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BASIC SCIENCE ARTICLE

aEEG and neurologic exam findings correlate with hypoxic–ischemic brain damage severity in a piglet survival model

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BACKGROUND: Newborn pigs offer theoretical advantages for studying newborn hypoxic–ischemic (HI) brain damage because of a development and structure similar to the human brain. However, the correlation between functional features and actual HI brain damage has not been reported.

METHODS: Newborn pigs were examined daily for 3 days after a HI insult using amplitude-integrated EEG (aEEG), and a neurobehavioral score enriched with stress and social and object interaction-driven activity evaluation. Brain damage was then assessed using histologic, immunohistochemical, and proton magnetic resonance spectroscopy studies. Brain concentration of several neurotransmitters was determined by HPLC.

RESULTS: HI insult led to aEEG amplitude decrease, muscle tone and activity impairment, eating disorders, poor environmental interaction, and increased motionless periods. Basal aEEG amplitude, muscle tone, and general behavior were the best predictive items for histological and biochemical (lactate/*N*-acetylaspartate ratio) brain damage. Hyperexcitable response to stress correlated inversely with brain damage. Motionless time, which correlated with brain damage severity, was inversely related to brain concentration of dopamine and norepinephrine.

CONCLUSION: Standard neurologic examination of brain activity and motor and behavioral performance of newborn pigs is a valuable tool to assess HI brain damage, thus offering a powerful translational model for HI brain damage pathophysiology and management studies.

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INTRODUCTION

Most effective neuroprotective therapies in animal models fail to demonstrate such efficacy when translated into clinical practice, one of the reasons being lack of a functional assessment when studying the outcome.[1] Although most animal models of newborn hypoxic–ischemic encephalopathy (NHIE) including neurobehavioral assessment have been reported in newborn rodents, large mammals such as newborn pig offer theoretic substantial advantages as a model of NHIE.[1] Piglets offer a unique tool for translational studies on newborn brain damage since piglet brain development, including gyral pattern, vascularization and moreover, white and gray matter distribution are far closer to humans than rodents.[2,3] It is of some interest too that the PK profile of most drugs are similar in pigs and humans.[2] Besides, piglets show just after birth some of the rich and complex neurobehavioral characteristics of pigs, including social and environmental interaction.[3,4] This, together with an opportunity for non-stressful early weaning[5] enables including a neurobehavioral assessment when using the piglet as a model of newborn brain damage. In addition, piglet head size allows using neuromonitoring similar to that in humans,[2] for instance amplitude-integrated EEG (aEEG).[6–8] These characteristics as a whole give piglet models a strong translational power for neuroprotection-oriented studies to bridge the gap between rodents and humans. There are different piglet models for studying NHIE. We have previously reported that a model combining hypoxia with carotid artery blood flow interruption leads to cerebral[6–8] and even extracerebral[9] damage comparable to that observed in human infants.

Despite the aforementioned characteristics, very few works describe neurobehavioral studies of newborn pigs after inducing acute brain damage. We reported[7] that HI insult leads to poor performance in a neurobehavioral test based on that reported by Leblanc et al.[10] more than two decades ago. However, that test was focused on motor domains with just some general references to neurovegetative status and behavior. Studies with reference to pure behavioral consequences of brain damage on piglets are scarce and most focus on traumatic brain injury.[4]

Although works reporting data from neuromonitoring and/or neurobehavioral assessment in piglets after acute brain injury also present some histologic and/or biochemical studies demonstrating the existence of brain damage,[4,7,10–12] none of those works actually correlates them. Thus, the actual value of the functional

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(neuromonitoring and neurobehavioral) assessment as a tool to evaluate or predict actual damage in acutely brain-injured piglets has not yet been established.

The aim of this work was to study the correlation between functional assessment by gathering data form neuromonitoring and standardized neuromotor examination test enriched with specific behavioral assessments and a comprehensive set of brain damage markers after HI insult in piglets.

