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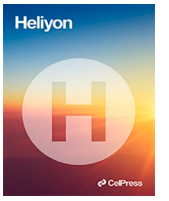

Research article

The effects of CO_2 levels and body temperature on brain interstitial pH alterations during the induction of hypoxic-ischemic encephalopathy in newborn pigs

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ABSTRACT

Brain interstitial pH (pH_{brain}) alterations play a crucial role in the development of hypoxic-ischemic (HI) encephalopathy (HIE) caused by asphyxia in neonates. The newborn pig is one of the most suitable large animal models for studying HIE, however, compared to rats, experimental data on pH_{brain} alterations during HIE induction are limited. The major objective of the present study was thus to compare pH_{brain} changes during HIE development induced by experimental normocapnic hypoxia (H) or asphyxia (A), elicited with ventilation of a gas mixture containing 6%O_2 or 6%O_2/20%CO_2, respectively for 20 min, under either normothermia (NT) or hypothermia (HT) (38.5 ± 0.5 °C or 33.5 ± 0.5 °C core temperature, respectively) in anesthetized piglets yielding four groups: H-NT, A-NT, H-HT, and A-HT. pH_{brain} changes during HI stress and the 60 min reoxygenation period were measured using a pH-selective microelectrode inserted into the parietal cortex through an open cranial window. In all groups, the pH_{brain} response to HI stress was acidosis, at the nadir pH_{brain} values dropped from the baseline of 7.27 ± 0.02 to H-NT:5.93 ± 0.30, A-NT:5.90 ± 0.52, H-HT:6.81 ± 0.27, and A-HT:6.27 ± 0.24 indicating that (1) H and A elicited similar, severe brain acidosis under NT greatly exceeding pH changes in arterial blood (pH_a dropped to 7.24 ± 0.07 and 6.78 ± 0.03 from 7.52 ± 0.06 and 7.50 ± 0.05, respectively), and (2) HT ameliorated more the brain acidosis induced by H than by A. In all four groups, pH_{brain} was restored to baseline values without an alkalotic overshoot during the observed reoxygenation, Our findings suggest that under NT either H or A – both commonly employed HI stresses to elicit HIE in piglet models – would result in a similar acidotic pH_{brain} response without an alkalotic component either during the HI stress or the early reoxygenation period.

Key Points Summary

- Hypoxia and asphyxia consistently resulted in similar, compared to pH_a severe pH_{brain} drops under normothermic conditions. The pH_{brain} recovery during reoxygenation was also similar without observing any alkalotic overshoot.
- Hypothermia almost fully ameliorated hypoxia- but not asphyxia-induced cerebral acidosis, suggesting temperature has a major impact on the pH_{brain} response to hypoxic/ischemic insults.

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- The dependence of pH_{brain} during hypoxic-ischemic insults on further physiological factors, such as corticocerebral blood flow, metabolites, CO_2 levels and their interactions are discussed.

1. Introduction

In term infants, hypoxic-ischemic insults around birth, importantly perinatal asphyxia can elicit hypoxic-ischemic (HI) encephalopathy (HIE), the most common form of neonatal encephalopathy [1]. HIE is characterized and diagnosed by the simultaneous presentation of signs of asphyxia (low Apgar scores, need for resuscitation, severe acidosis), clinical signs of encephalopathy (altered consciousness and reflexes, presence of seizures), and abnormal brain electrical activity [2,3]. Currently therapeutic hypothermia (TH) is being employed successfully to reduce mortality and to mitigate the deleterious long-term consequences of HIE in affected babies, however, the neuroprotection afforded by TH is incomplete. To develop adjunct/alternative neuroprotective therapies, the pathophysiology of neuronal injury during HIE development must be better understood using translational preclinical models.

During the primary hypoxic/ischemic insult such as birth asphyxia, one of the physiological hallmarks is the development of a severe combined (metabolic and respiratory) acidosis, in part due to the accumulation of organic acids by anaerobic processes and in part by the developing hypercapnia due to the failure of respiration. In contrast to the well-known effect of birth asphyxia on blood pH, much less attention has been devoted to the changes to the brain interstitial fluid pH (pH_{brain}) during asphyxia. However, pH_{brain} is known to greatly affect both neuronal excitability and viability as severe pH_{brain} alterations can lead to direct or indirect neuronal injury via the induction of seizures [4,5]. Although HI-induced cerebral acidosis seems to be a simple and straightforward response, in a recent elegant study Pospelov et al. showed that while experimental asphyxia induced by ventilation with hypoxic/hypercapnic gas mixtures elicited the expected acidotic pH_{brain} shift, nonetheless normocapnic hypoxia triggered instead an alkaline deflection of pH_{brain} in P6 rat pups [6]. In addition, there must be also interspecies differences among the preclinical HIE model species as well. For instance, using virtually the same hypoxic/hypercapnic gas mixture to elicit experimental asphyxia as in Ref. [7] vs [8]. (5%O_2/20% CO_2 vs 6%O_2/20%CO_2), compared to the P6 rat pups we found a much larger reduction in the pH_{brain} of newborn pigs (6.65 vs 5.94, respectively). Interestingly, body/arterial pH values were very similar (~6.8) in both models. This implies that in piglets a very significant (~0.8 pH unit) pH gradient developed across the blood-brain barrier during asphyxia that was apparently absent in rat pups. The direct comparison between the studies is further hindered by the fact that the rat pups were subjected to a longer asphyxia duration (45 vs 20 min) but at a lower body temperature (33.5 vs 38.5 °C).

The newborn pig is a well-established translational large animal model widely used in HIE research [9–11]. Compared to the lyssencephalic rodent brain, the gyrencephalic piglet brain displays a number of further similarities to that of the human infant: 1 the size of the brain at birth is virtually identical, 25% and 27% of the adult in pigs and humans, respectively, 2. there are many shared features in gross neuroanatomy and myelination, and 3. the neuronal growth spurt importantly coincides with birth in both species unlike the prenatal skewed growth spurt in monkey and sheep and the postnatal skewed spurt in rodents and rabbits [9,10,12–14]. In addition to these structural and neurodevelopmental analogies, cerebral energy metabolism, blood flow and cerebrovascular reactivity are all very similar in piglets and human neonates [15,16]. Furthermore, there are important genetic similarities between humans and pigs including synteny, gene order as well as DNA methylation patterns [11,17,18]. These advantages combined with the continuous availability of newborn pigs at a – compared to primates or lambs – less prohibiting cost makes the newborn pig an appealing large animal model allowing the monitoring of physiological parameters and the application of supportive therapy during HIE development that can rarely be employed in rodents [11]. In the piglet HIE models, HI insults are elicited by variable methodologies including ventilation with either normocapnic hypoxic or hypoxic/hypercapnic gas mixtures eliciting hypoxia or asphyxia, often combined with carotid artery occlusion.

