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Research Article

Relationship between Brain Injury and Clinical Biomarkers in Hypoxic-Ischemic Newborn Piglets

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Abstract

Background: The newborn affected by severe hypoxic-ischemic brain injury usually end up dying, and those who survive suffer neurodevelopmental handicaps. Biomarkers are required to identify the hypoxic-ischemic insult in order to determine the early rescue treatment method to be followed. The aim of the present study was to correlate relevant biomarkers to the pathophysiological process of hypoxic-ischemic brain injury at 6 h and 72 h in newborn piglets.

***Methods*:** Hypoxia-ischemia was induced in the piglets by clamping both the carotid arteries and reducing the inspired oxygen. Animals were assigned randomly into two groups: the

hypoxic-ischemic (HI) group and the sham group. In both the groups, 50% of the animals were sacrificed at 6 h, and the rest of the animals were sacrificed after 72 h. Brain tissue oxygen index (TOI) and amplitude-integrated electroencephalography (aEEG) were recorded. Fronto-parietal cortex sections stained using the Nissl method, TUNEL assay, and for glial fibrillary acidic protein (GFAP) were examined. Mean values were compared using the one-way analysis of variance for the unpaired data ($p < 0.05$).

***Results*:** A decrease in arterial pH, PaO_2, and base excess were observed during the hypoxic-ischemic injury, in the HI group compared to the sham group. A decrease in the TOI values was observed during insult, although the mean values of TOI were recovered afterward. aEEG was observed to remain stable throughout the 72 h in the sham group, while a decrease was observed in the HI group. Electroencephalographic traces displayed the occurrence of seizures at 6 h in 80% of the piglets. Nissl staining revealed a decrease in the number of normal neurons in the cortex at 6 and 72 h, as well as an increase in the number of pyknotic cells. In addition, in the cortex of the HI group animals, the number of TUNEL-positive cells at 72 h was higher than that in the sham group. GFAP staining revealed a decrease in the number of astrocytes in the cortex at 72 h.

***Conclusions*:** Results of the present study suggested that although both TOI and aEEG are suitable markers of the neuronal and astroglial cell damage caused by hypoxic-ischemic injury in piglets, the latter provides better outcomes at 72 h.

Keywords

Animal model; hypoxia-ischemia; newborn; piglet; biomarker; aEEG; brain injury

1. Introduction

Hypoxic-ischemic encephalopathy (HIE) of the newborn has been reported to occur in 1–8/1000 live full-term births [1], causing severe long-term neurodevelopmental deficits in the affected children. Approximately 50% of the newborn having moderate to severe HIE are at the risk of death during the newborn period or the risk of permanent neurodevelopmental impairments [2]. In spite of its great relevance in healthcare, the pathophysiology of HIE and the consequential events (primary failure, latent or lethargy, and secondary failure phases) are not completely understood [3]. There is significant evidence that brain cells die as a result of a profound or prolonged event of hypoxia-ischemia, during the initial energy failure (primary phase) [4]. However, several cells exhibit early partial or complete recovery (latent or lethargy phase). This transient recovery is followed by a secondary deterioration involving a failure of oxidative metabolism (secondary phase) [5-7], which is associated with excitotoxic edema, seizures, cerebral hyperperfusion, and ultimately, brain cell death [8]. The term HIE is preferred over perinatal asphyxia [9], and clinical markers, such as fetal heart rate variability [10], blood gases from umbilical cord or Apgar scores, among others, are required to determine the risk of developing brain injury [11]. These clinical markers are currently being used in the clinical setting with approximately 80% sensitivity and specificity [12].

The time period within which it is possible to have the brain damage reverted is unknown so far.

Experimental data have suggested that after the secondary energy failure (usually at 6 or 9 h), several brain cells exhibit specific cellular mechanisms [5, 6, 13] which may be contributing to cell death via necrosis or apoptosis. These data demonstrate that markers are required to properly identify the insult, prior to the application of any rescue treatment. Ideally, an optimal marker must be directly related to the neonatal brain damage (specificity), as this would allow the identification of the treatable babies. Several efforts have been put to establish markers that are suitable in terms of improvement of sensitivity and specificity [1, 14]. Several studies have attempted to correlate different markers with HIE, such as those identified in amplitude-integrated electroencephalography (aEEG) [15-18], near-infrared spectroscopy (NIRS) [19], brain perfusion [20, 21], magnetic resonance imaging (MRI) [22, 23], and those which are the components derived from oxidative stress of DNA and proteins, and several other biomarkers related to hypoxia-ischemia [1, 3, 24]. When compared to previously reported markers (pH, Apgar score, fetal heart rate variability, etc.), these specific markers also exhibit similar levels of sensitivity and specificity (80%). The availability of different markers for the pathophysiological process of hypoxic-ischemic brain injury, as well as their relationship with the therapeutic prospect, is of vital importance for the application of useful neuroprotectant therapies.

Animal models have provided information regarding the pathophysiology of brain damage, both in term [4, 5] and preterm animals [4, 6, 25]. These models represent the first step in the process of determining the mechanisms underlying brain damage, and in demonstrating the effectiveness of therapeutic interventions [25, 26]. Our research group has demonstrated that the immediate administration of cannabidiol after the hypoxic-ischemic event in the newborn piglets improves early neuronal rescue without causing adverse effects [26]. Furthermore, non-invasive markers, such as cerebral perfusion measured using NIRS and electrocortical brain activity measured using an aEEG were observed to have improved. These findings were reported for the early hours after the injury; however, it is also important to understand how these biomarkers are associated with HIE at the later phases of the disease. The experimental model used in the present study would allow the evaluation of brain damage prior to and post the secondary energy failure (at 6-9 h), and the determination of the neuroprotective effects of drugs and therapies [3, 13] at different phases after the injury. The aim of the present study was to correlate the pathophysiological process of HIE with biomarkers at 6 and 72 h in a newborn piglet model.

