using a custom-made stainless steel tissue puncher (3 mm) from the paraffin tissue blocks, sectioned at 4 µm, mounted on sylanized slides and processed for 8-OHdG immunohistochemistry using a LEICA BOND-MAX automated immunostainer (Leica Microsystems). In the immunostainer, the slides were dewaxed at 72°C, and antigen retrieval was performed at pH = 6. Then, slides were incubated with a 1:200 dilution of mouse monoclonal primary antibody against 8-OHdG (JaICA Inc., Fukuroi, Japan) for 20 min followed by horseradish peroxidase-conjugated rabbit anti-mouse secondary antibody for 15 minutes. 3,3'-diaminobenzidine was used to visualize the immunostaining, then the slides were counterstained with haematoxylin, to visualize the cell nuclei. The slides were covered with a coverslip then scanned in a slide scanner (Pannoramic MIDI, 3DHISTECH Ltd., Budapest, Hungary), and visualized on a personal computer using the Pannoramic Viewer software (3DHISTECH Ltd.) at 40× magnification. Homogenous, strong nuclear 8-OHdG immunoreactivity was considered as a sign of oxidative damage and the ratio of such nuclei to the total number of cell nuclei was determined and presented in the Results.

Neuron specific enolase (NSE) ELISA

One ml arterial blood samples were taken at regular intervals (baseline, 4 and 20 hour of survival) into EDTA-coated microcentrifuge tubes supplemented with 40 µl protease inhibitor cocktail procured following the manufacturer's directions (cOmplete, EDTA-free Protease Inhibitor Cocktail, Roche Diagnostics Gmbh, Mannheim, Germany). The blood samples were centrifuged at 2200 g and 4°C for 5 minutes (Model 5418R, Eppendorf, Hamburg, Germany) 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 (Elabscience Biotechnology Co., Ltd., Wuhan, China). The measurements were performed in duplicates following the manufacturer's instructions using a microplate reader (Fluostar Optima, BMG LABTECH, Gmbh, Ortenberg, Germany) at λ = 450 nm.

Laser-speckle contrast imaging and analysis (LSCI/LASCA) to study cortical blood flow (CoBF) changes

CoBF changes induced by PA were recorded (5 min baseline, 20 min PA, 10 min room air reventilation) and assessed by LSCI/LASCA in additional animals (*Fig. 1*, group LSCI, n = 5). Our custom-designed speckle imager and the LASCA software has been described recently in details (26). Briefly, LSCI/LASCA allows 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 is directly proportional to $1/\tau$. Anaesthesia and initial surgical procedures were similar to the other groups, however, in these animals after a circular craniotomy and removal of the dura mater, a closed cranial window with 3 injectible ports was inserted over the left parietal region as described in (36). The cranial window was sealed with bone wax, cyanoacrylate and dental acrylate (Lang Dental Manufacturing Co Inc, Wheeling, IL, USA). After 45 min incubation, the subarachnoidal space under the cranial window (~3 ml chamber volume) was filled with warmed, pH equilibrated artificial cerebrospinal fluid (aCSF) through the ports. The cranial window was illuminated with a near-infrared diode laser (λ = 808 nm) and speckle images were recorded (1 Hz, 2 ms) through an operating microscope by a PL-B771F monochrome camera (PixeLINK, Ottawa, Canada), visualised online with a custom-made software and stored on a personal computer. LASCA was performed offline; regions of interests (4 for each timepoint/animal) were selected over the cortical parenchyma not obstructed by surface pial vessels. The determined $1/\tau$ values were normalized for baseline, and data were then expressed as relative changes from baseline.

Statistical analysis

Results were analysed offline and plotted using SigmaPlot (v12.0, Systat Software Inc., San Jose, CA., USA) core temperature, saturation, HR and MABP as well as arterial blood gas and metabolic parameters, cell counts, relative CoBF changes

Table 2. Cortical neuronal damage was assessed using a neuropathology scoring system according to Foster *et al.* (35) based on the occurrence of the most severe pattern observed in 20 visual fields/regions. Higher scores represent more severe neuronal damage.

Score	Morphology of cortical damage	Ratio of the most severe pattern per area
0	No damage	
1	Scattered	< 20%
2		21 – 50%
3		> 50%
4	Grouped	< 20%
5		21 – 50%
6		> 50%
7	Panlaminar	< 20%
8		21 – 50%
9		> 50%

were expressed as mean ± S.E.M. EEG scores, EEG total power and neuropathology scores were expressed as median, 25 – 75 and 5 – 95 percentiles. Normality was tested with the Shapiro-Wilk test. Parametric data were compared 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. The significance of linear regression coefficients was tested with ANOVA. Level of significance (P) was set at 0.05.

RESULTS

Characterization of the perinatal asphyxia stress

The normoxic time control animals (CTR) had physiological core temperature, MABP, HR, oxygen saturation, arterial pH, blood gas values (37), and glucose levels (*Fig. 2*) as well as continuous EEG activity throughout the experiments.

In groups ASPH and ASPH + H_2, baseline MABP, HR, oxygen saturation, arterial pH, pCO_2, pO_2 and glucose values