We hypothesized that the differential pH_{brain} response: ie the alkalotic shift to normocapnic hypoxia and the acidotic shift to asphyxia described in rats [6] would also occur in newborn pigs and this would imply a significant difference about the mechanism of neuronal injury induction between the studies employing these two types of HI insults. Therefore, extending our previous study employing only asphyxia [8], we set out to investigate the pH_{brain} changes also during/after normocapnic hypoxia in piglets at a normal body temperature. Furthermore, we also tested the pH_{brain} alterations induced by either hypoxia or asphyxia in piglets at the hypothermic body temperature used in Ref. [6] to investigate if the difference in body temperatures could account for the observed differences in pH_{brain} response direction and magnitude to hypoxia or asphyxia.

2. Materials and methods

Ethical approval

All experimental procedures were approved by the National Animal Committee on Animal Experiments (ÁTET, I.74–7/2015) and the mandatory permit to obtain the animals was provided by the National Food Chain Safety and Animal Health Directorate of Csongrád county, Hungary (permit number: XIV./1414/2015). All animal experiments were in compliance with the guidelines of the Scientific Committee of Animal Experimentation of the Hungarian Academy of Sciences (updated Law and Regulations on Animal Protection: 40/2013. (II. 14.) Gov. of Hungary) and also complied with the ARRIVE guidelines 2.0 [19] and the EU Directive 2010/63/EU on animal protection used for scientific research.

2.1. Animals

Newborn (P0) male Landrace piglets (n = 15; body weight: 1.6 ± 0.3 kg) were obtained from a local company (Pigmark Ltd., Co., Szeged, Hungary). The animals were anesthetized with intraperitoneal sodium thiopental injection (45 mg/kg; Sandoz, Kundl, Austria) and their core temperature was maintained in the physiological range (38.5 ± 0.5 °C) with a servo-controlled heating pad (Blanketrol III, Cincinnati SUB-zero, Cincinnati, Ohio, USA). The animals were mechanically ventilated through a tracheal tube with humidified medical air setting the peak inspiratory pressure (PIP) to 12–14 cmH_2O, the respiration rate (RR) to 30-35 min^{-1} and the fraction of inspired oxygen (FiO_2) to 0.21. We catheterized the right carotid artery and the right femoral vein under aseptic conditions to record the mean arterial blood pressure (MABP) and to provide continuous infusion of anesthetics and supportive fluids (5% glucose, 0.45% NaCl 3 ml/kg/h), respectively. The anesthesia/analgesia was maintained with iv bolus injection of morphine (100 μg/kg; Teva, Petach Tikva, Israel) and midazolam (250 μg/kg; Torrex Pharma, Vienna, Austria), then switched to continuous infusion of morphine (10 μg/kg/h) and midazolam (250 μg/kg/h) throughout the whole experiment [8,20]. MABP and the heart rate (HR) were continuously monitored with an EDAN Im8 Vet Monitor (Edan Instruments Inc., Shekou, Nanshan, Shenzhen, China). Arterial blood samples (300 μl) were taken at baseline (−20 min), at the completion of 20 min asphyxia/hypoxia (0 min), then at 10, 30 and 60 min after asphyxia/hypoxia. The blood samples were analyzed for arterial pH (pH_a), pO_2, pCO_2, central oxygen saturation (cSO_2), base excess (BE(b)) and lactate levels with the epoc® Blood Analysis System (Epocal Inc., Ottawa, ON, Canada). At the end of the experiments, the animals were euthanized with an overdose of pentobarbital sodium (300 mg, iv; Release®; Wirtschaftsgenossenschaft deutscher Tierärzte eG, Garbsen, Germany).

2.2. Brain interstitial pH (pH_{brain}) measurements

After instrumentation, the head of the animals was fixed into a stainless steel stereotactic frame (RWD Life Science, Shenzhen, Guangdong Province, China). Over the left parietal cortex, we obtained an open cranial window for electrode insertion (Fig. 1). The coordinates were determined by stereotactic reference points measured from the bregma (posterior: 1.2–1.4 cm, lateral: 1.1–1.3 cm). We gained access to the cortex by opening the dura mater and if it was necessary, small bridging veins were cauterized. The glass-membrane H^+-sensitive pH microelectrode (pH-50 model, Unisense A/S, Aarhus, Denmark) with 50 μm tip diameter and the reference electrode (Ref-100 model, (Unisense A/S, Aarhus, Denmark)) with 100 μm tip diameter were mounted on stereotaxic manipulators. The electrodes were calibrated before each experiment in 3 different buffer solutions (38 °C, pH: 6.10, 7.10, and 8.10 containing 150 mmol/L NaCl and 40 mmol/L HEPES; the pH was adjusted with NaOH), then inserted ~1–2 mm deep into the cortex. The subarachnoideal space was filled with artificial cerebrospinal fluid (aCSF) containing 7.71 g/L NaCl, 0.22 g/L KCl, 0.221 g/L $CaCl_2$, 0.132 g/L $MgCl_2$, 0.665 g/L dextrose, 0.402 g/L urea and 2.066 g/L $NaHCO_3$, and was equilibrated with 6.3% O_2 and 6.2% CO_2 containing gas mixture (with 87.5% N_2, respectively).