METHODS

Experimental model

All procedures complied with European Directive (2010/63/EU) and Spanish (RD 53/2013) regulations for protection of experimental animals and were approved by the Animal Welfare Ethics Committee of Hospital Universitario Puerta de Hierro Majadahonda in Spain (ProEx 175/14). All experimental procedures were designed and carried out by personnel qualified in Laboratory Animal Science, following FELASA recommendations on categories B and C to reduce animal stress and to enhance animal welfare. All surgery was performed under adequate anesthesia and analgesia, and great effort was made to minimize suffering and reduce the number of animals used. Furthermore, all experimental procedures on animal welfare (anesthesia and analgesia, drug and substance administration) and euthanasia of the animals were conducted in compliance with FELASA recommendations. Since previous experiments by our group reported the item "NBS" as the one with the greater variance, sample size was calculated accordingly and based on those previous results[7] ($\alpha = 0.05$, power 80%, variance 16, accuracy 4).

The protocol was based on the model reported extensively elsewhere.[7] In short, 1-day-old male Landrace-White large piglets provided by a local certified farm the day the experiment started were intubated and then mechanically ventilated (Evita, Dräger, Germany) under sevoflurane anesthesia (5% induction and 1% maintenance) and received morphine chloride 1 0.1 mg/kg I.M. Piglets were then randomly assigned to Sham (SHM, $n = 5$) or Hypoxic–ischemic (HI, $n = 16$) group in a blind fashion. In the HI group, each carotid artery was exposed and surrounded by an elastic band, and a right jugular vein indwelling catheter was inserted to infuse dextrose 4 mg/kg/min. The catheter was kept in place over the entire experimental period for i.v. drug administration. Cardiac output (CO), heart rate (HR), mean arterial blood pressure (MABP), and central temperature were monitored (PiCCO Plus, Pulsion, München, Germany) by a femoral artery indwelling catheter (Ominare CMS24, HP, Göblingen, Germany). Body temperature was maintained at 37.5–38 °C by an air-warmed blanket. Arterial blood gases and glycemia were monitored throughout the experimental period and kept between normal limits. Finally, non-traumatic stainless-steel wires were placed into the piglet head's scalp to continuously monitor brain activity by aEEG (BRM3, BrainZ Instruments, Auckland, New Zealand). Quantitative changes in aEEG amplitude were recorded and the raw EEG traces manually reviewed for electric seizures (periods of rhythmic activity starting with sudden increase in voltage of at least 2 μV, accompanied by a narrowing of the band of aEEG activity, lasting at least 15 s).

After a 30-min period of stabilization, HI piglets underwent a 20-min-long cerebral HI insult by interrupting carotid blood flow by pulling out the carotid bands and reducing inspired oxygen fraction (FiO_2) to 10%. The 20-min countdown started when aEEG trace became flat. At the end of the HI period, carotid flow was restored and FiO_2 increased to 21%. Piglets were similarly managed but without HI insult were used as reference (SHM group).

Following HI and after fully regaining consciousness from the experimental procedure and anesthesia, piglets were extubated and transferred to the animal care facility. Piglets were housed in stainless-steel cages with 0.5 m^2 per animal in a well-controlled warm-temperature environment. The cage contained a pending rope and a plastic ball for environmental enrichment. From 24 h post-insult piglets were fed with artificial piglet formula (Nutrilac Milk Replacer for young piglets, Joosten Products B.V., Weert, The Netherlands) every 3–4 h by a catheter attached to the examiner's finger to assess suckling. The examiner was blinded to the experimental group. Ceftazidime 15 mg/kg i.v. was administered every 12 h to prevent infections and acetaminophen 15 mg/kg i.v. was administered every 8 h for pain control. Seventy-two hours after the HI insult piglets were killed by KCl infusion under anesthesia (5% sevoflurane). Both carotid arteries were cannulated to perfuse brains with heparin in cold saline until clean fluid flew from the sectioned jugular arteries. Then the brains were removed from the skull and sectioned. Brain slices from the left hemisphere were placed into 4% paraformaldehyde for histologic analysis whereas those from the right hemisphere were snap frozen in isopentane and stored at −80 °C for spectroscopy and biochemical studies.

Neurobehavioral assessment

Each morning from 8 to 10 am the piglets were studied in the room where its cage was placed and video-recorded by one examiner. All video recordings were subsequently evaluated and scored when appropriate by three researchers blinded to the experimental group to obtain a mean value of the different items. A neurobehavioral score (NBS: 8–36 pts) was carried out and video-recorded every 24 h, measuring alertness, behavior, muscle tone, standing, walking, and eating[7,10,12] (Table 1). Piglets were also video-recorded during free wandering for 5 min, to quantify the time spent in playful behavior with some known object (the sheet used for feeding) or caretakers. Time with no activity while standing and lasting more than 5 s was considered motionless time. After the neurobehavioral assessment, piglets were wrapped up with a blanket and held by an examiner sat in front of the aEEG device, to assess cerebral activity over 10 min Test start time and time when the piglet stopped fighting against immobilization (FAI) were recorded. That time of restlessness during slight restraint for aEEG performance was considered an indicator of piglet anxiety.