All surgical and experimental procedures were performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals provided by the National Institute of Health, and those in the European Communities directive 2010/63/EU regulating animal research. Experiment protocols used in the present study were approved by the Committee on the Ethics of Animal Experiments, Cruces University Hospital (Code: SEP#009-09 and SEP#012-11). All the surgical procedures were performed under the influence of anesthesia, and all efforts to minimize suffering were applied.

2. Materials and Methods

2.1 Animal Preparation and Neurophysiological Assessment

Piglets who were 1 to 3-days old (1.5-2.5 kg) were sedated using ketamine, atropine, and diazepam (15, 0.05, and 2 mg/kg i.m.), anesthetized using a perfusion of fentanyl, propofol, and midazolam in 5% dextrose (4, 3, and 0.5 mg/kg/h, respectively), and paralyzed using a perfusion of

vecuronium bromide (3 mg/kg/h) administered through a vein in the ear. The animals were intubated with an endotracheal tube (ID = 3-4 mm). Subsequently, the piglets were subjected to intermittent positive pressure ventilation (Bourns BP-200; Beard Medical Systems, Riverside, CA) with a fraction of 0.25 to 0.30 of inspired oxygen, at a ventilatory rate of 30 breaths/min, positive-end expiratory pressure of 0.3 kPa, and peak inspiratory pressure adjusted as necessary to provide an arterial pressure of oxygen (PaO_2) ranging from 10.0 to 15.3 kPa, an arterial pressure of carbon dioxide ($PaCO_2$) ranging from 4.7 to 6.0 kPa, and a pH ranged from 7.35 to 7.45.

Standardized lung tidal volume (V_T, mL/kg) and respiratory system compliance (C_{RS}, $mL/cmH_2O/kg$) were monitored using computerized pneumotacography (Chart5 Powerlab, ADI Instr., Colorado Springs, CO). The oxygenation index was calculated as the product of the mean airway pressure in cmH_2O and the inspired oxygen percentage and was standardized by PaO_2 [26].

Continuous three-lead electrocardiogram monitoring was used throughout the experiment. A 3-mm ultrasonic transit-time flow transducer (MA3PSB, Transonic Systems Inc., Ithaca, FL) was placed non-invasively around the right common carotid artery for the continuous measurement of carotid blood flow (CBF), which was a representation of the cerebral blood flow [27] and was expressed as milliliters per minute per gram tissue. Catheter was inserted into the caudal artery in order to measure the mean arterial blood pressure (MABP) (Ominare CMS24, HP, Göblingen, Germany), and to obtain blood samples for gas exchange (GemPremier 4000, Instrumentation Laboratory, Lexington, MA). In-dwelling catheters (5 Fr, PiCCO Plus, Pulsion Medical Systems, München, Germany) were inserted into the right jugular vein in order to determine the cardiac output (CO). Rectal temperature was maintained at 38°C with a in a servo-controlled radiant warmer (Electrodine, Becton-Dickinson, San Jose, CA).

In the midline of the frontoparietal region of skull, an NIRS sensor was placed and fixed using bandages. Tissue oxygen index (TOI) and variations in the total hemoglobin index (THI) were monitored (NIRO-200, Hamamatsu Photonics KK, Joko Cho, Japan) continuously. TOI represents tissue oxygen saturation and is expressed as a percentage. Normalized THI is an absolute figure of the total hemoglobin content in the brain. Furthermore, brain activity was monitored using a two-channel bed aEEG monitor (BRM2, BrainZ Instruments, Auckland, New Zealand). The electroencephalographic traces [28] were classified as: i) continuous normal voltage; ii) discontinuous normal voltage; iii) burst suppression; iv) continuous extremely low voltage; and, v) flat trace. In addition, traces were carefully reviewed to identify seizures, which were represented by periods of sudden increase in voltage, accompanied by a narrowing of the band of aEEG activity, and followed by a brief period of suppression [26].

2.2 Experimental Procedures

The animals were assigned randomly to the hypoxic-ischemic (HI) group (n = 12) or the sham group (n = 12) without injury. Hypoxic-ischemic brain injury was induced in the piglets through total interruption of CBF (tightening of two reversible occluders around both the carotid arteries) and reduction of the fraction of inspired oxygen up to 8%. Hypoxia-ischemia was considered for 30 min of the flat traces of aEEG (<4 μV) (grade V) [28]. At the end of hypoxia-ischemia, blood flow in the carotid arteries was restored and the fraction of inspired oxygen was returned to 21%. Six hours after the hypoxia-ischemia, or the equivalent period in the sham group without hypoxic-ischemic injury, half of the piglets in each group (n = 6) were sacrificed, while the other half were

allowed to recover spontaneously. The awake animals were fed on artificial piglet milk in the following 72 h. In this additional time, the animals were maintained in pairs (social group) at constant temperature and day-night cycles. Post sacrifice, the brain of the piglets was perfused with cold heparinized saline through the carotid arteries. One of the brain hemispheres was fixed with 4% formaldehyde and stored at room temperature, while the other hemisphere was frozen in liquid nitrogen and stored at -80°C.

2.3 Data Acquisition and Analysis

Blood gases were maintained at acceptable values (as previously described) using ventilator-adjusted control and/or by adding sodium bicarbonate as required. MABP and blood glucose levels were maintained within the normal range (over 50 mmHg, and over 40 mg/dL, respectively). Perfusions of dopamine (0.6 mg/mL in 5% dextrose) and/or 25% dextrose were administered if necessary. Physiological data of all the animals were registered at baseline (basal), post-injury in HI group or its temporal equivalent in the sham group (HI interval), 6 h post-injury, and 72 h post-injury.