2.3. Experimental protocol

1h stabilization period followed the surgical procedures, to obtain baseline values of each physiological parameter. The hypoxic/ischemic insults were induced by switching the ventilation from medical air (21% O_2, 79% N_2) to hypoxic or hypoxic/hypercapnic gas mixtures to elicit normocapnic hypoxia or asphyxia, respectively. Target body temperatures (33.5 ± 0.5 °C or 38.5 ± 0.5 °C) were

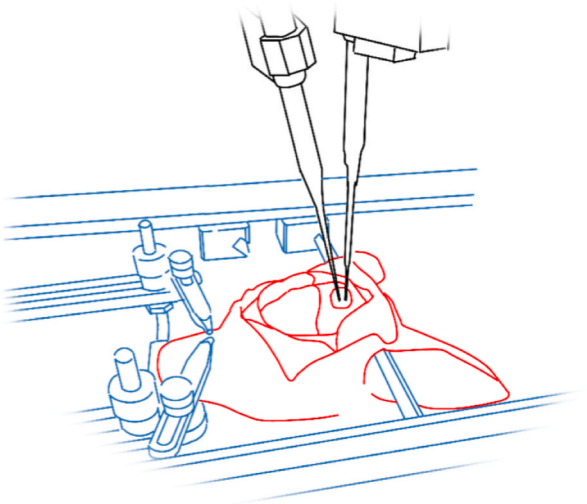

Fig. 1. Experimental design. The parietal cortex was exposed through a ~0.8 cm diameter open cranial window positioned according to stereotaxic reference points, posterior 1.2–1.4 cm and lateral 1.1–1.3 cm from the bregma. The electrodes were inserted ~1–2 mm deep into the cortex.

established 10 min before obtaining the baseline values and then applying the hypoxia or asphyxia.

The experimental groups were the following.

1. Hypoxia-normothermia (H-NT, n = 5) group: ventilation with hypoxic gas mixture (6% O_2 and 94% N_2) for 20 min, maintaining the normal RR (30 min^{-1}). The body temperature was maintained in the physiological range (38.5 ± 0.5 °C).
2. Hypoxia-hypothermia (H-HT, n = 5) group: the ventilation was the same as in H-NT group, while the body temperature was reduced to the level of therapeutic hypothermia (33.5 ± 0.5 °C).
3. Asphyxia-hypothermia (A-HT, n = 5) group: ventilation with hypoxic/hypercapnic gas mixture (6% O_2, 20% CO_2, 74% N_2) for 20 min, reducing the RR from 30 to 15 min^{-1}. while the body temperature was reduced to 33.5 ± 0.5 °C.

2.4. Data processing

pH_{brain} signals were recorded (4 Hz sampling frequency), digitized and stored using the Microsensor Multimeter and SensorTrace Logger software (Unisense A/S, Aarhus, Denmark). The signals were recorded in millivolts then converted to pH values by linear interpolation. The pH signals were converted to proton concentrations, averaged and followed by taking the negative base-ten log of each individual recording [6]. All data analysis was performed in MATLAB (RRID:SCR_001622, Mathworks Inc., Natick, MA, USA) with custom written scripts.

The data from the three experimental groups were compared to and analyzed together with data from the Asphyxia-normothermia (A-NT, n = 6) group from our recent publication [8].

2.5. Statistical analysis

All statistical analyses were performed in IBM SPSS Statistics 22.0 (RRID:SCR_019096, Armonk, NY: IBM Corp., USA) using two-way ANOVA with repeated measures, followed by the Tukey *post hoc* test. All data show the mean ± SD respective to the baseline. $p < 0.05$ was considered as significant. Multiple linear regression analysis between pH_a-pCO_2+lactate and pH_{brain}-pCO_2+lactate with multivariate ANOVA (MANOVA) was performed in IBM SPSS Statistics 22.0.

3. Results

3.1. Hemodynamic parameters and blood gases

Before the induction of hypoxia or asphyxia, MABP, HR, as well as arterial blood pO_2 cSO_2 and pCO_2 levels were all in their respective physiological ranges and were similar in all experimental groups (Table 1). Both types of insult resulted in severe, similar decreases in pO_2 and cSO_2 in all groups, whereas pCO_2 levels were profoundly higher in the asphyxia groups reflecting the high concentration of inhaled CO_2. One hour after the insults the assessed parameters were largely restored toward baseline levels and did not show consistent statistically significant differences among the different groups, although there was a tendency toward slightly lower pO_2/cSO_2 and slightly higher pCO_2 levels in the two groups subjected to asphyxia.

3.2. pH_a and pH_{brain} changes

Before the induction of hypoxia or asphyxia, both pH_a and pH_{brain} values were in their respective physiological ranges and were similar in all experimental groups (Fig. 2A and B and Fig. 3A and B). Both types of hypoxic/ischemic insults elicited acidotic changes in

Table 1
Mean arterial blood pressure (MABP), heart rate (HR), central oxygen saturation (cSO_2), pO_2, pCO_2 and blood glucose values in the four experimental groups. Values are shown at baseline, at the nadir of the hypoxia or asphyxia, and at 60 min after the completion of the insult (mean±SD). The hypoxic/ischemic insults elicited the expected hemodynamic changes, reduction in oxygenation, and increases in blood sugar levels that were similar in all experimental groups, except the significantly higher pCO_2 levels in the groups subjected to asphyxia. Please find the results of the statistical analysis in Table 2. Data for the A-NT group were taken from Ref. [8].

		MABP (mmHg)	HR (min^{-1})	cSO_2 (%)	pO_2 (mmHg)	pCO_2 (mmHg)	Glucose (mmol/L)
H-NT	Baseline	60.4 ± 15.7	134.0 ± 20.5	96.9 ± 1.7	83.8 ± 12.2	36.1 ± 8.9	5.34 ± 1.42
	Hypoxia	39.2 ± 8.2	189.8 ± 45.2	13.9 ± 1.7	17.5 ± 5.7	47.6 ± 10.3	10.16 ± 1.39
	60 min	51.6 ± 8.8	145.8 ± 17.4	96.4 ± 2.1	83.2 ± 12.2	37.3 ± 2.3	7.82 ± 2.35
H-HT	Baseline	64.6 ± 10.8	151.4 ± 29.0	96.0 ± 1.6	73.8 ± 5.4	34.9 ± 7.0	5.20 ± 0.63
	Hypoxia	58.0 ± 18.4	176.5 ± 7.8	17.9 ± 11.2	11.9 ± 3.4	45.8 ± 9.5	9.08 ± 2.03
	60 min	54.5 ± 0.7	130.5 ± 3.5	97.0 ± 0.5	73.4 ± 3.2	35.6 ± 2.5	8.20 ± 3.13
A-NT	Baseline	66.3 ± 6.9	139.1 ± 19.4	94.4 ± 4.1	63.4 ± 13.7	36.7 ± 11.8	5.43 ± 1.53
	Asphyxia	49.8 ± 15.7	172.7 ± 42.2	12.6 ± 3.6	18.5 ± 3.3	161.6 ± 23.4	10.56 ± 2.78
	60 min	54.9 ± 8.5	164.5 ± 18.2	90.5 ± 3.8	65.2 ± 10.6	49.4 ± 12.3	7.33 ± 1.75
A-HT	Baseline	61.4 ± 21.4	142.2 ± 18.2	90.8 ± 8.9	61.2 ± 24.3	43.5 ± 16.8	4.47 ± 0.49
	Asphyxia	43.6 ± 9.7	125.6 ± 6.5	6.6 ± 0.9	14.5 ± 8.6	141.4 ± 19.2	7.85 ± 0.33
	60 min	44.8 ± 11.3	106.2 ± 30.4	89.7 ± 6.8	48.2 ± 20.3	44.8 ± 9.4	7.33 ± 0.28

both pH_a and pH_{brain} under all experimental conditions, albeit the absolute and the relative magnitude of these changes were remarkably different in the various experimental conditions.