Histologic analysis

Histological studies were performed in 4-μm thick coronal sections obtained from fixed brain hemispheres, as reported previously.[7,8] Areas of 1 mm^2 in the central three lobes of the parietal cortex were examined by two skilled investigators blinded to the experimental group. Neuronal necrosis was identified by Nissl staining.[7,8] Immunohistochemistry studies were performed in the same brain areas as reported previously[8] to detect and quantify mature neurons (NeuN 1:600; Merck Millipore, Billerica, MA), astrocytes (GFAP-Cy3 1:1000; Sigma-Aldrich, Madrid, Spain), and microglia (Iba1 1:500; Wako Chemicals GmbH, Neuss, Germany). Using the ImageJ 1.43u software (NIH, Bethesda) neuronal and glial cell density were calculated; in addition, the areal percentage of GFAP-immunoreactive or Iba1-immunoreactive cell bodies and processes were calculated to determine cell mean size and process mean length. Astrocytes were considered activated when they showed a highly ramified morphology with hypertrophic processes whereas microglial cells were considered activated when they presented an ameboid shape with enlarged soma and thickened and retracted branches. Cell death was further studied by TUNEL staining (DeadEnd Colorimetric TUNEL System, Promega, Spain) as reported elsewhere.[7] Samples were visualized and photographed with a confocal TCS SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany).

Proton magnetic resonance spectroscopy (H+ -MRS)

Ex vivo 1H spectrum was performed on a Bruker Avance 11.7 Tesla spectrometer (Bruker BioSpin, Karlsruhe, Germany) equipped with

Table 1. Neurobehavioral score (Leblanc, Schubert, Lafuente)

Mental status	
Coma	0
Stupor	1
Lethargy	3
Awake	4
Behavior	
None	0
Weak	1
Aggressive	3
Normal	4
Pupils	
Non-reactive	1
Slow, assimetric	2
Normal	3
Vestibulo-ocular reflex	
Absent	1
Nistagmus	2
Normal	3
Stepping	
None	1
Just fore/hind paws	2
Normal	3
Righting	
Absent	1
Present	2
Muscle tone	
Atonic/hypertonic	1
Partially atonic	2
Partially hypertonic	3
Normal	4
Standing	
No	1
Paresis	2
Unsteady	3
Normal	4
Walking	
No	1
Paresis	2
Falling	3
Normal	4
Feeding	
No suckling reflex	1
Weak reflex, tube feeding	2
No appetite	3
Brief suckling	4
Normal	5

a 4 mm triple channel 1H/13C/31P HR-MAS (High Resolution Magic Angle Spinning) resonance probe. H-MRS was performed in the MRI Unit of Instituto Investigaciones biomédicas "Alberto Sols" (CSIC-UAM, Madrid, Spain). Frozen cortex samples (5–10 mg weight) were introduced into 50 µL zirconia rotor (4 mm OD) with 50 µL D2O and spun at 5000 Hz at 4 °C to prevent tissue degradation processes. Two types of monodimensional proton spectra were acquired using a water suppressed spin echo Carr Purcell Meiboom Gill (CPMG) sequence with 36 ms and 144 ms echo time and 128 scans. Data were collected into 64 k data points using a spectral width of 10 kHz (20ppm) and water presaturation during a relaxation delay of 2 s, total acquisition 16 min. All spectra were analyzed using the LC Model software,[13] a prior knowledge spectral fit software; only peak concentrations obtained with a standard deviation lower than 20% were accepted. The content of glutamate (Glu), lactate (Lac), myo-inositol (mI), and *N*-acetylaspartate (NAA) was normalized to the creatine content.

Brain neurotransmitter concentration

Brain concentration of norepinephrine (NE), dopamine (DA), and serotonin (5-HT) was measured in homogenate from frozen brain tissue by HPLC, and expressed as fmol/100 mg.