In the first 6 h, TOI, THI, and aEEG were recorded continuously. In the awake piglets (both sham and HI groups), the measurements were performed in a duration of 30 min at 12 h and 72 h post-insult. At the same intervals, the neurobehavioral function of the newborn piglets was also analyzed by means of an adapted standardized test score, in which the scores for normal functioning ranged from 34 to 36 (Table 1) [29].

Table 1 Neurobehavioral score for neonatal pigs [29].

Item	Description	Scale (Score points)
Vigilance		
	Mental status (consciousness)	Coma (1); stupor (2); lethargy (3); awake (4)
	Behavior (defense on manipulation)	No (1); weak (2); aggressive (3); normal (4)
Cranial nerves		
	Pupils (light reaction, corneal reflex)	Non reactive (1); sluggish (2); normal (3)
	Oculo-vestibular reflex	Absent (1); nystagmus (2); present (3)
Reflexes		
	Stepping (wheel barrowing)	Absent (1); forelimb/posterior (2); integral (3)
	Righting (to move or roll over)	Absent (1); present (2)
Motor		
	Tone in trunk and limb muscles	Atonic/hypertonic (1); partly atonic (2); partly hypertonic (3); normal (4)
	Standing	No (1); paretic (2); bears weight unsure (3); normal (4)
Coordination		
	Walking	Not possible (1); creeping/paretic (2); walks but falls (3); normal (4)
	Feeding	No swallowing reflex (1); has to be fed (3); normal (5)

2.4 Histological Study

Fixed brain hemispheres (at 6 and 72 h) were cut into slices (5 mm in width) and embedded in paraffin. Coronal sections (4 µm) were cut and mounted on a glass slide for staining. Fronto-parietal cortex has been reported to be particularly sensitive to hypoxic-ischemic damage [26, 29]. Coronal sections were obtained in accordance with the stereotaxic atlas of pig brain [30].

Neuronal necrosis was determined in the consecutive pairs of brain sections and through staining with the Nissl method as described in a previous study [26]. Briefly, an investigator, who was blinded to the randomization assignment, evaluated the presence of damaged cells in the layer II-III of the frontoparietal cortex using an optical microscope. The mean of five high-power fields, which are defined as the observed area using a 10x eyepiece and a 20x objective lens, was used to obtain the mean pyknotic cell number per mm^2. The apparently normal neurons were identified through the presence of a typical nucleus with scattered chromatin and a clear nucleolus, surrounded by a purple-stained cytoplasm. The presence of necrotic-like neurons was considered when the neurons exhibited a retracted aspect, with eosinophilic cytoplasm and a loss of nuclear detail [4].

In order to determine the presence of apoptotic cells, brain sections were stained using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay, performed using the ApopTag Kit (ApopTag® In Situ Apoptosis Detection Kit, Millipore, MA) [26]. The mean TUNEL-positive cell number per mm^2 was calculated.

Glial fibrillary acidic protein (GFAP)-positive cells in the frontoparietal cortex were identified using immunohistochemistry. Tissue sections were washed in phosphate-buffered saline (PBS), followed by overnight incubation with GFAP-Cy3-conjugated antibody (1:1000; Sigma-Aldrich, St. Louis, MI) at 4°C. After the incubation, the sections were washed again with PBS and placed in an aqueous medium with Vectashield (Vector Laboratories, Burlingame, UK). The samples were observed under a confocal Nikon Eclipse C1 coupled to a Nikon 90i microscope and a DXM1200F camera (Nikon, Haarlem, Netherlands). The GFAP-positive cell surface area was quantified using the ImageJ 1.43s software (NIH, Bethesda, MA) [29]. The mean GFAP-positive cell surface area was calculated by dividing the total stained surface area by the number of GFAP-positive cells and was expressed as μm^2 per astrocyte.

2.5 Biochemical Markers

Frozen brain samples were homogenized with PBS and EDTA in order to quantify the concentration of malondialdehyde (MDA) using ELISA (OxiSelect TBARS Assay Kit, Cell Biolabs, San Diego, CA). MDA was used as a representative of oxidative damage. The concentrations of neuronal-specific enolase (NSE) and S100β protein (CanAg NSE and S100β, Fujirebio Diagnostics, Göteborg, Sweden), which represented the neuronal injury biomarkers in the cerebrospinal fluid samples, were determined using ELISA. Cardiac troponin T (cTnT), a cardiac failure biomarker (Elecsys Analyzer, Roche Diagnostics, Mannheim, Germany), was measured in the venous blood sample. All the referred biomarkers were determined at basal, HI post-injury, 6 h post-injury, and 72 h post-injury.

2.6 Statistical Analysis

Data were expressed as mean ± standard error of the mean (SEM). Variables were compared with the Brown-Forsythe test to confirm the homogeneity of the variance between the groups and with the Shapiro-Wilk W test for normality. Comparisons between the groups were analyzed using one-way analysis of variance (ANOVA) with Bonferroni-Dunn's correction as the function of the group (GraphPad Prism version 6.01; GraphPad Software, San Diego, CA). A p-value of <0.05 was accepted as the significance threshold.

