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$MgSO_4$ treatment preserves the ischemia-induced reduction in S-100 protein without modification of the expression of endothelial tight junction molecules

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Summary. The aim of this work was to evaluate the effect of magnesium sulphate ($MgSO_4$) administration on blood-brain barrier (BBB) permeabilization after cerebral hypoxia-ischemia (HI) induced by partial occlusion of the umbilical cord of premature fetal lambs. We also characterized BBB dysfunction in terms of the levels of expression of a panel of BBB proteins; Occludin, Claudin, Zona Occludens-1, Zonula Occludens-2, VE-cadherin and ß-catenin. Lambs were assigned to: *Control group*: non-injured animals, *0 h post-partial cord occlusion (0h-PCO) group*: animals subjected to 60 min HI and sacrificed just after the insult, *3h-PCO group*: HI injured animals resuscitated and managed on ventilation for 3 hours and *$MgSO_4$ group*: animals which received a dose of 400 mg/kg $MgSO_4$ after the HI event and managed on ventilation for 3 hours. Brains were fixed and blocks processed for S-100 protein immunohistochemistry. Other brains were dissociated and processed for S-100 and BBB protein immunochemistry for analysis by flow cytometry. The percentage of S-100 positive cells was found to be dramatically reduced in all studied brain tissues in the 3h-PCO group with respect to the other groups. No differences were found in the percentage or mean intensity of BBB protein immunolabeled cells among the groups. In the $MgSO_4$ group, the percentage of S-100 positive cells 3 h after the HI event was similar to the control group. These results suggest that $MgSO_4$ treatment preserves the ischemia-induced reduction in S-100 protein without modification in the expression of endothelial tight junction molecules. We speculate that $MgSO_4$ treatment confers neuroprotection by restoration of blood brain permeability in hypoxia-ischemia.

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Introduction

The Central Nervous System is one of the most sensitive systems in mammals. The blood-brain barrier (BBB) is a specialized structure to isolate its cells from plasma and cerebrospinal fluid in order to maintain a stable and controlled ionic environment which is necessary to sustain the vital activities of brain cells (Møllgard and Saunders, 1986; Saunders et al., 1991). Endothelial cells, pericytes, basal lamina and astrocytes are the constitutive elements of the BBB and serve to protect the brain from sudden changes of plasma composition and to regulate exchanges between parenchymal and vascular compartments (Engelhardt, 2003). Hypoxia ischemia sets in motion a series of events (Hilario et al., 2006) which lead to disruption and increased permeability of the BBB. The tight junctions of the endothelial cells are the main component of the BBB (Mazzetti et al., 2004; Alvarez et al., 2007; Kierszenbaum, 2007), consisting of integral membrane proteins (claudin and occludin), adhered junction proteins (CE-cadherin and ß-catening) and a number of cytoplasmic accessory proteins (including ZO-1, ZO-2, ZO-3). Tight junctions diminish the intercellular diffusion of substances, and therefore limit the paracellular diffusion of hydrophilic molecules (Rubin and Staddon, 1999; Kim et al., 2006). They seem to be the most susceptible component of the BBB, being readily modified under pathological circumstances, such as ischemia, inflammation or tumor growth (Ballabh et al., 2004). BBB dysfunction is typically accompanied by an increased permeability of the barrier allowing the diffusion of water, proteins and solutes.

Alterations in BBB permeability (Büttner et al., 1997) contribute to neuronal damage in many conditions (Banks, 1999) and participate in the etiology of a variety of neuronal disorders (Grant et al., 1999; Strelow et al., 2001). The S-100 protein is a major component in the cytosol and processes of astrocytes. The function of S-100 in the Central Nervous System is only poorly understood, having a neurotrophic role in neurons and glial cells. The appearance of this protein in plasma may be a consequence of the opening or disruption of the BBB. Thus, S-100 is an early marker of BBB disruption although it is not necessarily related to neural damage (Kapural et al., 2002; Stroick et al., 2006). It may precede neuronal damage and therefore can influence the choice of therapeutic strategy (Ingebrigtsen et al., 1999).