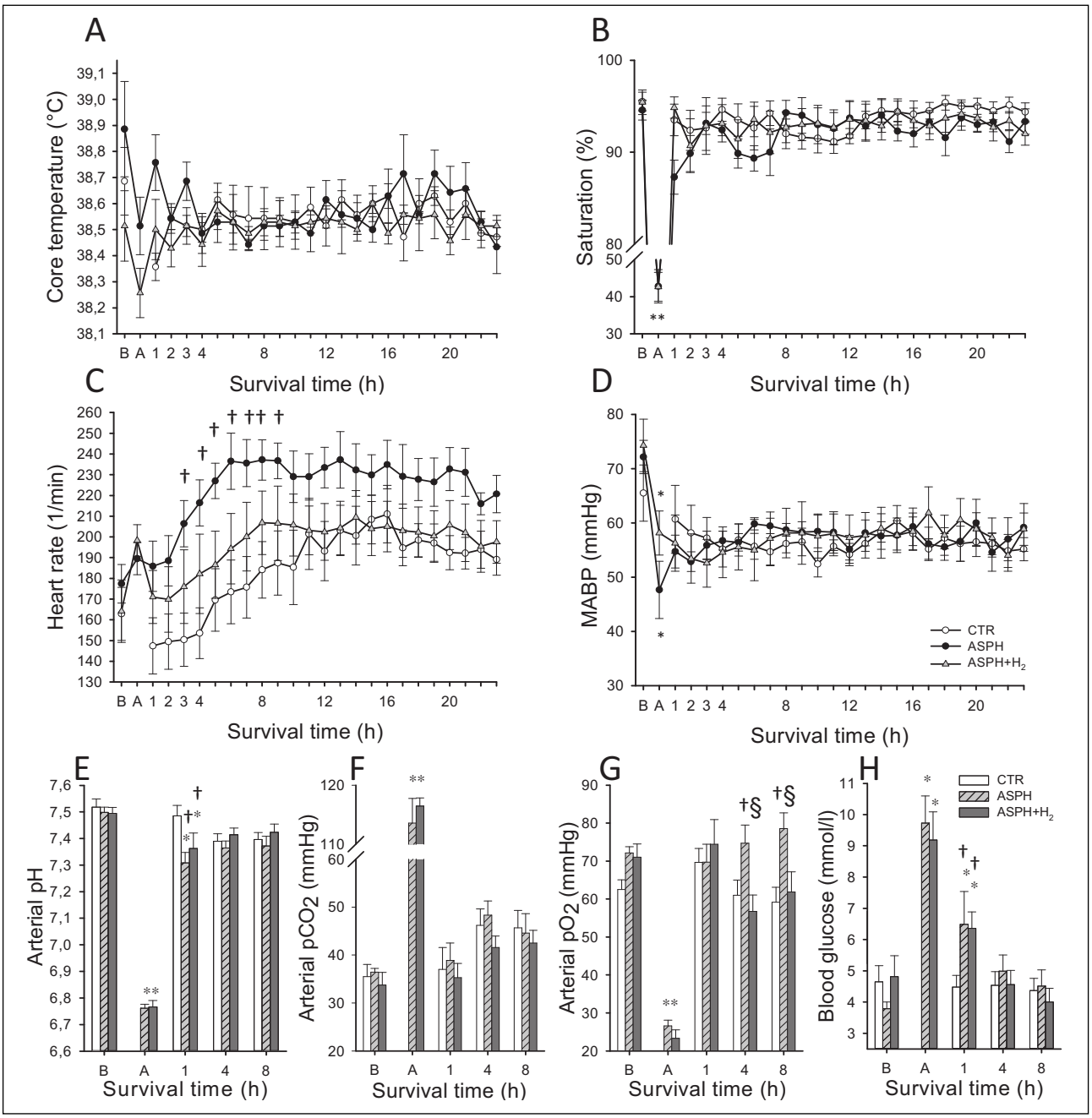

Fig. 2. Monitored physiological parameters in group CTR (n = 7), group ASPH (n = 7) and group ASPH + H_2 (n = 7) during baseline (B), in the last minute of 20 min asphyxia (A) and the survival period: core temperature (Panel A), oxygen saturation (Panel B), heart rate (Panel C), and mean arterial blood pressure (MABP) (Panel D). On the bar graphs arterial blood pH (Panel E), partial pressures of carbon dioxide (Panel F) and oxygen (Panel G), as well as glucose levels (Panel H) are presented only up to 8 hours of reventilation as there was no difference among the experimental groups at later time points and they remained in the physiological range. Asphyxia induced similar degree of acidosis, hypercapnia and hypoxia in groups ASPH and ASPH + H_2. The asphyxiated groups did not differ markedly in the monitored parameters during the survival period except that heart rate was significantly higher in group ASPH during the first 10 – 12 hours of survival (Panel C). Data are shown as means ± S.E.M. $P < 0.05$. * versus baseline, † versus time control group and § versus ASPH + H_2 at given time point.

were all similar to the time controls (*Fig. 2*). After the onset of asphyxia, however, MABP and HR were markedly raised as oxygen saturation rapidly fell (*Fig. 3*). The severe reduction in oxygen saturation in addition to the values obtained by pulsoxymetry were confirmed by blood gas analysis, in the arterial blood sample taken at the end of PA, this central oxygen saturation also fell from 94 ± 5 to 13 ± 4% (data from group LSCI). The EEG became isoelectric within 1 – 2 min after the onset of PA. In additional animals (group LSCI) using LSCI/LASCA, we found that this stress resulted in a modest (~30 – 50%) increase in CoBF (*Fig. 4*). Reventilation resulted in quick restoration of oxygen saturation with a simultaneous further increase in MABP and HR that was gradually restored (*Fig. 3*). CoBF increased also further thus showing a strong reactive hyperaemic response (*Fig. 4*).

Asphyxia resulted in severe acidosis, hypercapnia and hypoxemia along with hyperglycemia that were similar in groups ASPH and ASPH + H_2 (*Fig. 2*). In addition to the hypercapnia-induced respiratory acidosis, marked metabolic component of the acidosis was also demonstrated as blood lactate levels were profoundly elevated from 1.80 ± 0.55 to 12.12 ± 0.83 mmol/l. Blood lactate was moderately elevated at 1 hour (5.28 ± 1.06) but returned to baseline (1.33 ± 0.10) by 4 hour of reventilation (group ASPH, n = 7).