3. Results

3.1 Physiological Data

The results related to pulmonary gas exchange, hemodynamics, and respiratory lung function are presented in Table 2. In the post-injury interval, different mean arterial pH values were noted between the groups (7.37 ± 0.04 vs 7.18 ± 0.08, $p < 0.05$), although normal pH values were recovered in almost all the animals at 6 h after insult. During the hypoxia-ischemia, a decrease in the mean values of PaO_2 was observed in the HI group (14.4 ± 0.9 vs 4.5 ± 0.3 kPa, $p < 0.05$). Later, no differences were observed in the mean PaO_2 values between the groups. In regard to $PaCO_2$, the mean values were observed to increase significantly at the post-injury time-point (5.2 ± 0.1 vs 6.9 ± 0.3 kPa, $p<0.05$), although no such differences were observed after the event. After the hypoxia-ischemia, lower mean values of the base excess were observed in the HI group in comparison to the sham group (-0.7 ± 2.1 vs. -12.9 ± 4.8 mE/L) (Table 2).

Hypoxic-ischemic injury produced a three-fold decrease in MABP (91 ± 6 vs 34 ± 1 mmHg), which was partially recovered (84% of the mean values in the sham group) at 6 h. This partial recovery (84% of the mean values in the sham group) was maintained until 72 h. However, both groups did not exhibit any differences in relation to heart rate (232 ± 4 vs 232 ± 6 bpm). After the unclamping of both common carotid arteries, reactive and transient hyperemia to the brain occurred, as demonstrated by the normalization of the carotid blood flow (Table 2), which remained similar afterward. However, at 72 h after the hypoxia-ischemia, the HI group exhibited a 40% decrease in CBF compared to the sham group (150 ± 28 vs 91 ± 25 ml/min/100mg). In regard to the respiratory function, animals were subjected to similar ventilation conditions at the same tidal volume or oxygenation index (Table 2).

Table 2 The results of blood gas, hemodynamic, and respiratory data.

	Basal	HI	6 h	72 h
Blood gases data				
pH				
Sham group	7.33 ± 0.02	7.37 ± 0.04	7.39 ± 0.03	7.44 ± 0.03
HI group	7.32 ± 0.01	7.18 ± 0.08 [a]	7.39 ± 0.02	7.43±0.02
PaO_2 (kPa)				
Sham group	13.1 ±0.8	14.4 ± 0.9	12.0 ± 1.9	11.3 ± 0.4
HI group	14.7 ± 0.5	4.5 ± 0.3 [a]	11.6 ± 1.2	11.9 ± 0.7
$PaCO_2$ (kPa)				
Sham group	5.3 ± 0.3	5.2 ± 0.1	6.0 ± 0.7	5.7 ± 0.3
HI group	5.2 ± 0.1	6.9 ± 0.3 [a]	6.5 ± 0.4	5.2 ± 0.4
Base excess (mEq/L)				
Sham group	-4.0 ± 2.2	-0.7 ± 2.1	2.0 ± 1.8	3.0 ± 1.9
HI group	-5.7 ± 1.3	-12.9 ± 4.8 [a]	3.5 ± 1.1	1.5 ± 2.5
Hemodynamic data				
MABP (mmHg)				
Sham group	94 ± 6	91 ± 6	77 ± 4	75 ± 8
HI group	90 ± 3	34 ± 1 [a]	65 ± 2 [a]	64 ± 7 [a]
H (bpm)				
Sham group	237 ± 5	232 ± 4	215 ± 4	181 ± 9
HI group	230 ± 6	232 ± 6	231 ± 5	176 ± 11
CBF (ml/min/100g)				
Sham group	159 ± 28	147 ± 15	160 ± 25	150 ± 28
HI group	134 ± 16	0 ± 0 [a]	172 ± 16	91 ± 25 [a]
CO (ml/kg)				
Sham group	1.1 ± 0.1	1.0 ± 0.1	1.2 ± 0.1	1.2 ± 0.1
HI group	0.9 ± 0.1	1.4 ± 0.1 [a]	1.1 ± 0.1	1.3 ± 0.1
Respiratory data				
OI				
Sham group	12.2 ± 1.3	11.4 ± 1.6	16.9 ± 1.7	14.7 ± 1.5
HI group	13.6 ± 2.2	15.6 ± 2.0 [a]	20.2 ± 1.8 [a]	13.9 ± 1.4
V_T (ml/kg)				
Sham group	7.2 ± 1.6	6.9 ± 0.1	6.8 ± 0.1	9.8 ± 1.0
HI group	7.2 ± 0.2	7.0 ± 0.5	6.7 ± 1.1	7.4 ± 0.5 [a]
C_{RS} (ml/kPa/kg)				
Sham group	8 ± 2	8 ± 1	8 ± 1	14 ± 3
HI group	9 ± 2	7 ± 1	7 ± 1 [a]	12 ± 2

PaO_2: arterial pressure of oxygen; $PaCO_2$: arterial pressure of carbon dioxide; MABP: mean arterial blood pressure; H: heart rate; CBF: carotid blood flow; CO: cardiac output; OI: oxygenation index; V_T: standardized tidal volume; C_{RS}: standardized respiratory system compliance. Data are shown as mean ± SEM. (a) $p < 0.05$ versus sham group.

3.2 Neurophysiological Assessment

In the HI group animals, a decrease in TOI was observed during the hypoxic-ischemic insult compared to the animals in the sham group (Figure 1A). Later, the mean values of TOI reached the basal level again. In the HI group, the mean aEEG values also exhibited a decrease (88%) at the post-injury interval, being 30% lower than the values obtained for the sham animals at 72 h (Figure 1B). In the HI group, electroencephalographic traces at 6 h showed the presence of seizures in 8 piglets (Figure 2). Finally, at 72 h, the aEEG amplitude in the HI group remained 57% lower than its mean values at the basal level ($p < 0.05$), and the presence of seizures was noted in 4 animals.