Concerning the negative pH_a shifts during the insults, they were consistently and significantly milder in animals subjected to hypoxia than to asphyxia at either body temperature (Fig. 3A and B, Table 2). However, this difference in pH_a drops induced by the two insults decreased in hypothermia: compared to the normothermic condition, asphyxia- but not hypoxia-induced blood acidosis was significantly ameliorated in hypothermia.

In contrast, at least under normothermic conditions, the acidotic pH_{brain} shifts induced by either hypoxia or asphyxia were very similar, therefore, in both groups a very large pH difference across the blood-brain barrier developed (Fig. 3A and B), especially in the H-NT group, where the drop in pH_a was smaller. Hypothermia affected pH_{brain} changes also differentially, however, unlike in the case of pH_a, hypothermia significantly ameliorated pH_{brain} drops in the animals subjected to hypoxia, but not to asphyxia.

After the completion of the hypoxic/ischemic insults, both pH_a and pH_{brain} values were quickly restored toward the baseline values with similar dynamics in all groups.

3.3. Markers of metabolic acidosis

Both hypoxia and asphyxia elicited similar, severe metabolic acidosis corresponding with the diagnostic criteria of HIE (Fig. 4A and B, Table 2). Specifically, both insult types resulted in similar, major negative shifts in blood base excess with simultaneous increases in lactate levels that were also similar in magnitude under normothermic conditions. Hypothermia could significantly ameliorate both ΔBE(b) and lactate changes only induced by asphyxia but not by hypoxia, although restoration of lactate levels after hypoxia was indeed found to be also promoted by hypothermia in the H-HT group. Reoxygenation resulted in gradual restoration of ΔBE(b) and lactate levels towards baseline values, except lactate levels remaining elevated in the A-NT group.

Multiple linear regression analysis (MANOVA) revealed a rather close but variable correlation between pH_a or pH_{brain} and blood lactate levels before and during the hypoxic/ischemic insult in all four experimental groups (Fig. 5A and B). Under normothermic conditions, the correlation between lactate, pCO_2 and pH_a was high in the animals subjected to either hypoxia ($R^2 = 0.925$, $F = 100.37$, $p < 0.0001$) or asphyxia ($R^2 = 0.832$, $F = 27.376$, $p < 0.0001$). In hypothermia, the strength of correlation was only slightly changed to $R^2 = 0.886$ ($F = 63.256$, $p < 0.0001$) and $R^2 = 0.932$, ($F = 111.109$, $p < 0.0001$), respectively. Concerning pH_{brain}, however, under normothermic conditions blood lactate levels correlated better in the group exposed to asphyxia ($R^2 = 0.892$, $F = 44.879$, $p < 0.0001$) than to hypoxia ($R^2 = 0.668$, $F = 17.10$, $p = 0.0001$). Interestingly, hypothermia appeared to only slightly affect the strength of pH_{brain}–lactate + pCO_2 correlation ($R^2 = 0.830$, $F = 40.084$, $p < 0.0001$) in the asphyxia group, but it was further decreased to ($R^2 = 0.461$, $F = 7.844$, $p = 0.001$) in the hypoxia group.

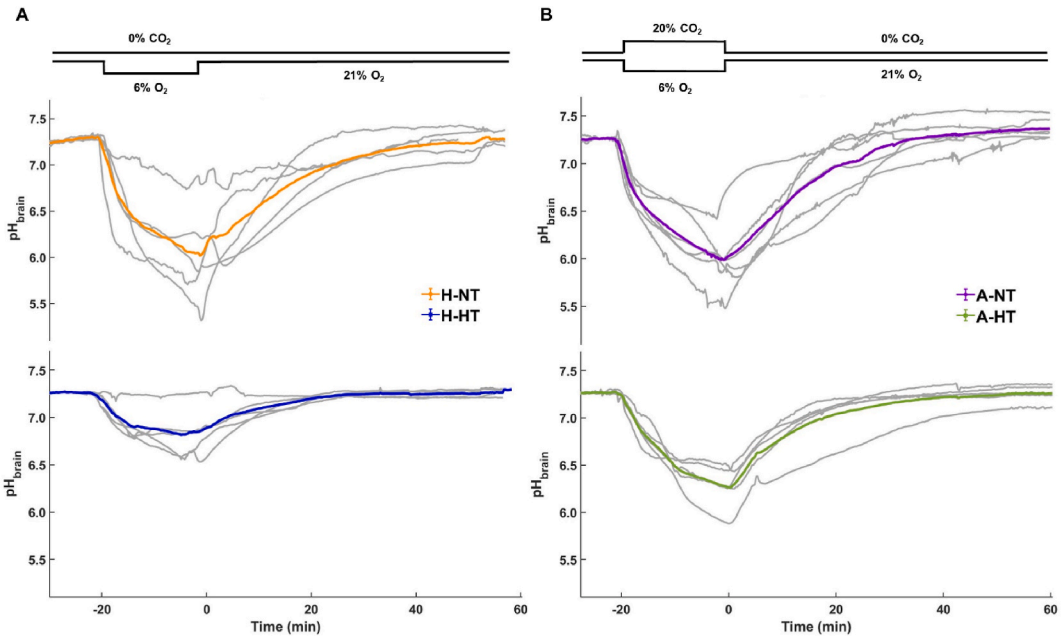

Fig. 2. pH_{brain} recordings (grey lines-individual traces, color lines –group means) in the experimental groups exposed to either hypoxia (Panel A) or asphyxia (Panel B) at normal body temperature (top plots) or at therapeutic hypothermia (bottom plots). The initiation of hypoxic/ischemic insult resulted in an acidotic pH_{brain} shift in every animal, regardless of the type of insult or the body temperature. There was a conspicuous attenuation of hypoxia-induced pH_{brain} drops in hypothermic animals. Data for the asphyxia-normothermia group are used from Ref. [8].