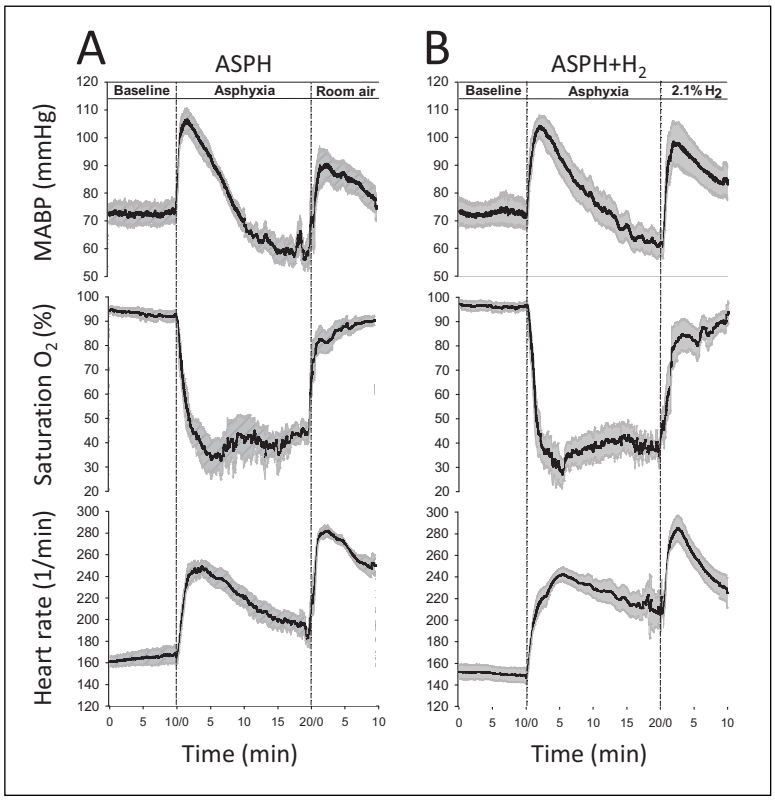

Fig. 3. Hemodynamic response to asphyxia in groups ASPH and ASPH + H_2. Asphyxia induced a biphasic response with an acute increases in mean arterial blood pressure (MABP) (top panel), and heart rate (HR) (bottom panel) as oxygen saturation (middle panel) fell indicating the onset of hypoxia. This initial increase in MABP was however not maintained, beyond 10 min moderate MABP reduction was recorded. Reventilation resulted again in a marked increase in both MABP and HR that were gradually returning towards baseline values. The hemodynamic response was nearly identical in group ASPH (n = 7; Panel A) and group ASPH + H_2 (n = 7; Panel B) indicating that hemodynamic consequences of asphyxia were similar in both groups and that hydrogen did not affect hemodynamics in the early reventilation period. Data are presented as means ± S.E.M. (black lines and the gray shaded areas, respectively).

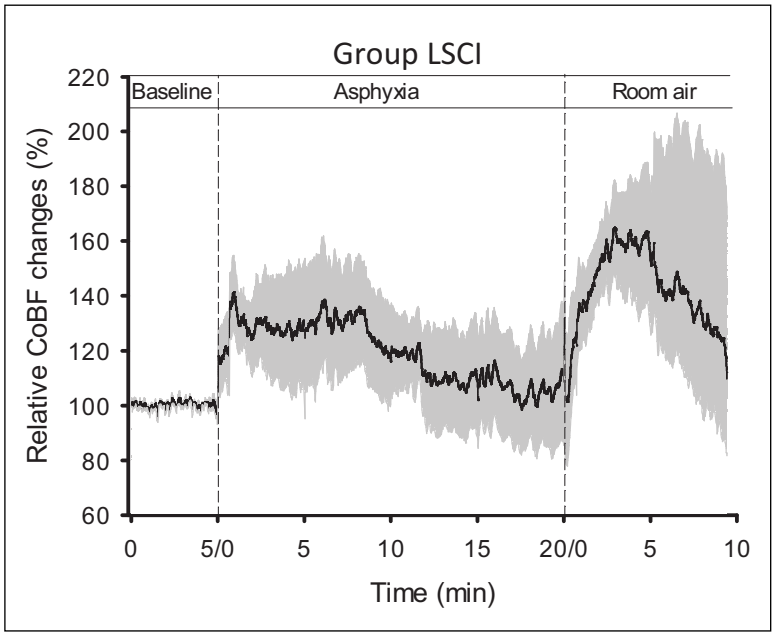

Fig. 4. Cortical blood flow (CoBF) response to asphyxia/reventilation. Laser Speckle Contrast Imaging and Analysis in group LSCI (n = 5) revealed that asphyxia elicited somewhat variable, but always moderate increases in CoBF, hypoperfusion or cortical ischemia did not develop during the stress. However, reventilation resulted in further CoBF increases representing marked reactive hyperaemia paralleling the systemic hemodynamic response. Data are presented as mean ± S.E.M. (black line and the grey shaded areas, respectively).

Hypoxic-ischemic encephalopathy development

Mortality of PA was 12.5% (2/16) as 1 – 1 piglet in groups ASPH and ASPH + H_2 were lost at 8 and 12 hours of survival, respectively, due to cardiorespiratory failure. The data from these animals were excluded from the study.

After PA, in groups ASPH and ASPH + H_2, normocapnia was restored by 30 minutes after the onset of reventilation ($PaCO_2$: 39 ± 2 mmHg and 39 ± 4 mmHg, respectively) and core temperature, MABP, oxygen saturation as well as arterial blood gas and glucose values were maintained in the respective physiological ranges and were statistically not different from the