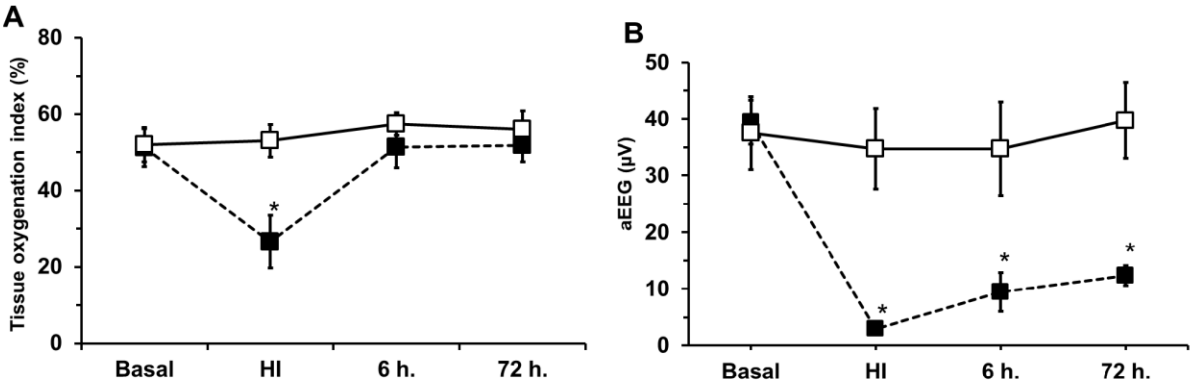

Figure 1 Neurophysiological changes induced by hypoxia-ischemia. **A.** Tissue oxygenation index (TOI). **B.** Amplitude-integrated electroencephalography (aEEG) in sham (open square) and hypoxic-ischemic groups (solid square). (*) $p < 0.05$ versus sham group. Data are shown as mean ± SEM.

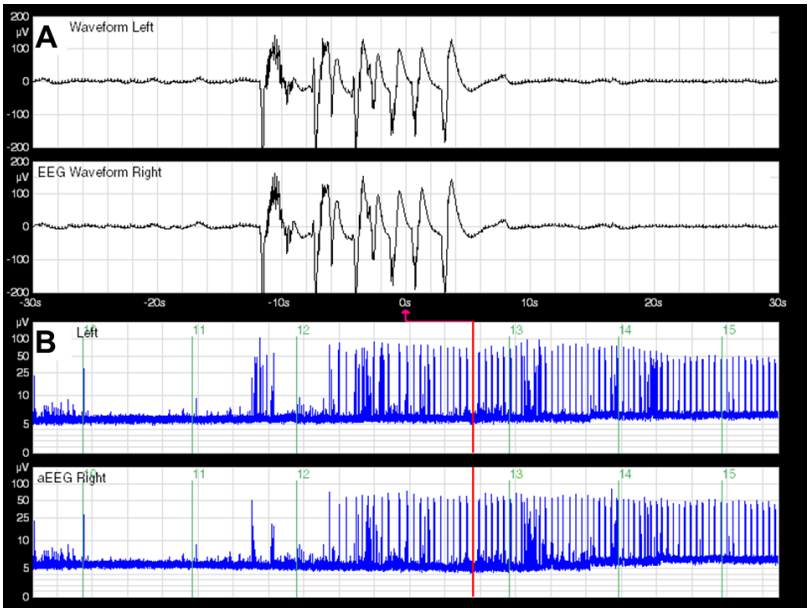

Figure 2 Representative electroencephalographic traces of an HI animal. Seizures were defined as periods of a sudden increase in voltage, accompanied by a narrowing of the band of aEEG activity and followed by a brief period of suppression. **A**. Seizures can be observed on the top of the figure. **B**. Repetitive convulsive episodes were seen in a time-compressed print-screen for 3 h.

3.3 Neurobehavioral Studies

All animals exhibited a normal neurobehavioral score, scoring 36, prior to the experimental procedure (Figure 3A). In the early phase after the hypoxic-ischemic injury, all the animals (sham and HI groups) were in a recovery phase from the effect of anesthesia applied during the experimental procedure. Although it was not noticeable, the first neurobehavioral score was possibly obtained at 12 h (14.5 ± 1.0 in the sham animals vs. 14.2 ± 1.2 in the HI ones). At 72 h, the sham group reached a near-normal score (35.1 ± 0.5). while the HI animals exhibited an impairment in neurobehavior, recording a score which was 30% lower than that achieved for the sham group (Figure 3A). All the items, i.e., vigilance or mental status (Figure 3B), cranial nerves (Figure 3C), reflexes (Figure 3D), motor activity (Figure 3C), and coordination (Figure 3F), were observed to have been affected.