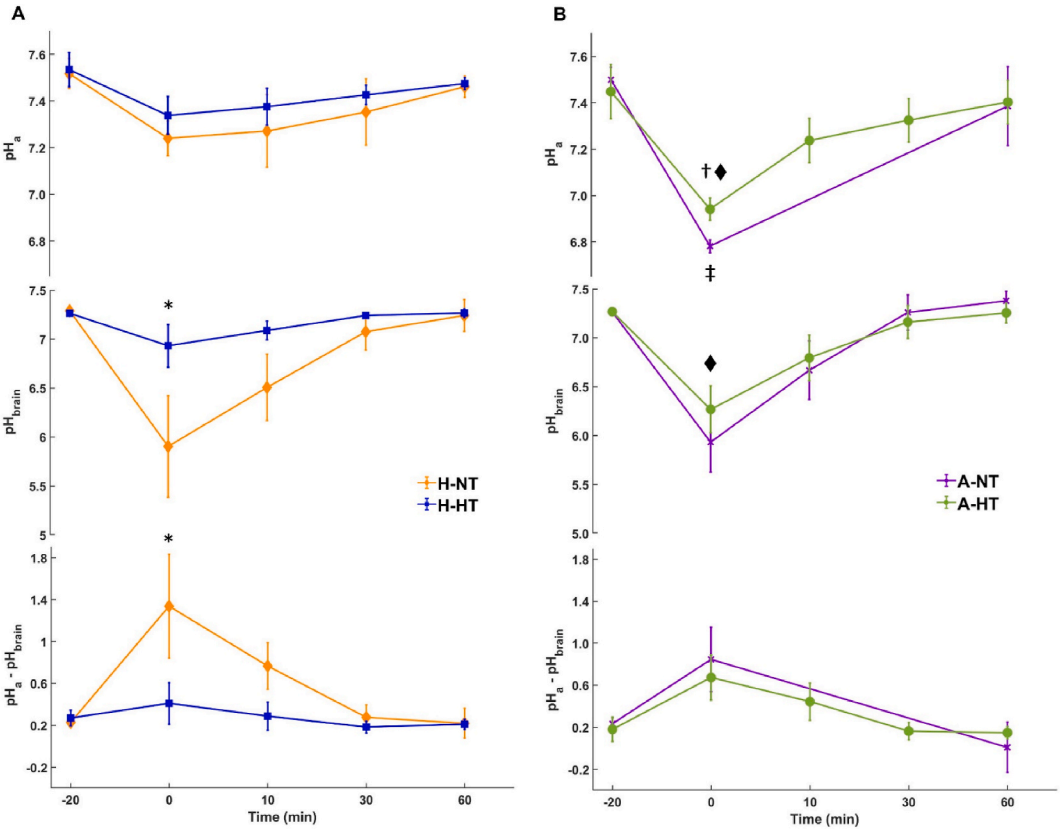

Fig. 3. pH_a, pH_{brain}, and pH_a-pH_{brain} changes in the experimental groups subjected to either hypoxia (Panel A) or asphyxia (Panel B) at normal body temperature or at therapeutic hypothermia. Hypoxia caused a significantly smaller drop in pH_a than asphyxia at both temperatures. Furthermore, hypothermia significantly ameliorated the blood acidosis induced by asphyxia but not by hypoxia. In contrast, the degree of cerebral acidosis induced by either hypoxia or asphyxia was similar in normothermia. In hypothermia, the drop in pH_{brain} induced by either hypoxic/ischemic insult was significantly reduced, however, the amelioration was much more robust in the H-HT group, thus creating a significant difference in pH_{brain} values at the nadir of the insults. The pH_a-pH_{brain} difference representing the pH gradient developing across the blood brain barrier were greatly increased by the hypoxic/ischemic insults in all experimental groups with the exception of the H-HT group mainly due to the robust amelioration of the change in pH_{brain}. All data show mean±SD. Data for the A-NT group are used from Ref. [8]. *H-NT vs H-HT, † A-NT vs A-HT, ‡ H-NT vs A-NT, ◆ H-HT vs A-HT.

4. Discussion

In this study, we assessed the effects of HI stress on pH_{brain} - one of the key pathophysiological factors determining neuronal damage – in a major large animal HIE model, the newborn pig. We were especially interested, if the two major HI insult models commonly used in HIE research in this species, namely (normocapnic/hypoxic) hypoxia and (hypercapnic/hypoxic) asphyxia would trigger a differential pH_{brain} response, more specifically an alkalotic pH_{brain} shift to hypoxia and an acidotic shift pH_{brain} to asphyxia similar to a previous report in P6 rats [6]. One of the most important results of the present study unequivocally demonstrates that both HI insults, hypoxia and asphyxia, trigger similar, severe acidotic pH_{brain} shifts greatly exceeding the acidosis measured in the arterial blood (pH_a). To address the obvious discrepancy between our results in piglets with that of the rat data, we repeated our experiments at the hypothermic body temperature that was used in that study. Although still no hypoxia-induced alkalotic pH_{brain} shift could be recorded in piglets, the cerebral acidosis to hypoxia but not to asphyxia was virtually ameliorated by hypothermia suggesting that the discrepancy can be attributed more to selected experimental conditions than to interspecies difference. In the subsequent paragraphs we carefully discuss the potential mechanisms of the observed differences in the pH_{brain} responses to hypoxia or asphyxia elicited at normothermia or hypothermia and the relevance of our findings in preclinical HIE research.

Brain pH changes during/after hypoxic/ischemic insults have been studied previously in piglet HIE models. However, direct comparison of these reports to the findings of the present study are confounded by the employed hypoxic/ischemic stress type that can directly affect the degree, and the mechanism of brain pH alterations. In a set of elegant studies, ^{31}P and ^{1}H nuclear magnetic resonance spectroscopy was established [21] then employed to study changes in intracellular pH (pH_i), lactate levels and energy metabolites in the cerebral cortex of piglets subjected to ischemia [22,23]. In these studies, partial or near-complete cerebral ischemia (~60–75% vs. 90% reduction in cerebral blood flow, respectively) was elicited by phlebotomy reducing mean arterial blood pressure below the lower limit of cerebral blood flow autoregulation combined with either bilateral carotid artery occlusion [22,23] or neck compression [24] –

Table 2
Statistical analysis of data shown in Table 1, Fig. 3A and B and Fig. 4A and B at the nadir of the respective hypoxic/ischemic insult and 60 min after reoxygenation. Highlighted cells show p-values indicating a significant difference in the pairwise comparisons.