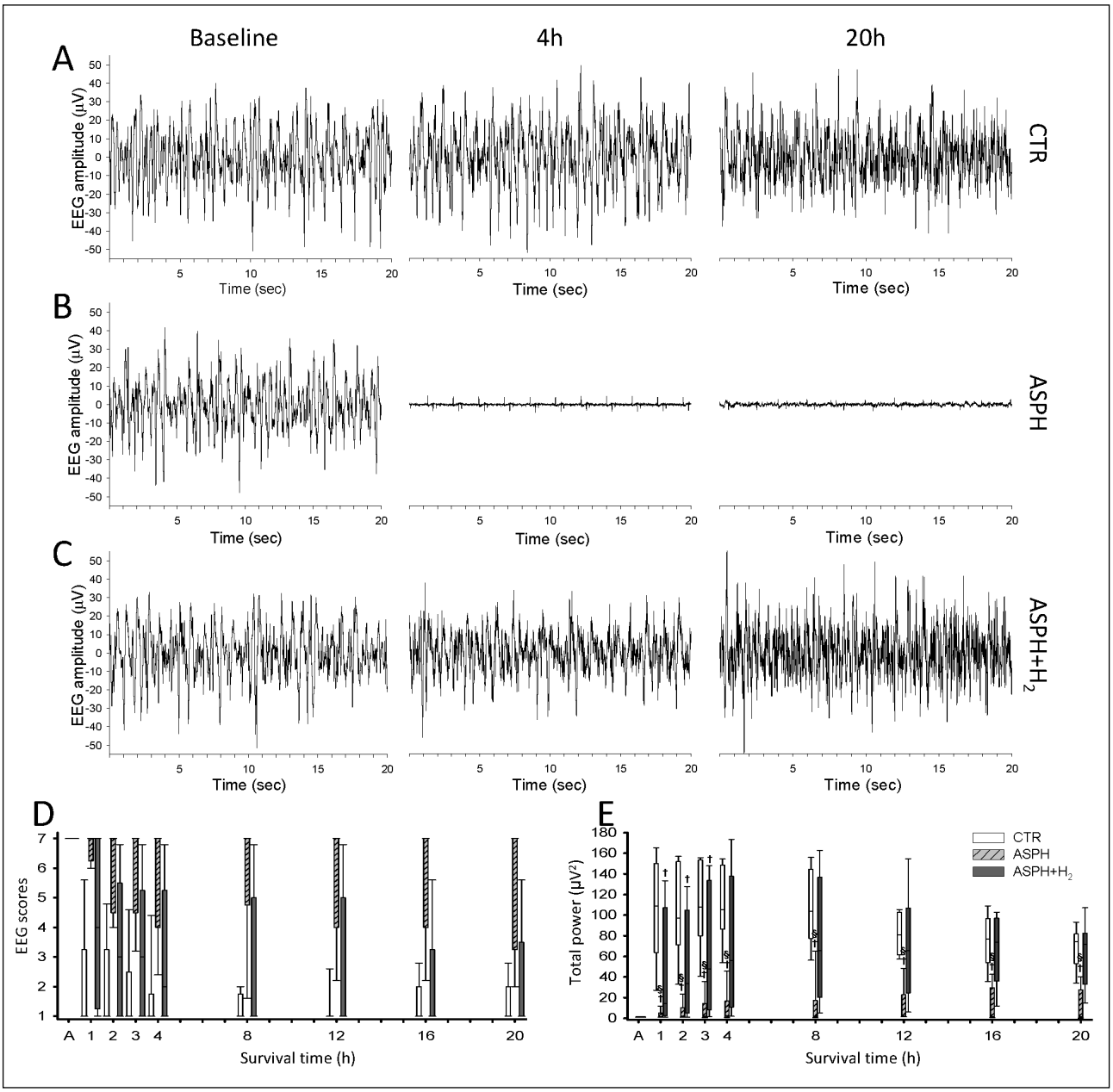

Fig. 5. Representative EEG tracings (occipital electrode, 20 – 20 second epochs displayed from the analyzed 10 – 10 minutes tracings at the indicated time points) registered in animals representing the median EEG score values of each group on Panels A, B and C. Similar continuous EEG activity (> 25 μV) can be observed at baseline in all subjects. The control animal from group CTR (Panel A) maintained high amplitudes throughout the observation period meanwhile 20 minute asphyxia in the piglet from group ASPH (Panel B) elicited severe electrocortical depression. Interestingly, ECG artefacts became prominent over the isoelectric EEG background pattern in this severely injured animal. Molecular hydrogen resulted in restoration of the EEG by 4 hours in this piglet from group ASPH + H_2 (Panel C). The EEG recordings were analyzed using two methods. First, a custom-made amplitude-based scoring system was created (*Table 1*), where higher scores represent progressive deterioration of electrical activity (Panel D). Second, total EEG power (Panel E) was determined. EEG tracings of time control animals (n = 7, white) were characterized by continuous EEG activity and high total power during the entire experiment. Asphyxia (A on Panels D, E), induced isoelectric EEG that was followed by slow regeneration of electrical activity. Regeneration of EEG was more rapid and more complete in the hydrogen-reventilated animals of group ASPH + H_2 (n = 7, dark grey) shown by lower EEG scores and higher EEG power, as compared to those of group ASPH (n = 7, light grey, hatched) that were reventilated with air only. Data of Panels D and E are shown as median, 25^{th} – 75^{th}, and 5^{th} – 95^{th} percentiles (line, box, and error bars, respectively). $P < 0.05$. † versus time control group at respective time point, § versus ASPH + H_2 at respective time point.

time control group during the survival period (*Fig. 2*). Only HR was significantly elevated in group ASPH compared to time controls during the early reventilation (*Fig. 2*), but significant differences of the monitored physiological parameters between the asphyxiated groups (groups ASPH and ASPH + H_2) were not observed even then. Some piglets (5 versus 7 versus 4 animals in groups CTR, ASPH and ASPH + H_2, respectively), required higher FiO_2 to maintain similar oxygen saturation that was responsible for the difference in PaO_2 during the early reventilation period (*Fig. 2G*). Brain electrical activity remained virtually isoelectric in the ASPH group that resulted in low total power and high EEG scores (*Fig. 5*). Two animals in group ASPH manifested electro-clinical convulsions appearing at 9 and 13 hours of survival, respectively. Seizures were identified by repetitive trunk/limb muscle contractions and characteristic EEG patterns. Hydrogen administration facilitated the restoration of the EEG activity and most of the animals (5 out of 7) presented continuous background activity by the end of the survival period with higher power values (*Fig. 5*), furthermore, none of the animals in group ASPH + H_2 showed electrographic seizure activity. 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 ASPH, n = 6).

Neuropathology

In the normoxic time control group, no significant neuronal damage was noted except for the low-frequency occurrence of scattered damaged neurons. In contrast, neuropathology analysis revealed marked cortical neuronal damage in group ASPH that is