To determine the amount of neurotransmitters in tissue, high-performance liquid chromatography was used.[14] In short, tissues were ultrasonically homogenized in 0.4 M perchloric acid with 5 mM sodium metabisulfite, 8.3 µM cysteine and 0.3 mM of EDTA. Samples were then centrifuged at 12,000×*g* for 30 min at 4 °C. The three neurotransmitters were separated using an Ultrasphere 3-µm column (7.5 cm × 0.46 cm, Beckman, San Ramon, CA) and detected with a Hewlett-Packard amperometric detector (Palo Alto, CA). The mobile phase was comprised of 0.15 m KH2PO4, 0.46 mm octyl sodium sulfate, 0.5 mmEDTA (pH 2.8 adjusted with phosphoric acid) and 12% methanol, and was pumped at a rate of 0.6 mL/min.

Statistical analysis

Normality of data distribution was assessed by Kolmogorov–Smirnov test. All data are presented as mean (standard error of the mean, SEM). Paired values were compared using Student *t*-test. Correlation was studied using Pearson's test. $p < 0.05$ was considered to be statistically significant. StatPlus:mac v6 (AnalystSoft Inc., Walnut, CA) software was used to perform all statistical analyses.

RESULTS

Mortality

From 16 HI piglets four died before completing 72 h follow-up, which represents a mortality of 25%, and accordingly their data were excluded.

Assessment of HI-induced brain damage

Physiologic perturbations occurring during HI are described in Supplementary Table S1, showing that HI led to lactic acidosis and hyperglycemia associated to decreased MABP and increased HR. Different consequences of the HI insult as assessed 72 h after HI are shown in Table 2.

Histology

HI led to a reduction in density of surviving neurons (NeuN+ cells) in the cortex, in parallel to the increase in necrotic neurons (Suppl. Fig S1). HI induced a marked increase in the number of TUNEL+ cells. HI led to an astrogliotic response, increasing the number of astrocytes as well as the percentage of activated astrocytes (Suppl. Fig S1). In the case of microglial cells there were no differences between sham and HI animals in the density of Iba1+ cells. However, the percentage of microglial cells with activated phenotype was increased in HI piglets (Suppl. Fig S1).

H^+-MRS

HI insult led to brain metabolic derangement, as reflected by the increase in Lac/NAA ratio.[15] In addition, HI led to astrocyte function impairment, as reflected by the decrease in mI/Cr ratio.[16] At the time of H^+-MRS study (72 h after HI insult) there was a measurable increase in excitotoxicity as reflected by the higher Glu/Cr ratio value in HI than in SHM animals.

Table 2. Group characteristics

	Sham ($n = 5$)	HI ($n = 12$)
Weight (kg)	1.72 (0.08)	1.73 (0.06)
Histology		
NeuN+ cells (*n*)	4.1 (0.4)	2.7 (0.3)*
Necrotic neurons (%)	1.8 (0.2)	10.7 (2.7)*
TUNEL+ cells (*n*)	2.8 (1.4)	187.3 (63.8)*
GFAP+ cells: *n*	28.6 (8.8)	61.1 (5.9)*
Activated (%)	9.1 (4.5)	24.4 (4.9)*
Iba1+ cells: *n*	94.8 (11.5)	107.6 (14.3)
Activated (%)	5.8 (2.2)	38.3 (13.1)*
H^+MRS		
Lac/NAA	0.86 (0.04)	2.20 (0.52)*
mI/Cr	1.59 (0.14)	1.27 (0.09)*
Glu/NAA	0.69 (0.06)	1.32 (0.25)*
aEEG		
Basal amplitude (μV)	10.1 (0.1)	8.1 (0.6)*
Mean amplitude (μV)	18.1 (0.1)	13.4 (1.3)*
Electrical seizures	0/5	5/12
NBS		
Global (points)	35.5 (0.5)	29.1 (1.6)*
Motor		
Tone (points)	4 (0)	2.8 (0.2)*
Walking (points)	4 (0)	3.1 (0.3)*
Eating		
Points	4.6 (0.3)	3.3 (0.3)*
Milk intake (mL/kg)	150.4 (6.3)	110 (10.8)*
Behavior		
Points	4 (0)	2.6 (0.3)*
Anxiety: FAI (min)	3.5 (0.5)	6.5 (1.2)*
Activity		
Playfulness		
Object (%)	24.5 (1.3)	18.5 (5.3)
Social (%)	54.9 (3.7)	18.5 (6.5)*
Motionless (%)	2.9 (0.6)	12.1 (3.8)*