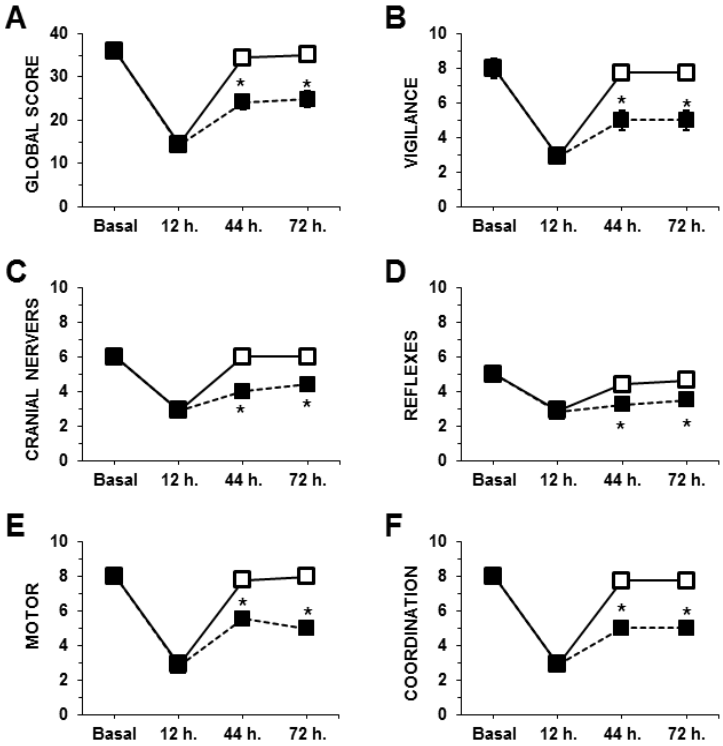

Figure 3 Neurobehavioral score of newborn piglets in the sham and hypoxic-ischemic groups (open and solid square, respectively) performed at basal, 12, 44, and 72 h intervals after hypoxia-ischemia, or equivalent experimental time in the sham animals. **A.** Global score; **B.** Vigilance score (mental status and behavior); **C.** Cranial nerves score (pupils and oculo-vestibular reflex); **D.** Reflexes score (stepping and righting); **E.** Motor score (tone in trunk and limb muscles and standing); **F.** Coordination score (walking and feeding). (*) $p < 0.05$ versus sham group. Data are shown as mean ± SEM.

3.4 Histological Analysis

The Nissl staining of the brain slices revealed a decrease in the number of apparently normal neurons in the frontoparietal cortex, both at 6 and 72 h (Table 3), as well as an increase in the number of pyknotic cells (percentage of pyknotic cells with respect to the total amount of stained cells: 52 ± 4%; Figure 4). TUNEL-positive cells were not detectable at 6 h in either group. However,

in the cortex of HI animals, the number of TUNEL-positive cells at 72 h was observed to be 6-fold higher than that in the sham group (Figure 5A and 5B).

Table 3 Histological results of normal and pyknotic neurons, terminal deoxynucleotidyl transferase dUTP nick end labeling positive cells and total number and mean area of glial fibrillary acidic protein-positive cells in the frontoparietal cortex.

	6 h	72 h
Apparently normal neurons (cell/mm^2)		
Sham group	653 ± 6	613 ± 36
HI group	371 ± 21 [a]	404 ± 42 [a]
Pyknotic neurons (cell/mm^2)		
Sham group	5 ± 2	27 ± 8
HI group	260 ± 44 [a]	212 ± 86 [a]
TUNEL positive cells (cell/mm^2)		
Sham group	ND	24 ± 4
HI group	ND	154 ± 88 [a]
GFAP-positive cell number per area (cell/mm^2)		
Sham group	111 ± 38	111 ± 8
HI group	110 ± 15	92 ± 3 [a]
Mean GFAP-positive cell surface area (µm^2)		
Sham group	825 ± 245	825 ± 58
HI group	862 ± 155	459 ± 68 [a]

ND: non-detectable. Data are shown as mean ± SEM. (*a*) $p < 0.05$ versus sham group.

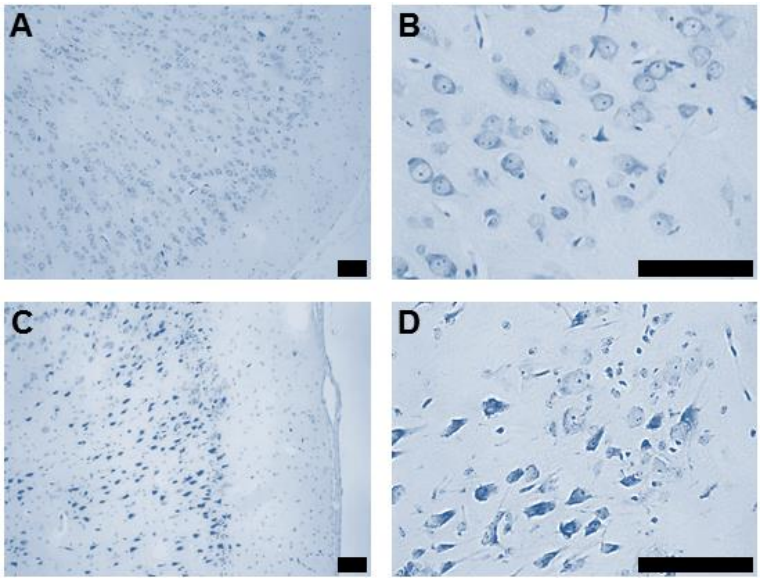

Figure 4 Representative light microphotographs of the Nissl-stained brain sections of the sham and hypoxic-ischemic piglets at 72 h after injury. The frontoparietal cortex of a sham piglet at x100 (**A**) and x400 (**B**). The frontoparietal cortex of a hypoxic-ischemic piglet at x100 (**C**) and x400 (**D**). Bar: 100 µm. There is a decrease in the number of viable neurons and an increase in the number of pyknotic cells.