Thus, the astroglial S-100 protein represents an established early and sensitive biochemical marker of brain damage in adults and children after head trauma, infection, ischemia, and perinatal asphyxia (Gazzolo et al., 1999; Böttiger et al., 2001; Blennow et al., 2001; Thorngren-Jerneck et al., 2004; Bracci et al., 2006). Recently, it has been reported that hypoxemia with associated acidemia in sheep fetuses leads to persistent elevation in S-100 concentrations in plasma (Giussani et al., 2005). The presence of S-100 after hypoxia/asphyxia may be due to combined leakage out of necrotic glial cells and passage through an impaired blood-brain barrier (Büttner et al., 1997). However, no study to date has reported changes in the concentration of S-100 protein in the fetus at a cellular level and how these changes may correlate with the maintenance of BBB integrity.

The aim of the present work was to evaluate the neuroprotective efficacy of $MgSO_4$ treatment using protein S-100 as a BBB disruption marker after HI injury induced by partial cord occlusion in premature lambs. We also aimed to characterize the levels and intensity of expression of integral membrane proteins and cytoplasmic accessory proteins in order to further characterize the events which underlie BBB disruption.

Materials and methods

Experimental animals

The study was carried out using 40 fetal lambs at 86-92% of gestation (125-133 days of developmental age; term, 145 days). 20 animals were destined for immunohistological studies and the other half were used for flow cytometry analysis. Experimental procedures were carried out in accordance with European Union regulations for animal research (EU 86/609).

Experimental procedures and physiological parameters

We employed a previously described model of intrauterine asphyxia of the fetus by means of partial cord occlusion (Hilario et al., 2005). Briefly, sheep were anesthetized and fetal lambs were exposed by left laparotomy. The head was exteriorized and an endotracheal tube was inserted. Catheters (XRO umbilical catheter, Vygon, France) were inserted into the right jugular vein and into the left axillary artery. Core temperature was monitored and kept between 37-38°C with a temperature blanket (Digiterm S542, JP Selecta, Spain).

A non-invasive Doppler flow sensor (T106, Transonics, Ithaca, FL, USA) was placed in the umbilical cord to determine both venous and arterial blood flow in real time. A vascular occluder was placed around the umbilical cord and properly compressed to produce a reduction of umbilical blood flow to lower than 50% of the basal value, as indicated by the Doppler flow sensor monitor. HI endpoint was established after 60 min of such partial cord occlusion and fetal arterial pH, blood gases, mean systemic arterial pressure and heart rate were determined.

Experimental groups

Both in histological and in cytometric studies, lambs were randomly assigned to four different experimental groups by means of the closed envelope method. Control group: after cesarean section without asphyxia, lambs were managed on conventional mechanical ventilation for 3 hours (n=5 for immunohistochemistry, n=5 for flow cytometry) and sacrificed. *0 hours post-Partial Cord Occlusion Group (0h-PCO group)*: lambs were sacrificed after 60 minutes of asphyxia. (n=5 for immunohistochemistry, n=5 for flow cytometry). *3 hours post-Partial Cord Occlusion Group (3h-PCO group)*: after 60 minutes of asphyxia, lambs were resuscitated and managed on conventional mechanical ventilation for 3 hours (n=5 for immunohistochemistry, n=5 for flow cytometry), and sacrificed. *Magnesium sulfate Group ($MgSO_4$ Group)*: after 60 minutes of asphyxia, lambs were resuscitated and managed on conventional mechanical ventilation for 3 hours (n=5 for immunohistochemistry, n=5 for flow cytometry) and a dose of 400 mg/kg of $MgSO_4$ (15% $MgSO_4 \cdot 7H_2O$ solution) was infused slowly for the first 20 minutes after asphyxia (Greenwood et al., 2000).