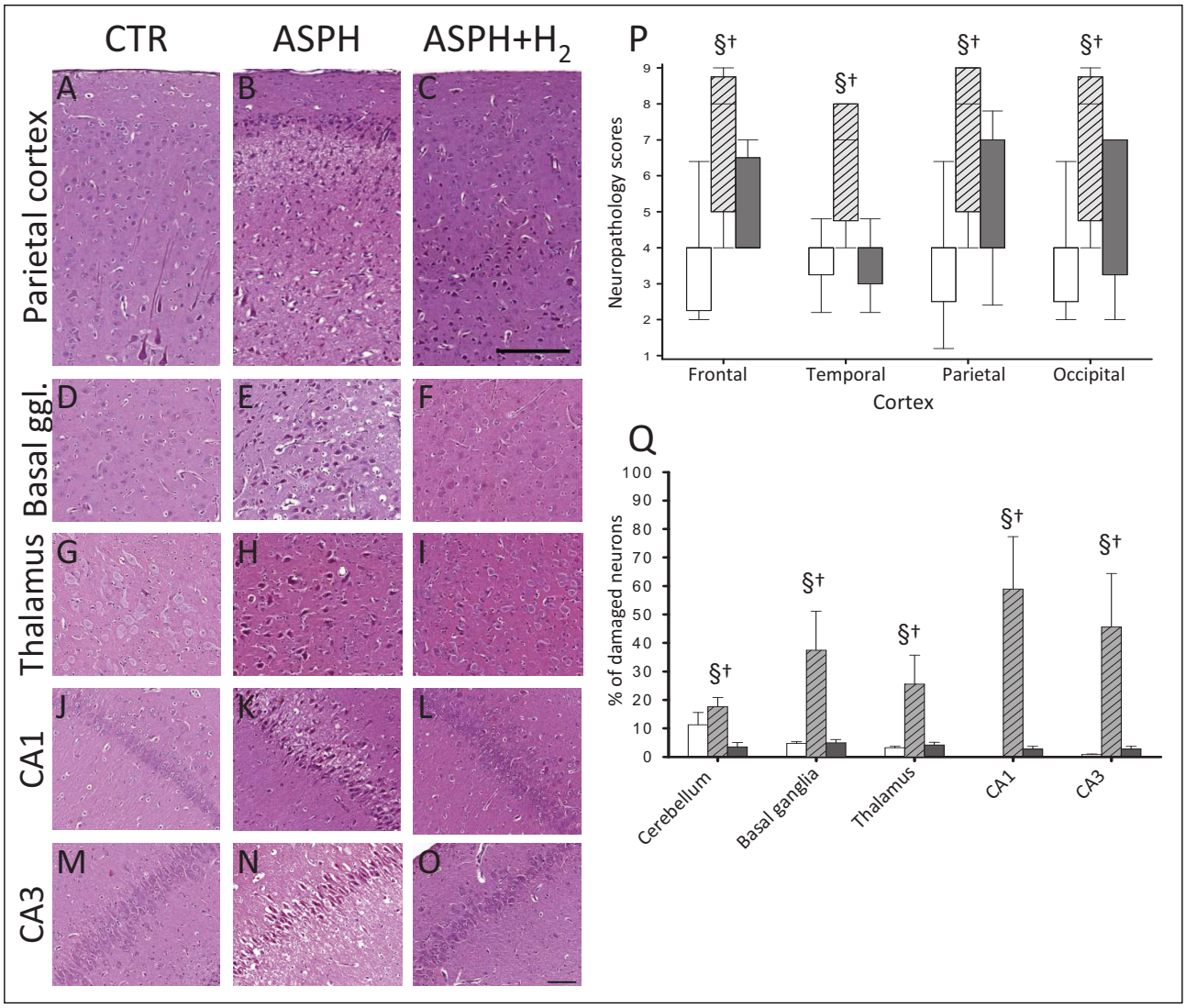

Fig. 6. Histology results and representative photomicrographs taken from subjects representing the median of the groups. Neuronal damage induced by PA in group ASPH and neuroprotection afforded by H_2 may be observed in the selected cortical and subcortical areas. Scale bar for photomicrographs A – C: 200 µm (top bar), for D – O: 100 µm (bottom bar). In all assessed cortical regions (Panel P), the hydrogen-reventilated animals (ASPH + H_2, n = 7, dark grey) had significantly smaller cortical damage as compared to air-reventilated (ASPH, n = 7, light grey, hatched) piglets. Brain damage was minimal in the time control animals (CTR, n = 7, white). In other brain regions, including the ganglionic cell layer of the cerebellar cortex; the basal ganglia, the thalamus and the CA1 and CA3 regions of the hippocampus (Panel Q) the neuronal damage was determined by cell counting. Similar to the cortex there was a striking neuroprotective effect afforded by hydrogen, especially marked neuroprotection was observed in the hippocampus and the basal ganglia. Data are shown as median, 25^{th} – 75^{th}, and 5^{th} – 95^{th} percentiles (line, box, and error bars, respectively for Panel P, and as mean ± S.E.M. for Panel Q. $P < 0.05$. † versus group CTR and § versus group ASPH + H_2.

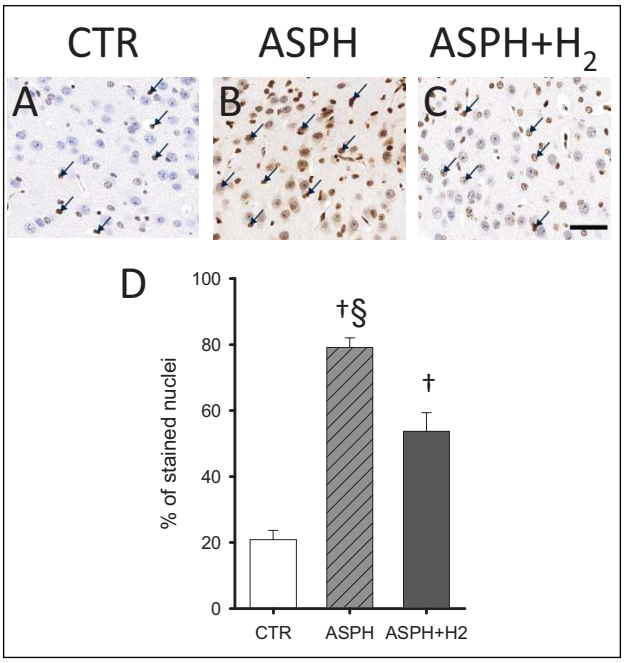

Fig. 7. Hydrogen reduces oxidative stress after PA. Representative photomicrographs (Panels A – C) of 8-OHdG immunopositive nuclei from the parietal cortex. Time control animals (group CTR) exhibited only few strongly stained immunopositive nuclei (arrows). 20 minute asphyxia markedly increased DNA oxidation, resulted in strong immunoreactivity against 8-OHdG at 24 hour of survival (group ASPH). Molecular hydrogen (group ASPH + H_2) significantly reduced oxidative damage after PA. (Panel D). $P < 0.05$. † versus group CTR and § versus group ASPH + H_2 , n = 14 – 28 regions of interests/ group, scale bar 100 µm.