Mean (SEM)
HI hypoxic–ischemic insult, *H^+MRS* proton magnetic resonance spectroscopy, carried out 72 h after HI, *Glu* glutamate, *Cr* creatine, *Lac* lactate, *NAA* *N*-acyl aspartate, *aEEG* amplitude-integrated EEG, carried out 72 h after HI, *NBS* neurobehavioural score, carried out 72 h after HI, *FAI* fighting against immobilization
*Student's *t*-test $p < 0.05$ vs. Sham

Functional studies

aEEG. HI insult led to a decrease in brain activity, as shown by the decrease in both basal and mean aEEG amplitude. aEEG background pattern in three HI piglets corresponded to continuous low voltage, in one HI piglet to discontinuous normal voltage and in the other piglets to continuous normal voltage. There was no relationship with histological, biochemical or neurobehavioral parameters.

In addition, none of SHM (0/5) but nearly half of HI piglets (5/12) revealed electric seizures in the aEEG, although such a difference did not attain statistical significance. Seizure burden in those piglets was 19.3 ± 7.6% of the recording time, ranging from 1.6 to 41.6%.

NBS. HI insult led to a remarkable decrease in NBS global score (Fig. 1). Such a decrease was because of the decrease in motor and behavioral scores as well as eating score (Fig. 1), which

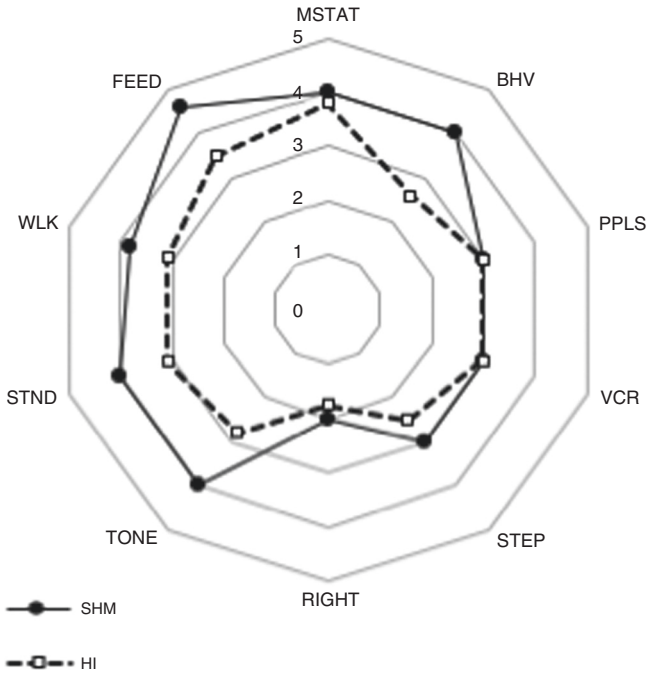

Fig. 1 Radial graph showing the mean value for the different items from a neurobehavioral test performed in newborn pigs 72 h after a hypoxic–ischemic (HI) insult or equivalent period in Sham piglets. BHV behavior, FEED feeding, MSTAT, mental status, PPLS pupils, RIGHT righting, STEP stepping, STND standing, TONE muscle tone, VOR vestibulo-ocular reflex, WLK walking

determined a reduced milk intake. Moreover, HI piglets showed increased anxiety as reflected by a near twofold increase in the time spent fighting against immobilization. During free activity periods, HI piglets showed a severely reduced time devoted to social contact with researchers compared to SHM animals. In addition, HI piglets showed a marked increase in time of no activity.

Correlation between HI-induced brain damage and functional studies

Coefficients and statistics of the correlation between functional studies and the most representative parameters reflecting brain damage (percentage of dead neurons by Nissl staining, density of TUNEL+ cells and Lac/NAA ratio) are shown in Table 3.