Tissue collection

In all cases, the brain was flushed with Ringer lactate solution at 4°C via the carotid arteries before perfusion with 500 ml of 4% paraformaldehyde. Then, brains were removed and dissected into different cerebral regions: cerebral cortex (frontal, parietal, temporal and occipital), hypothalamus, thalamus, hippocampus, basal nuclei, cerebellum (cortex and deep cerebellar nuclei) and pons. For immunohistological studies, tissue blocks were embedded in paraffin wax and sectioned (5 micron thick). Samples also obtained for histology were embedded in paraffin wax and

sections stained with hematoxylin-eosin (H&E).

S-100 protein analysis

The S-100 protein is a dimeric acidic calcium-binding protein constituting a mayor component of the cytosol of astrocytes and Schwann cells. This protein was used as a marker of Central Nervous System damage.

Flow cytometry

Tissue blocks from different brain regions were disaggregated in microplates by means of 70 µm cell-straining (BD Falcon, Becton Dickinson, San Jose, CA, USA) in a 0.25% HCl-pepsin solution. Afterwards, mouse anti protein S-100 (1:200, Sigma) primary antibody was added to each cell-suspension and incubated over-night. After primary incubation, cell-suspensions were incubated with Alexa Fluor anti-mouse secondary antibody (1:300, Molecular Probes, The Netherlands) for one hour. As a control, parallel cell-suspensions were processed as above except for the omission of the primary antibody. The percentage of immunopositive cells and the means of fluorescent intensity were measured in an EPICS ELITE Flow Cytometer. To exclude debris and cellular aggregates, samples were gated based on light scattering properties in the side scattering (SSC) which correlates with cell complexity, and forward scattering (FSC) which correlates with cell size, and 10,000 events per sample within a gate (R1) were collected. Events within R1, which corresponded to individual cells, were plotted for their fluorescence at 525 nm. An unstained sample was used as a control to establish autofluorescence background values.

Immunohistochemistry

For immunohistological staining, paraffin sections were dewaxed and rehydrated. Endogenous peroxidase was blocked with H_2O_2 (1%). Afterwards, tissue sections were incubated overnight with mouse anti-protein S-100 primary antibody (1:200, Sigma) and then peroxidase labeled second antibody (HRP anti-mouse 1:300, Zymed) for one hour. The sections were stained with peroxidase-diaminobenzidine (DAB) for 10 min and counterstained with hematoxylin.

Blood-brain barrier proteins

In order to examine BBB integrity, we studied the levels of expression of several proteins present in the tight and in the adherens junctions of the blood-brain barrier. Occludin and Claudin are transmembrane proteins that play a regulatory role in tight junctions. Zonula Occludens-1 (ZO-1) and Zonula Occludens-2 (ZO-2) are cytoplasmic accessory proteins of tight junctions. VE-cadherin and ß-catenin are adherens junction proteins.

Flow cytometry

Tissue blocks from the different brain regions were dissociated into microplates by means of a 70 µm cell-strainer (BD Falcon, Becton Dickinson, San Jose, CA, USA) in a 0.25% HCl-pepsin solution. Cell suspensions were incubated overnight with the following primary rabbit antiserum, all diluted at 1:100 and purchased from Zymed (San Francisco, CA, USA): anti-ZO-1 and anti-ZO-2, anti-occludin, anti-claudin, anti- ß-catenin and anti-VE-cadherin. Afterwards, cell-suspensions were incubated with secondary antiserum (Alexa Fluor anti-rabbit 1:300, Molecular Probes) for one hour and analyzed in an EPICS ELITE Flow Cytometer (Coulter Electronics, Inc., Hialeah, Florida, USA). As a control, parallel cell-suspensions were processed as above except for the omission of the primary antiserum. In order to exclude debris and cellular aggregates, samples were processed as in the S-100 assay (see above).