reflected by high damage scores (*Fig. 6*) corresponding to laminar or even confluent panlaminar necrotic lesions virtually in all cortical areas observed at least in some of the assessed fields of view. We compared the cortical neuronal damage induced by our new PA method to our previous (8 min tracheal tube occlusion) PA model, by determining also the neuropathology scores of the same cortical regions obtained in our previous study (19). In summary, we found that in group ASPH, the summated damage scores of the four assessed cortical regions were 32; 20; 36 (median; 25th; 75th percentile), whereas in our previous PA model the values were significantly lower: 17; 10; 21 ($P = 0.026$) indicating a much more robust cerebrocortical damage in the present model. Accordingly, very severe neuronal loss and destructed neuropil were found in the CA1 and CA3 hippocampal regions in group ASPH of the present study, and significant neuronal loss was also obvious in the basal ganglia, the thalamus and the cerebellar Purkinje cells (*Fig. 6*). Molecular H_2 applied in the early reventilation (group ASPH + H_2) significantly improved neuronal viability in virtually all cortical and subcortical regions studied. Importantly, the hippocampal CA1 and CA3 subfields preserved their structural integrity and most of its cells. Similar preservation of viable neurons was noted in the cerebellum, thalamus and the basal ganglia in the hydrogen treated group ASPH + H_2 (*Fig. 6*).

In the parietal cortex, the number of 8-OHdG immunoreactive cell nuclei was low in group CTR, however, strong, significantly elevated staining was characteristic of group ASPH and ASPH + H_2. However, there was also a significant difference between the ratios of 8-OHdG immunoreactive nuclei between group ASPH and ASPH + H_2, indicating reduced oxidative stress in the hydrogen treated group (*Fig. 7*).

DISCUSSION

In the present study, we introduce a novel piglet PA/HIE model that is relatively simple to induce but still faithfully reproduces all major symptoms associated with PA (isoelectric EEG, severe hypoxemia, hypercapnia, lactacidemia) and elicits significant encephalopathy shown by severely depressed EEG activity and neuronal damage at 24 hours survival. By characterizing our model we showed that this level of asphyxia did not induce severe cerebral ischemia during the PA stress but was able to induce more severe brain damage as compared to our previous PA model (8 min tracheal occlusion) (19). This more robust model thus enabled us to discover the marked

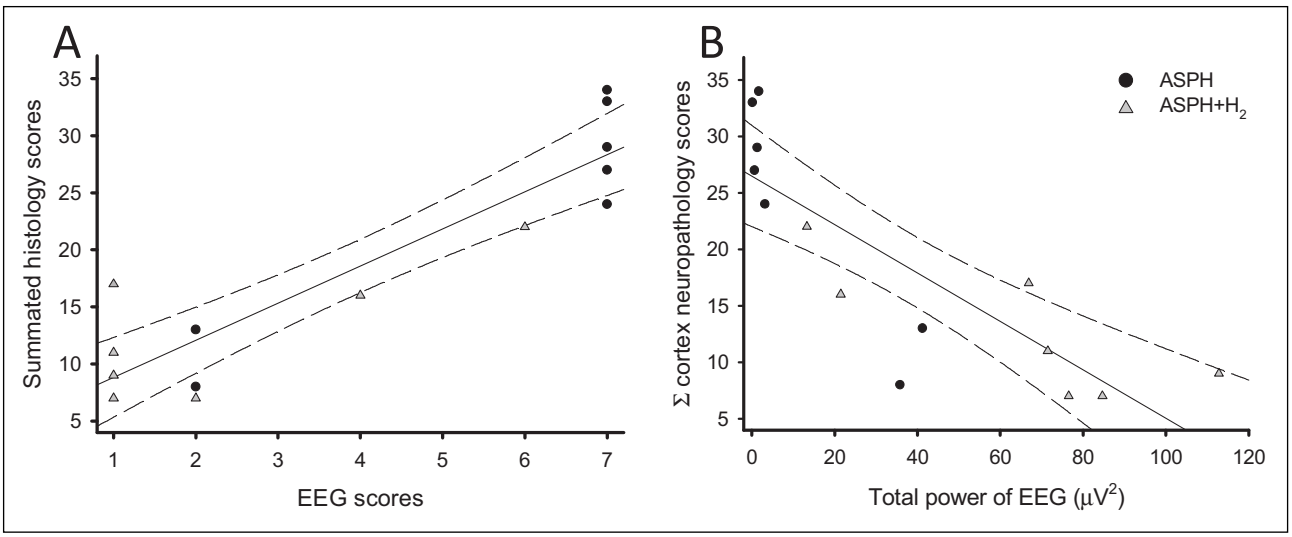

Fig. 8. EEG activity at 20 hour of survival shows good correlation with cortical neuronal damage assessed with neuropathology in the animals subjected to PA. Linear regression analysis shows significant correlations between both EEG scores (Panel A) or total EEG power (Panel B) and the sum of histopathology scores from 4 cortical regions. Solid lines represent the regression line (r^2: 83.06% of Panel A and r^2: 69.90% of Panel B; $P < 0.05$) whereas dashed lines the 95% confidence intervals.

neuroprotection afforded by molecular H_2: 4 h inhalation of 2.1% H_2 started together with the reventilation following PA facilitated the recovery of brain electric activity, and ameliorated neuronal damage in numerous cortical and subcortical regions.

Approximately 13 million newborns require assisted ventilation at birth worldwide while 1 million newborns die annually due to PA complications (2). Despite the introduction of therapeutic hypothermia, HIE induced by PA remains a major health care problem, and translational animal models are warranted to test further putative neuroprotective therapies. PA has multiple aetiologies, and unfortunately, the onset, cause, severity and duration of PA are often difficult to determine under clinical conditions, so the exact pathophysiology of human PA is still poorly understood challenging researchers whilst designing PA/HIE animal models. The newborn piglet has become a widely accepted large animal model for studying PA/HIE as its brain developmental stage, structure and metabolism is similar to the term neonate (38). Furthermore, its size, cost and availability are also in favour of its application in PA/HIE research. During the past few decades, numerous piglet PA/HIE models were tested with variable clinical feasibility (20-25). In our previous paper assessing the effects of molecular H_2 on neurovascular unit integrity after PA (19), we used 8 min tracheal tube occlusion to induce PA. This method was very useful to show delayed attenuation of cerebrovascular reactivity, however, the neuropathology revealed only limited number of irreversibly damaged neurons at 24 hour of survival. Increasing the duration of PA by tracheal occlusion to induce more severe HIE proved to be impossible because of the high occurrence of lethal cardiac complications (personal observations). There is a multitude of piglet PA models in the literature that are elicited by ventilating the animals with hypoxic gas mixtures. These models vary considerably in the applied FiO_2 and whether the gas mixture contains also CO_2 or not. Moreover, bilateral carotid artery occlusion is often combined with hypoxic ventilation. We chose the PA model employed in the present study based on two lines of experimental evidence. First, we found that bilateral carotid artery occlusion during normoxic or hypoxic conditions did not induce reductions in CoBF (26). Second, Helmy *et al.* (30) showed that in rat pups hypoxia alone (9% O_2) did not whereas hypoxia combined with hypercapnia (9% O_2 and 20% CO_2) elicited seizures - an important hallmark of HIE. Therefore, our task was to develop a PA injury that elicits more severe HIE than our previous (tracheal occlusion) PA model (18, 19).