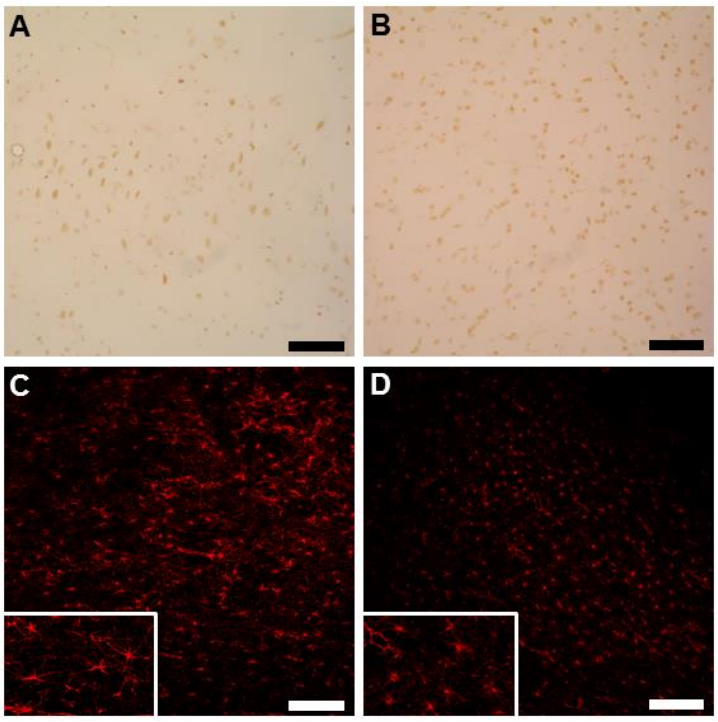

Figure 5 Representative light microphotographs of the frontoparietal cortex of sham. (**A**) and hypoxic-ischemic; (**B**) piglets at 72 h after injury, stained with terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay (TUNEL). Glial fibrillary acidic protein (GFAP) positive cells of frontoparietal cortex of sham; (**C**) and hypoxic-ischemic; (**D**) piglets at 72 h after injury. Original magnification: x200. Bar: 100 µm. Details of GFAP positive cells of both animals are inserted in small images at the inferior left corner at x400.

GFAP immunohistochemical staining did not reveal any differences at 6 h between the sham and HI groups, neither did the number of astrocytes or their morphology (Table 3). However, at 72 h, the number of astrocytes in the cortex of the HI animals was observed to have decreased, and the surviving astrocytes appeared smaller, with a rounded cell body (with shorter and lesser processes), compared to those in the cortex of sham animals (Figure 5C and 5D).

3.5 Brain and Cardiac Biomarkers

Hypoxia-ischemia produced a 50-fold increase in the NSE concentration (Figure 6A) and a 4-fold increase in the S100β concentration (Figure 6B), at 72 h. After the hypoxic-ischemic injury, an increase in cTnT was observed in the HI group, both at 6 and 72 h (Figure 6C). However, no differences in the concentration of MDA were observed both at 6 or 72 h after the hypoxic-ischemic injury (Figure 6D).

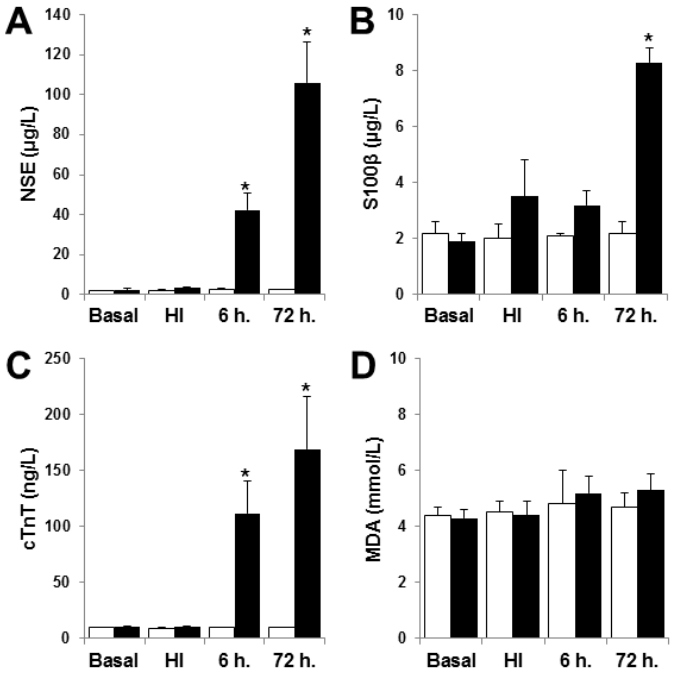

Figure 6 Biochemical markers induced by hypoxia-ischemia**. A.** Mean values of neuron-specific enolase (NSE); **B.** S100β protein; **C.** Cardiac troponin T (cTnT); **D.** Malonaldehyde (MDA) in the sham (white bars) and HI animals (black bars) as a representation of neuronal, blood-brain barrier, cardiac failure, and oxidation biomarkers. (*) $p < 0.05$ versus sham group. Data are shown as mean ± SEM.

4. Discussion

In the present work, during the acute phase of cerebral hypoxia-ischemia, we observed brain-compromised functionality with poor oxygen perfusion (CBF, TOI) as well as electrical brain activity (aEEG). Further, HI animals demonstrated a misbalance of hemodynamics (MABP, CO), biochemical markers (NSE, cTnT), and electrical brain activity during the initial recovery phase (6 h), but oxygenation-associated factors were only marginally disturbed, which included oxygenation index, gas exchange, pulmonary function, and brain tissue oxygen index. Moreover, neonatal seizures were mainly observed at this early phase. Finally, at 72 h, electrical brain activity (aEEG) and neurobehavioral score demonstrated permanent misbalance, associated with a relatively high proportion of electrical convulsive patterns (neonatal seizures). In addition, alteration of biomarker levels (cTnT, NSE, S100β) was present at 72 h. Taken together, our results describe the physiological findings of HIE as a function of time during the initial 72 h, and how biomarkers, indicative of HIE injury, were associated with this process at 6 and 72 h. Thus, aEEG showed a good correlation with cerebral damage, revealing to be a good marker of cerebral damage.

In clinical practice, unlike experimental studies, the hypoxic-ischemic insult is not always clearly defined in infants [24, 31]. First, the injury begins many hours before birth and often involves repeated or prolonged exposure to hypoxia-ischemia. Thus, injury may evolve before the time of birth, which may lead to a very short or unsuccessful phase for some neuroprotective treatment to be beneficial, including therapeutic hypothermia. Further, the duration of the secondary latent phase is modulated by the severity of the insult, shortening potential window of opportunity for treatment.