Global NBS correlated with percentage of dead neurons but not with other brain damage parameters. When analyzing specific items from the NBS, motor tone showed a good relationship with the different brain damage parameters with poorer muscle tone being associated with more severe brain damage. Feeding disturbances, either qualitative or quantitative (milk intake volume) correlated with histologic but not with biochemical parameters of brain damage. The item considering behavior showed a good correlation with histologic and biochemical brain damage, so that hypoactive or weak animals showed more severe brain damage. In agreement, anxiety response quantified as FAI time inversely correlated with brain damage parameters; thus, the more irritable the piglet the less severe the brain damage. Time spent in playful activity had no correlation with brain damage. However, motionless time did correlate with brain damage so that longer periods of no activity correlated with more severe brain damage.

Piglets showing seizures showed a decrease muscle tone score (2.4 ± 0.4 vs 3.6 ± 0.1 points in piglets having or not seizures, respectively, $p = 0.04$), but there were no differences in the other neurobehavioral items. Global NBS was similar in both

Table 3. Pearson's correlation between functional parameters and brain damage markers

	% Dead neurons			TUNEL+ cells			Lac/NAA		
	Coef	*t*-value	*p*-value	Coef	*t*-value	*p*-value	Coef	*t*-value	*p*-value
aEEG									
Basal amplitude (μV)	−0.71	−3.19	0.009	−0.55	−2.12	0.05	−0.90	−4.68	0.005
Mean amplitude (μV)	−0.13	−0.42	0.67	−0.61	−2.41	0.03	−0.93	−5.89	0.002
NBS									
Global (points)	−0.72	−3.31	0.007	−0.46	−1.64	0.13	−0.56	−2.14	
Motor									
Tone (points)	−0.64	−2.66	0.02	−0.62	−2.52	0.02	−0.61	−2.31	0.03
Walking (points)	− 0.68	−2.99	0.01	−0.52	−1.97	0.07	−0.49	−1.69	0.12
Eating									
Points	−0.70	−3.15	0.01	−0.10	0.34	0.73	−0.53	−1.4	0.21
Milk intake (mL/kg)	0.37	1.13	0.29	−0.66	−2.81	0.01	−0.40	−1.3	0.21
Behavior									
Points	−0.63	−2.58	0.02	−0.72	−3.34	0.007	−0.97	−5.40	0.001
Anxiety: FAI (min)	−0.46	−1.47	0.17	−0.78	−3.14	0.01	−0.73	−2.90	0.02
Activity									
Playfulness									
Object (%)	−0.01	−0.02	0.97	− 0.37	− 1.07	0.31	− 0.11	− 0.27	0.79
Social (%)	−0.01	0.03	0.97	− 0.19	− 0.50	0.62	− 0.57	−1.7	0.13
Motionless (%)	−0.07	−0.19	0.84	0.76	2.65	0.04	0.78	3.08	0.02

Lac lactate, *NAA N*-acyl aspartate, *aEEG* amplitude-integrated EEG, carried out 72 h after HI, *NBS* neurobehavioural score, carried out 72 h after HI, *FAI* fighting against immobilization

groups (26.2 ± 4.05 vs 33.3 ± 1.37 points in piglets having or not seizures, respectively, $p = 0.10$). Since motionless periods could correspond to seizures, we studied whether piglets having electrical seizures during aEEG record had longer motionless periods. Motionless periods were similar in piglets having or not seizures (motionless periods: 8.3 ± 3.1% vs 13.4 ± 4.3% time of recorded activity for piglets having or not seizures, respectively, $p = 0.32$).

There was no correlation between seizure burden and histological or neurobehavioral studies, although this could have been affected by the reduced number of seizing animals.

Correlation between motionless and neurotransmitter brain concentration

There was no relationship between motionless time and brain 5-HT concentration (Pearson's $R = 0.43$, $t = 1.27$, $p = 0.24$). However, a statistically significant inverse correlation was detected between motionless time and NE ($R = -0.75$, $t = -3.07$, $p = 0.01$) and DA ($R = -0.73$, $t = -2.85$, $p = 0.02$) brain concentration.