		Nadir of insult				60 min of reoxygenation			
		H-NT vs H-HT	A-NT vs A-HT	A-NT vs H-NT	H-HT vs A-HT	H-NT vs H-HT	A-NT vs A-HT	A-NT vs H-NT	H-HT vs A-HT
MABP (mmHg)	mean diff.	18.80	6.12	10.58	14.33	2.90	10.13	3.33	9.70
	95% conf. int.	−14.30–51.90	−19.05–31.29	−10.02–31.19	−21.78–50.45	−17.79–23.59	−2.64–22.90	−9.44–16.11	−10.99–30.39
	p value	0.407	0.903	0.492	0.687	0.980	0.154	0.887	0.574
HR (min^{-1})	mean diff.	13.30	35.53	28.60	50.83	15.30	58.26	18.66	24.30
	95% conf. int.	−110.63–137.23	−58.15–129.21	−47.89–105.09	−84.38–186.05	−31.83–62.43	29.18–87.35	−10.42–47.75	−22.83–71.43
	p value	0.990	0.718	0.727	0.724	0.806	<0.0001	0.310	0.496
cSO_2 (mmHg)	mean diff.	3.99	6.03	1.32	11.34	0.58	0.75	5.96	7.30
	95% conf. int.	−6.45–14.43	−2.95–15.02	−7.67–10.30	0.89–21.78	−6.08–7.24	−5.27–6.77	0.42–11.51	0.23–14.36
	p value	0.714	0.270	0.976	0.030	0.995	0.985	0.032	0.041
pO_2 (mmHg)	mean diff.	5.62	2.24	3.60	0.74	9.80	17.06	18.04	25.30
	95% conf. int.	−4.41–15.65	−7.79–12.27	−7.16–14.36	−9.29–10.77	−13.51–33.12	−5.26–39.38	−4.28–40.36	1.98–48.62
	p value	0.405	0.918	0.775	0.997	0.638	0.171	0.138	0.031
pCO_2 (mmHg)	mean diff.	1.74	20.29	114.09	95.54	1.70	7.43	14.93	9.20
	95% conf. int.	−32.03–35.51	−7.53–48.11	86.27–141.91	61.76–129.31	−17.46–20.86	−8.36–23.22	−0.86–30.72	−9.96–28.37
	p value	0.999	0.213	<0.0001	<0.0001	0.995	0.575	0.069	0.559
Glucose (mmol/L)	mean diff.	1.08	2.71	0.40	1.23	0.38	0.99	1.49	0.87
	95% conf. int.	−2.89–5.06	−0.85–6.28	−2.87–3.68	−2.98–5.45	−4.49–5.25	−3.34–5.33	−2.48–5.47	−4.29–6.04
	p value	0.876	0.182	0.986	0.852	0.996	0.920	0.732	0.966
pH_a	mean diff.	0.10	0.16	0.46	0.40	0.01	0.02	0.07	0.07
	95% conf. int.	−0.01–0.20	0.06–0.26	0.36–0.56	0.29–0.50	−0.17–0.20	−0.16–0.19	−0.10–0.26	−0.12–0.26
	p value	0.082	0.002	<0.0001	<0.0001	0.997	0.993	0.646	0.711
pH_{brain}	mean diff.	1.03	0.33	0.03	0.66	0.02	0.12	0.13	0.01
	95% conf. int.	0.41–1.64	−0.92–0.25	−0.55–0.62	0.05–1.27	−0.17–0.22	−0.06–0.31	−0.05–0.32	−0.19–0.21
	p value	0.0009	0.393	0.999	0.031	0.984	0.295	0.212	0.999
pH_a-pH_{brain}	mean diff.	0.93	0.17	0.49	0.26	0.01	0.14	0.21	0.06
	95% conf. int.	0.34–1.51	−0.38–0.73	−0.06–1.05	−0.32–0.85	−0.26–0.28	−0.12–0.40	−0.05–0.47	−0.21–0.33
	p value	0.002	0.815	0.097	0.58	0.999	0.451	0.135	0.917
BE(b) (mmol/L)	mean diff.	3.40	5.92	4.38	1.86	2.38	1.87	3.89	0.36
	95% conf. int.	−1.66–8.46	0.86–10.98	−0.68–9.44	−3.20–6.92	−5.11–9.87	−5.30–9.04	−3.28–11.06	−7.13–7.85
	p value	0.258	0.019	0.102	0.722	0.803	0.880	0.437	0.999
Lactate (mmol/L)	mean diff.	2.11	5.54	0.49	2.95	1.34	4.58	3.18	0.07
	95% conf. int.	−1.56–5.78	2.03–9.06	−3.02–4.00	−0.72–6.62	−1.14–3.81	2.22–6.95	0.81–5.55	−2.40–2.54
	p value	0.386	0.002	0.978	0.142	0.439	0.0002	0.007	1.000