Despite the advances in perinatal medicine occurring in the last 20 years, the detection and prevention of hypoxic-ischemic injury continue to demonstrate high morbidity and mortality. Invasive techniques, such as measurement of venous oxyhemoglobin saturation, cerebral oxygen consumption, measurement of metabolites by radiological techniques, or microdialysis, are not clinically reliable techniques at "patient's bedside". On the other hand, non-invasive techniques at "patient's bedside", such as neurological examination, Doppler ultrasound or electroencephalography, are useful to determinate the brain damage but they offer low specificity for the immature brain [20, 28]. For this reason, the lesion is diagnosed too late after the HI event in some cases [32, 33].

The hypoxic-ischemic insult led to a decrease in aEEG amplitude, remaining lower than sham animals [27]. Immediately after the injury, a depressed aEEG was described as a poor outcome in piglets [28], as also described in humans [34]. TOI represents the tissue saturation of oxygen whereas nTHI provides an absolute measure of total hemoglobin in brain, which reflects changes in cerebral blood volume [35]. In the context of neurobehavioral and histological results, the changes in TOI and nTHI suggest poor outcomes in HI animals, associated with the deterioration of brain metabolic activity and CBF that, at least, was not recovered at 72 h after hypoxia-ischemia. Although TOI and nTHI are considered to correlate with CBF and cerebral blood volume, they are not considered good markers [35]. Our results of aEEG and NIRS data correlate to histological results. Hypoxia-ischemia led to severe neuronal damage, as reflected by the increase in NSE concentration observed in cerebrospinal fluid samples. The increase in the NSE concentration in cerebrospinal fluid is an early marker of HI neuronal damage in newborns and could be associated with an abnormal outcome but validation studies are required [36]. There was a loss of viable neurons in the HI group together with an increase in the necrotic and apoptotic neurons. This features started just 6 h post-injury and were even more apparent at 72 h. Apoptosis is a preeminent feature of hypoxic-ischemic damage in immature brain, appearing shortly after insult and coexisting with necrosis [37]. NIRS is a non-invasive technology, capable of measuring cerebral oxygen saturation and oxygen consumption at the "patient's bedside". Earlier studies in asphyxiated neonates have described that hypoxic-ischemic events decrease cerebral oxygen saturation and fractional tissue oxygen extraction [21]. Even so, difficulties with the use of NIRS in critical care could hinder its potential diagnostic and predictive utility in HIE.

In other similar models, severe disabilities have been defined as the inability to ambulate, minimal awareness of the surroundings along with decreased sensation and tone, low level of consciousness, sensory function, gait and gross motor tone [38]. Similarly, our data show a neurobehavioral score that suggests an adverse outcome. At 72 h, HI animals demonstrated a low score (approx. 25 points) in all items: lethargy (3); weak behavior (2); sluggish on vigilance (2); present ocular reflex (3); stepping in only anterior or posterior forelimbs (2); need to be fed (3); righting (2); partly atonic muscle tone (2); unsure standing (3); and walks but falls (3). The normalization of the neurological examination after several weeks is one of the best predictors of the good outcome in asphyxiated newborns [39]. Although there was a modest recovery of neurobehavioral performance (behavior, motor performance or coordination) in the HI group, we can rule out the total recovery of the basal behavior. Nevertheless, since we are unable to maintain long-lasting husbandry of animals in our facilities, we could not assess long term follow up. It must be taken into account that all animals showed a low neurobehavioral score at 12 h due to the experimental procedure (recovery from anesthesia), and thus, the score did not reflect the mental

status of animals. Moreover, this neurobehavioral score could present some limitations in order to establish the baseline value (10 points). However, it has been used in many studies by other authors [29, 40-42].

Our model is reproducible in a dose-dependent way with histologic brain damage similar to moderate-severe HIE infants. There is clinical and aEEG evidence of encephalopathy that correlates with subsequent neuropathology [43]. We have used a neurological score that is associated with the pathologic damage in the cortex and hippocampus at 72 h [29]. This model should be suitable for examining mechanisms of damage and repair, and for testing possible neuroprotective treatments. However, no animal model is perfect in reflecting the complexity of the human brain but can be used as an approximation to the clinical trials. Thus, we used a model of 1 to 3 day-old piglets because the anatomy and neurophysiology are quite similar to newborns. Also, the cerebral maturation and myelinization of the newborn pig are comparable to the human neonate [44]. Another limitation is that the current model does not allow long-term follow-up in its current form. It should also be noted that sampling method (brain biopsies) is not reproducible in clinics due to ethical concerns but venous and cerebrospinal fluid samples were collected when they were possible. Histological findings in this animal model confirm considerable brain damage as early as 6 h after the insult [45], and so it could be adequate to establish a relationship between biomarkers and its appearance as a function of time.

5. Conclusions

Our results suggest that aEEG and TOI, in a lesser manner, are good markers of cerebral damage after hypoxic-ischemic injury in newborn piglets. Moreover, the former provides a better prognostic value of brain injury at 72 h.

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Author Contributions

FJA and AAA have designed the experimental design in accordance with international ARRIVE guidelines. HL, AAA, JLB, DAA; FS and FJA have performed the in vivo experimental phase. EH, AAA and FJA have performed the in vitro experimental phase. DAA, FS, JLB and FJA have contributed with reagents/materials/analysis tools. AAA, HL and FJA have been responsible for data analysis. AAA, EH and FJA have collaborated and approved the final manuscript version.

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Competing Interests

The authors have declared that no competing interests exist.

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