DISCUSSION

We have presented in this work a piglet model inducing brain damage and reproducing many of the clinical findings in terms of neuromonitoring and neurologic assessment, observed in HI newborns. We have reported the correspondence of those clinical findings with the actual histologic and/or biochemical brain damage, which not only leads to insights into the value of those findings for estimating brain injury in piglets but also supports the utility of some of those findings for assessing HI infants. The strength of this work is that we present a tool for piglet neurologic assessment that is easy to learn and use, feasible, simple and affordable for every research team, which enriches a well-known neuromotor test[10] with some basic neurobehavioral parameters. Piglets can be neurobehaviorally assessed reproducing most paradigms used in rodents such as open field, different mazes, or object recognition.[5,17] However, those tests demand a vast specifically dedicated space, apparatus not easily available and complex technology. By contrast, the model presented herein could be easily carried out in any facility appropriate for breeding piglets. Our model explores different items related to motor and neurovegetative domains, behavior and eating, reproducing findings from more sophisticated tests[4] related to activity, anxiety, and environmental interaction. As a whole, our model meets the main criteria for neurofunctional tests in piglets:[2] it has provided indices for different domains (motor, cognitive, and motivational), tapped ecologically relevant behaviors (foraging, eating, interacting, socializing) and was automated.

Our model demonstrated remarkable translational power. Despite the fact that our piglets were able to spontaneously breathe, eat and even walk, post-HI mortality in our experiment attained 25%, which was comparable to that described for similar models in piglets[10,12,18] but slightly superior to that reported for asphyxiated newborns with moderate encephalopathy[19] likely because in the experimental model piglets were not benefited from post-HI sustained critical care. In agreement, the HI insult led to a remarkable increase in brain cortex of cell death as well as Lac/NAA ratio—a well-known surrogate for HI brain damage in newborn animals and humans.[8,15] Moreover, we detected increased astroglial proliferation and activation after HI insult associated with a decrease in mI/Cr ratio in the H-MRS study. Proliferation of activated astrocytes is associated with post-HI brain damage[20] and decrease in mI reflects astrocyte functional impairment and corresponds to cell edema and cytolysis in acute brain damage.[16] Astrocyte dysfunction compromises natural mechanisms of post-insult repair.[20] We also detected increased activated microglial cell density. Microglial cells have a double-edged role after HI brain insults, but the activated type, with a characteristic ameboid shape like we observed in HI piglets, is associated with increased inflammation-related brain damage.[21]

Together with inflammation, excitotoxicity is one of the major factors leading to brain damage after a HI insult, in particular in immature brain hypersensitive to these factors.[22] In agreement, we detected increased concentrations of glutamate in brains from HI piglets. Thus, brain tissue from HI piglets in our model exhibited all the histologic and biochemical characteristics typical from moderate-to-severe HI brain damage.

aEEG has become a usual tool for bedside neurologic monitoring of HI infants. In our model, we observed a quantitative decrease in aEEG voltage maintained over the 72 h follow-up. HI piglets show severe abnormalities in aEEG voltage in the first hours after HI[6,8] but we have now observed that it was the decrease in aEEG voltage, in particular basal aEEG voltage, as observed 72 h after the HI insult which revealed a lineal relationship with histologic and biochemical parameters of brain damage. aEEG abnormalities after HI correlated with brain damage in HI newborns and although findings over the first 6 h after HI have good predictive value,[23] the best correlation between aEEG disturbances and brain damage severity is observed when those disturbances are still detected 48 h or more after HI insult.[24] However, most research has been focused on the value of aEEG background pattern rather than on voltage values, although it is well-known that patterns associated with low basal amplitude are related to worse outcomes after HI.[24] aEEG amplitude in asphyxiated infants correlates inversely with HIE clinical severity.[25] In a study on asphyxiated newborns, receiving hypothermia mean aEEG discontinuity correlated with severe MRI disturbances and unfavorable neurodevelopmental outcome.[26] Seizures are a common and worrying complication observed in the following hours to HI insults.[24] We observed a trend towards increase in aEEG-detected seizures in HI piglets, although that increase did not attain statistical significance. Muscle tone score was reduced in piglets showing seizures with no differences in other neurobehavioral items. There was no relationship between seizure burden and histological or neurological assessment. Since we studied aEEG periodically instead of continuously as is usual in HI infants, it is conceivable that some electric seizure episodes could have been overlooked in HI piglets. The lack of correspondence between background pattern or seizure burden and histological or neurological assessment could be determined by the fact that seizures and abnormal patterns are associated with increased severity in HI piglets[18] but in our model more severely affected piglets died.