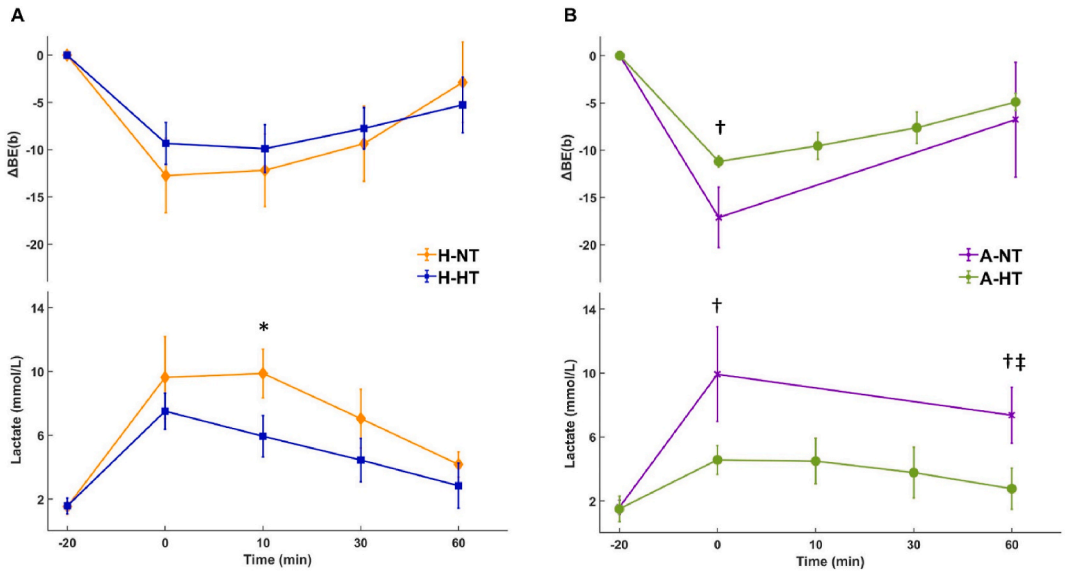

Fig. 4. Blood base excess (ΔBE(b)) and lactate level changes in the experimental groups subjected to either hypoxia (Panel A) or asphyxia (Panel B) at normal body temperature or at hypothermia. In normothermia, both hypoxia and asphyxia elicited marked drops in ΔBE(b) and robust elevations in lactate indicating the developing metabolic acidosis. Interestingly, hypothermia could significantly reduce the developing base deficit and attenuate lactate levels in the animals subjected to asphyxia but not to hypoxia. Nevertheless, the restoration of lactate levels appeared to be significantly quickened by hypothermia in the H-HT group as well, and by 60 min after reoxygenation, lactate levels were restored to baseline except in the A-NT group. All data show mean±SD. Data for the A-NT group are used from Ref. [8]. *H-NT vs H-HT, † A-NT vs A-HT, ‡ H-NT vs A-NT, ◆ H-HT vs A-HT.

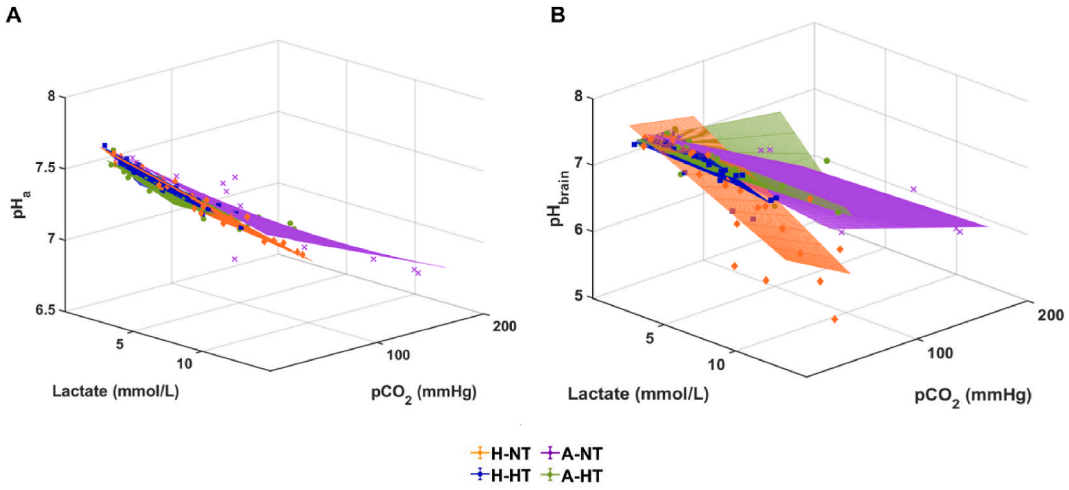

Fig. 5. Results of multiple linear regression analysis showing correlation between pH_a (Panel A) or pH_{brain} (Panel B) and blood lactate + pCO_2 levels in the experimental groups subjected to either hypoxia or asphyxia under normothermic or hypothermic conditions. The striking difference between the similar, strong correlation of lactate + pCO_2 with pH_a in all experimental groups and the weaker and in the different groups much more variable correlation with pH_{brain} can be readily observed. Data for the A-NT group are used from Ref. [8].

the latter perhaps to mimic strangulation by the umbilical cord. At the same time, normocapnia was rigorously maintained during the ischemia by adjusting the settings of the mechanical respiration. Under these conditions, the developing cerebral acidosis was intimately coupled to the accumulation of lactic acid that was found critically dependent on preischemic [22] and intraischemic [23] blood glucose levels – clearly glucose delivery to the brain to fuel anaerobic lactate production was a limiting factor during partial cerebral ischemia. The degree of acidotic pH_i shift induced by near complete ischemia could also be manipulated to more severe values by preischemic administration of glucose [24].

In our experimental design, neither hypoxia nor asphyxia elicit cerebrocortical ischemia, instead, a pronounced (80–100%) or a moderate (10–40%) increase in cerebrocortical blood flow was demonstrated, respectively, using laser-speckle contrast imaging [25,

26]. In our present study, the control and the intra-insult levels of blood glucose and lactate (Table 1, Fig. 4A and B) were similar during either hypoxia or asphyxia to those reported before/during near-complete ischemia (glucose: 6.2 ± 0.8–9.7 ± 3.6 mmol/L; lactate: 1.9 ± 0.5–6.9 ± 2.4) [24]. However, under these conditions, the minimal pH_i (6.32 ± 0.10) was much less acidic than the pH_{brain} in our study either to hypoxia (5.90 ± 0.52) or asphyxia (5.93 ± 0.30). In the case of asphyxia, the cause of the difference seems obvious, as the brain acidosis to ischemia lacked the obvious respiratory component elicited by the severe hypercapnia of asphyxia. Indeed, both pH_i and pH_{brain} were shown to have similar linear correlation with arterial pCO_2 under normoxic/normothermic conditions in the piglet model as well [8,27]. However, the cause of the much lower pH_{brain} value developing also to isolated hypoxia in our study is intriguing. Interestingly, in the study by Corbett et al., a very similar minimal pH_i value (5.89 ± 0.11) was recorded in piglets manipulated to have very high blood glucose values (21.6 ± 9.1) during the ischemic insult [24]. This lower pH_i coincided with much higher brain extracellular lactate levels (18.8 ± 4.6 vs 13.2 ± 2.8 mmol/L) but was not at all reflected in blood lactate levels as these were virtually identical (6.9 ± 3.4 vs 6.9 ± 2.4 mmol/L) compared to the animals not receiving glucose. Although a major limitation of our study is that we could not measure the brain extracellular lactate levels, but based on the available pH_{brain}, blood sugar/lactate levels, and cerebrocortical perfusion data, we can carefully speculate that despite the more modestly elevated blood sugar levels in our study during hypoxia, the almost doubled cerebrocortical blood flow delivered similar amounts of glucose to the hypoxic brain fueling similar rates of lactate production and accumulation resulting in the more pronounced cerebral acidosis – similar to that achieved only under hyperglycemic conditions in the near-complete ischemia model.