We performed a number of preliminary experiments in which we used EEG recovery as a primary indicator of HIE severity. In our previous study, none of the ASPH group animals displayed continuous (normal) EEG pattern in the first 4 hours of reventilation. We assumed therefore, that any PA stress allowing full recovery of the EEG faster than 4 hours likely induced less severe neuronal injury, and therefore more severe PA was still warranted. We first induced PA using 9%O_2 – 20% CO_2 gas mixture with increasing durations of 30, 40, and 60 mins (n = 2–2–2) as these PA injuries were not severe enough, we reduced FiO_2 to 6% and performed additional experiments with 35, 25, and 15 min duration (n = 2, 2, and 3, respectively). Animals subjected to 35 – 25 min PA were presented with isoelectric/very low voltage EEG at 24 h survival and were all dependent on inotropics to keep their blood pressure at 40 – 45 mmHg indicating cardiac complications. In contrast, all three animals subjected to 15 min of PA had quickly recovered their electrical brain activity. Our experiments using PA lasting for 20 min produced consistently animals that showed both stable hemodynamics and EEG signs of moderate/severe brain injury forming later group ASPH of the present study. We would like to note that pH and blood gas values taken at the last minute of PA using a given gas mixture were very similar to each other indicating that in this model likely not the absolute degree of hypoxia, hypercapnia or acidosis itself rather than its duration determines the degree of elicited brain damage. However, hypercapnia appears to be an important determinant of HIE severity. Goodwin *et al.* (39) reported that newborns with P_aCO_2 values greater than 140 mmHg, manifested major neurological deficits and good correlation to seizure activity. Moreover, in an other study by Belai *et al.* (40), HIE did not develop in all infants with P_aCO_2 below 140 mmHg. A marked advantage of our current model is also the stability of systemic hemodynamic parameters, virtually no animals were lost during the PA/immediate reventilation period and none of the animals required pharmacological treatment for hypotension afterwards. This is in sharp contrast with the PA model published by Chalak *et al.* (41), who ventilated the animals with 5.3% O_2 and 7.5% CO_2 gas mixture and decreased the RR by 10 1/min every 15 minute and reached asystole in order to produce blood gas and pH values that are similar to our results and in accordance with those reported from term newborns undergoing severe PA (40, 42). We found a moderate increase in CoBF during PA in the present study, so our PA model clearly does not induce cortical ischemia, however, the increase in CoBF was still 2 – 3 times less (~35% versus ~100%) than in animals ventilated with the 10% O_2 combined with bilateral carotid artery occlusion (26). As we previously reported (43) that 10 minute hydrogen ventilation did not influence cortical microcirculation in newborn piglets, hence we did not test the effect of the neuroprotectant in a separate group in the present study.

Unfortunately, in the clinical setting precise prediction of neurodevelopmental outcome in infants affected by PA/HIE is difficult in most cases. A recent meta-analysis suggested that neurophysiology tests (i.e. serial EEG-s) within the first week of life may prove to be promising tools in this effort (44). Moreover, magnetic resonance spectroscopy (MRS) and imaging (MRI) offer an invaluable aid for assessing morphologic and metabolic changes in the neonatal brain as early as the first days of life (45) but its accessibility, the facility to serial imaging as well as the necessary human and material resources are not available everywhere. Bedside neonatal EEG monitoring provides continuous information about the impaired cortical electrical activity after PA and was reported to be a useful tool to predict late outcome as early as 24 hours (46, 47). Although, it is widespread in neonatal intensive care as its application and maintenance does not require special conditions, however, trained staff is required to interpret the EEG patterns. The clinical evaluation of EEG signals is predominantly based on the amplitudes of waves, the presence/absence of sleep-wake cycles, seizures, repetitive signals; the quantification of given periods is more challenging. Consequently, custom-made scoring scales were introduced and were widely accepted in the literature (48), thus first, we established our own amplitude-based scoring system according to international recommendations (34), but we also simultaneously determined the total power of the recorded EEG at given time points. Our PA model elicited similar, severe electrocortical depression within 1 – 2 minutes in groups ASPH and ASPH + H_2 that remained practically isoelectric (< 5 µV, high EEG scores, low total power) in the asphyxia-only animals whereas EEG activity was at least partially restored in the hydrogen treated group. Interestingly, EEG scores and the total power of EEG of all asphyxiated animals (groups ASPH and ASPH + H_2) at 20 hours showed strong correlation (*Fig. 8.*, r^2: 83.06% and 69.9%; respectively, $P < 0.05$) to the summated histopathological scores characterizing cortical brain damage, thus EEG activity this time point might be predictive for indicating the severity of brain injury in our present model. Indeed, the recovery of EEG amplitudes correlated well with the preservation of the cerebral cortex after hydrogen ventilation.

Neuronal injury was assessed by a previously reported scoring system as cell counting alone does not represent the pattern of cortical damage: a single panlaminar necrosis may elicit more severe clinical outcome compared to similar number of injured neurons distributed along the 20 fields of vision. Concerning the other brain regions studied, it is worth noting that the hippocampal CA1 and CA3 regions as well as the basal ganglia suffered the most extensive neuronal damage in our model in accordance with early MRI findings in PA/HIE survivors (49), and that H_2 could exert marked neuroprotective effect in these areas as well. Despite marked neuronal injury, we could not detect increases in plasma NSE levels after asphyxia. This finding is in accordance with those of Nagdyman *et al.* (50) who reported also unaltered NSE levels in asphyxiated term newborns during the first day of life. Admittedly, Kecskes *et al.* (51) reported NSE elevations in piglets at 24 hours, but only following a longer PA stress (40 min hypoxic ventilation combined with arterial hypotension) that likely severely affected at least transiently the integrity of the blood brain barrier.