The neuromotor test we used in this work has been reported to be affected by HI insults.[7,10–12] In accordance, we observed that HI piglets scored worse than SHM animals for all items except pupils and oculovestibular reflex, which were unaffected in all piglets. After looking for a relationship between the different NBS items and parameters of brain damage, the most predictive was motor tone with decreased tone and gait impairment as observed 72 h after HI insult correlating with more severe brain damage. Muscle tone impairment including gait disturbances have been considered part of moderate-to-severe neurologic disability in piglets.[27] In humans, the relationship between decreased muscle tone and severity of brain HI damage has long been known and is included in historic scales for clinical assessment of asphyxiated infants.[28,29] Although hypothermia has altered the prognostic value of early clinical assessment of asphyxiated newborns, motor abnormalities sustained for more than 3 days after birth correlate with more severe brain damage as observed by MRI[30] and adverse clinical outcome.[23] Interestingly, eating as assessed either qualitatively or quantitatively (volume of milk eaten) correlated with brain damage. Previous studies have reported feeding abnormalities as a part of severe neurologic impairment in piglets,[27] a similar finding observed in HIE infants in which feeding problems antecede poor short and long-term outcome and correlate with the severity of brain damage.[31] Feeding abnormalities after HI insults might be due to oromotor dysfunction because of brainstem damage but also gastroesophageal mobility disturbance[31] neither of which could be assessed in this work.

The neuromotor test used in our experiments originally include an item assessing neurobehavioral performance.[10] Although that item score was clearly affected in HI piglets and correlates with brain damage, its qualitative nature makes it subjective and highly dependent on the examiner's skills. Thus, we aimed to obtain some quantitative and objective parameters. We based ourselves on some basic behavioral characteristics of piglets such as playfulness, social interaction and anxiety responses to immobilization[2,5,17] which can easily and inexpensively be assessed. We observed that those items were affected by the HI insult so that HI piglets showed increased anxiety and poorer object and social interaction. Shorter interaction with objects is an open-field behavior affected in piglets after traumatic head injury associated with histologic brain damage,[4] but unlike our results such impairment is observed just one day but not 4 days after trauma. By contrast, poor social interaction after traumatic injury is still observable 4 days after trauma[4] similar to our results. However, we did not find a statistically significant correlation between those items and brain damage markers. This is likely because as a difference respecting motor impairment, such complex cognitive patterns are unrelated to focal damage but with dysfunctional brain networks. Interestingly, we did find a relationship between motionless time and post-HI brain damage in newborn piglets. Motionless time was clearly increased in HI piglets and correlated with TUNEL+ staining and Lac/NAA. Motionless has been reported in newborn piglets after isolation stress and interpreted as a sign of depression.[32] Interestingly, apathy is a component of post-stroke depression (PSD), a complication from stroke-related to greater post-stroke morbidity and mortality.[33,34] Studies in humans have linked PSD and brain damage severity.[34] Recent reports have linked PSD to monoamine disturbances in brain, in particular 5-HT, NE, and DA.[33,34] We analyzed brain concentration of 5-HT, NE, and DA in our piglets and detected no relationship between motionless and 5-HT but a significant inverse relationship between motionless time and NE or DA levels. In adult humans after stroke, low 5-HT levels seem to be related to emotional problems whereas low NE and DA levels are linked to apathy,[33] which is in correspondence with the results from our work, the first one reporting on post-HI motionless linked to monoamine brain levels disturbances in newborn animals. There are no reports on a PSD-like picture in HI newborns. However, motionless periods could also correspond to seizures. Although motionless periods were similar in piglets having or not seizures during the aEEG record, since we did not studied EEG during the motionless periods we cannot rule out that possibility.

In conclusion, the model presented in this work led to HI brain damage in newborn pigs, affecting neurons and astrocytes in a manner related to increased neuroinflammation and excitotoxicity, resulting in functional and behavioral disturbances. In this model, aEEG voltage and neurological exam 72 h after the HI correlated with the severity of histologic and biochemical brain damage. Most predictive items for brain damage from the neurologic exam were poor muscle tone and feeding as well as motionless time. These results support the utility of the model and simple neurobehavioral testing presented here for future experiments on neonatal HI pathophysiology and therapeutics. Besides, they support the value of a parameter currently used in human newborns as the Lac/NAA ratio as an effective biomarker of HI brain damage.

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AUTHOR CONTRIBUTIONS

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ADDITIONAL INFORMATION

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