Furthermore, comparison of our pH_{brain} data from normothermic animals subjected to hypoxia and asphyxia suggest that the more pronounced carbonic acidosis during asphyxia likely attenuates anaerobic lactate production in our model. We showed previously that normoxic hypercapnia (21% O_2, 20% CO_2) alone reduces pH_{brain} to 6.77 ± 0.05 [8] thus, if lactate production/accumulation levels were similar, a more profound drop in pH_{brain} during asphyxia than during hypoxia would be expected, however, these minimum pH_{brain} values were virtually identical in these groups suggesting reduced lactate contribution to the acidosis in asphyxia. This inverse relationship between pCO_2 and brain lactate levels induced for instance by hypo or hyperventilation has long been known [28,29] and were shown to be dependent on local changes of the cerebral metabolic rate of glucose [30]. In summary, under normothermic conditions, a complex interaction of a number of factors appear to determine the pH_{brain} response to hypoxic/ischemic insult: a more severe hypoxia, higher blood sugar level and higher cerebral blood flow all strongly promote acidosis, while less severe hypoxia, lower blood glucose and brain ischemia tend to reduce pH_{brain} drops. Rise in CO_2 levels on one hand promote carbonic acidosis but appear to simultaneously limit metabolic/lactic acidosis, and in our experimental model these opposing actions seem to neutralize each other resulting in the virtually identical pH_{brain} responses to hypoxia and asphyxia. Moreover, the pH_i/pH_{brain} values obtained in various piglet HIE models are also in accordance with these assumptions [22,23,31].

After completion of hypoxia or asphyxia, restoration of pH_{brain} showed similar dynamics returning towards baseline levels within 40–60 min. In our previous study we showed that after restoration, pH_{brain} levels after asphyxia were stable without secondary acidotic/alkalotic shifts for 24h [8]. Restoration of pH_{brain} depends on the normalization of arterial pCO_2 and lactic acid extrusion or metabolism. Astrocytes have a major role in removing the lactate from the brain. They express both the monocarboxylate transporter types 1 and 4 (MCT1 and MCT4). MCT4 is a high lactate affinity transporter which is able to reduce the lactate from high lactate concentration microenvironment during hypoxia [32,33]. The cerebral microvessels also express functional MCT1 and hydroxycarboxylic acid receptor 1 (HCAR1) the latter being a lactate receptor both playing an important role in brain lactate signaling [34]. MCT4 but not MCT1 may be upregulated by hypoxia [35] via the hypoxia-inducible factor 1α (HIF-1α) [36]. In addition to MCTs, hemichannels made up of connexins/pannexin have been reported to also participate in hypoxia-induced lactate release *ex vivo* [37]. Importantly, peripheral lactate may be transported into the brain, and the metabolic consumption of exogenous lactate by the newborn brain has been found neuroprotective in a rat HIE model [38].

In contrast to the consistent, similar magnitude of acidotic pH_{brain} shifts during either hypoxia or asphyxia that was restored to normal levels without alkalotic overshoot in our piglet studies, P6 rat pups were found to respond with a cerebral alkalosis, ie pH_{brain} elevation to hypoxia and with the expected acidosis to asphyxia [6,39]. The hypoxia and the asphyxia was induced with ventilation with a hypoxic/hypercapnic gas mixture very similar to the ones employed in our piglet studies (5% vs 6% O_2 in the rat vs the piglet study and 20% CO_2 in both studies), while alkalotic shift appears in response to hypoxic (5% O_2) stress. We noted that the rat pups were cooled to core body temperatures consistent with TH likely in order to survive the 40 min long hypoxic/ischemic insult. To check whether the temperature or perhaps interspecies difference is responsible for the different direction pH_{brain} response to hypoxia, in the second part of our study we repeated the insults at hypothermia. This level of hypothermia is known to reduce both cerebral metabolic rate and cerebral blood flow more than 50% in newborn pigs, although relative cerebrovascular reactivity to arterial pCO_2 was preserved [40]. In accordance with the increased oxygen availability due to hypothermia-induced reductions in oxygen consumption and reduced cerebral blood flow abrogating anaerobic lactate production, acidotic pH_{brain} shifts were reduced in piglets exposed to asphyxia, and nearly but not fully abolished in piglets exposed to asphyxia. In addition, increase in blood lactate levels were attenuated in piglets exposed to asphyxia but not hypoxia. The remaining difference in the observed pH_{brain} response between piglets and P6 rat pups exposed to hypoxia under hypothermic conditions may be due to the difference in ventilation, the piglets were mechanically ventilated enabling stable respiration during the hypoxic period, whereas the rat pups were breathing spontaneously under urethane anesthesia [6] and likely hyperventilated during the hypoxia leading to further reductions in arterial pCO_2 pushing the pH_{brain} towards alkalosis. This explanation for the observed difference is feasible and possibly clinically important as cooling is often associated with hypocapnia in human HIE patients as well [41].

5. Conclusion

We conclude that both normocapnic hypoxia and hypercapnic asphyxia that are commonly employed HI stress types in piglet HIE models, result in similar levels of severe cerebral acidosis during the HI insult and show a similar dynamic of pH_{brain} restoration upon reoxygenation. These findings suggest that the two HI stress types have similar impact on cerebral neurons in this aspect (pH_{brain} alterations) during HIE development in our experimental settings. The similarity of pH_{brain} responses to hypoxia or asphyxia was shown to depend heavily on the body temperature and maybe other yet unexplored factors that could affect the neurological outcome and the translational value of the piglet HIE model.

Data availability

All experimental data are available at Open Science Framework: DOI: 10.17605/OSF.IO/TKARF. All previously published experimental data included in the recent manuscript are available at Open Science Framework: DOI: 10.17605/OSF.IO/MUTGA.

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CRediT authorship contribution statement

Gábor Remzső: Writing – original draft, Visualization, Formal analysis, Data curation, Conceptualization. **Viktória Kovács:** Validation, Methodology, Data curation, Conceptualization. **Valéria Tóth-Szűki:** Validation, Project administration, Methodology, Data curation, Conceptualization. **Ferenc Domoki:** Writing – review & editing, Validation, Supervision, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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