Therapeutic hypothermia has become the gold standard care to combat HIE development (8), however, not all of the babies may benefit from total body cooling (52) thus supplementary neuroprotective efforts are urgently required. Furthermore, 'therapeutic' hypothermia has recently been reported not to prevent white matter damage following PA in piglets, but hypothermia was in fact shown to per se induce white matter damage in normoxic controls, raising concerns about the safety of this approach (53). Molecular hydrogen was introduced as a novel hydroxyl radical scavenger by Ohsawa *et al.* (13) and since then, its antioxidant, anti-inflammatory and anti-apoptotic effects were proved in numerous preclinical and clinical studies (14). Among the animal models of PA/HIE, first Cai *et al.* (15) reported neurological improvement after molecular hydrogen (2% H_2 in medical air) resuscitation in a neonatal rodent HIE model. This finding was in accordance with the neuroprotective effect of H_2 in a transient global ischemia rat model showing improved neuronal survival (2% H_2 in medical air) and cognitive functions in rats (54). Surprisingly, Matchett *et al.* (17) reported no benefit from H_2 administration in a neonatal rat HIE model, in contrast, H_2 pretreatment even appeared to enhance hypoxic/ischemic brain damage. To the best of our knowledge, our research group was the first who reported the beneficial effects of hydrogen in a large animal PA/HIE model (18, 19). The current study importantly expands our previous knowledge in that aspect that H_2 is not only able to reduce injury in a mild but also in a severe PA/HIE model. The effect of H_2 is quite robust in a translationally feasible administration route: administration started after the PA together with the reventilation efforts. Furthermore, we showed in the present study for the first time in this large animal model that molecular hydrogen alleviated the level of oxidative stress, indicated by reduced 8-OHdG levels in the parietal cortex, similar to method and findings of Zhai *et al.* (55) who reported the antioxidant capacity of hydrogen in an adult stroke rat model.

The direct molecular target/ mechanism of action of this relatively inert gas is still unknown, although several biochemical markers have shown altered expression followed by hydrogen administration (56). However, it is often difficult, or virtually impossible, to decipher *in vivo* if the observed effect has a causative role in hydrogen-induced neuroprotection or rather is the consequence of the hydrogen-induced protection achieved through an independent mechanism. Nevertheless, very recently hypoxia-induced elevations in cerebrocortical interleukin 18 mRNA levels have been shown to be inhibited by ventilation with 2% H_2 in piglets (57) suggesting that in addition to the putative reactive oxygen species scavenging capacity, anti-inflammatory effect of hydrogen can contribute to reduced neuronal death after PA in the newborn brain.

Molecular hydrogen was administered as before in our current model for two reasons. First, the brevity of the therapeutic window demands the introduction of a potential neuroprotective intervention up to 6 hours, secondly, reventilation/reperfusion-induced ROS generation (58) is the most important contributor to hypoxic-ischemic brain damage (59, 60). Hydrogen resuscitation facilitated the recovery of the EEG amplitudes in our model, moreover, prevented structural neuronal damage assessed at 24 hour of survival.

The present study has some limitations. Although this 24 hour observation period likely spans the time frame in which molecular hydrogen can afford protection, it does not allow conclusions on long-term neuroprotection or late neurodevelopmental outcome with certainty. A longer, at least 48 hour survival would likely allow us to study if anti-apoptotic mechanisms played also a role in the neuroprotective effect of molecular H_2, using for instance cleaved caspase 3 immunohistochemistry that has already been shown in piglets to indicate anti-apoptotic effects elicited by hypothermia or melatonin at this timepoint (61, 62). Moreover, 48 hours survival would also allow us to study altered neurotoxic activation of microglia by determining microglial ramification index or CD86 mRNA levels that have also been found useful to characterize anti-inflammatory neuroprotection in this animal model (61, 62). Second, the neuroprotective effect of molecular hydrogen in combination with therapeutic hypothermia was not tested, although the latter became the gold standard care after PA. Unfortunately, the use of hypothermia with this survival period was not feasible; although cooling can be achieved within an hour, rewarming must be not faster than 0.5°C/h (53) that would last more than 10 hours, allowing only very limited time in the therapeutic temperature range likely causing underestimation of its neuroprotective potential. As molecular hydrogen was found in the present study to be effective against moderate/severe HIE stress, combining the therapeutic effect of molecular hydrogen with total body hypothermia using an even longer (48 – 72 hours) survival period remains our future direction. The third limitation is the use of anaesthesia/analgesia during the experiments, especially during PA complicating translation of the model and the results to the human clinical situation. Adequate anaesthesia is mandatory to meet ethical concerns using laboratory animals, however, we carefully chose an anaesthethic/analgesic regimen that is often used in neonatology (63) to support HIE patients: morphine to combat mechanical ventilation-induced pain and stress (64), and midazolam is routinely administered to suppress neonatal seizure activity. Furthermore, midazolam has been shown to unaffect the EEG and not jeopardising amplitude-based EEG analysis (65).

In conclusion, we introduce a new piglet PA/HIE model that faithfully represents the human clinical and metabolic hallmarks of PA and results in moderate/severe brain injury. The degree of HIE has been characterized by depressed EEG activity and robust histopathology findings at 24 hour of survival confirming a more severe asphyxic insult compared to our previous PA model (tracheal occlusion) (19). Molecular hydrogen administered in this severe PA/HIE model displays marked neuroprotection shown by facilitating the restoration of brain electrical activity and preserving structural integrity virtually in all assessed brain regions.

Authors' contributions: Janos Nemeth: performing *in vivo* experiments, EEG scoring, neuropathology evaluation, writing the manuscript; Valeria Toth-Szuki: performing *in vivo* experiments, histology; Viktoria Varga: performing *in vivo* experiments, neuropathology evaluation; Viktoria Kovacs: performing *in vivo* experiments; Gabor Remzso: performing *in vivo* experiments, EEG scoring; Ferenc Domoki: performing *in vivo* experiments, statistics, writing the manuscript.

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