Immunohistochemistry

Paraffin sections were dewaxed and rehydrated for immunohistological staining. For tissue permeabilization, sections were pre-incubated overnight with anti-ZO-1 and anti-ZO-2, anti-occludin, anti-claudin, anti-ß-catening and anti-VE-cadherin primary rabit antiserum (Zymed, San Francisco, CA, USA) all diluted at 1:100 and then incubated with secondary antiserum (Alexa Fluor anti-rabbit 1:300, Molecular Probes) for one hour. Samples were studied by Confocal Microscopy (Olympus FV500).

Statistical analyses

Values are presented as mean ± standard deviation (SD). Results were analyzed with the Levene test to confirm the homogeneity of variance between the different treatments and with the Kolmogorov-Smirnoff test for normality. One factor analysis of variance (ANOVA) with the Bonferroni-Dunn correction was performed to assess differences in protein S-100 labeling and in integral membrane protein labeling as a function of group (Statview, Abacus Corp., USA). Comparison between parameters was performed by two factor ANOVA for repeated measurements as a function of brain zones and groups. A $p \leq 0.05$ was considered to be significant.

Results

Physiological parameters

Umbilical cord occlusion did not cause a decline in fetal arterial pressure in any experimental group. Mean

arterial blood pressure was 55±3 mmHg (control group), 55±4 mmHg (in both the 0h- and 3hPCO groups) and 49±10 mmHg ($MgSO_4$) group. Fetal heart rate increased ($p<0.005$) after HI injury (214±11 beats/min in both partial cord occlusion groups and 221±38 beats/min in the $MgSO_4$ group) in comparison to the control group (159±5 beats/min). However, at 3 hours post-HI, no difference in heart rate was observed between the 3h-PCO group, the $MgSO_4$ group and the control group (249±43 beats/min and 227±25 beats/min vs. 180±37 beats/min respectively).

Partial cord occlusion caused an increment in P_{CO2} at the HI endpoint in both PCO groups and in the $MgSO_4$ group (8.9±0.7 and 9.4±0.9 kPa) in comparison to the control group (7.7±0.7 kPa). Immediately after HI injury, lambs showed a significant decrease in arterial pH to below 7.1 (7.02±0.07 in PCO groups and 7.04±0.04 in the MgSO4 group with respect to 7.27±0.02 in the control group) and a base excess less than -10 mM in the PCO groups and MgSO4 group in comparison to the non-injured group (-14.6±2.8 vs. -10.1±4.2 vs. -0.9±1.5 mM respectively) (Ikeda et al., 1998). After 3 h, no differences in these variables were apparent between the control, 3h-PCO and $MgSO_4$ groups.

Histological observations

Samples were verified to be histologically well preserved by means of hematoxylin-eosin and Nissl staining, and corresponded to an 8-9 on the van Reempts scale (Van Reempts, 1984). The control group did not show histological alterations. In both PCO groups and in the $MgSO_4$ group, some isolated neurons showed a retracted aspect with eosinophilic cytoplasm and a loss of nuclear detail. Almost all these necrotic-like neurons were observed mainly in the cerebellum, pons basal nuclei and mesencephalon. These observations are coincident to those previously described by the authors (Goñi de Cerio et al., 2007), indicating that, at least in our experimental model, necrosis is a direct consequence of the hypoxic–ischemic assault which leads to sudden death immediately after the injury.

S-100 astroglial protein

The immunohistological study revealed a similar pattern of distribution of stained cells in all experimental groups studied. The number of stained cells was similar in all groups, with the exception of the 3h-PCO group, which showed a decrease in the number of S-100 immunolabeled cells (Fig. 1).

Flow cytometry revealed that at 0 hours after partial cord occlusion (0h-PCO group), the percentage of astroglial protein S-100 positive cells was similar to that of the control group in all studied zones. Both in the 0h-PCO group and in the control group, more than 40% of the cell populations were found to be S-100 immunoreactive. In contrast, 3 hours after partial cord occlusion (3h-PCO group), the percentage of S-100 positive cells was only 7-10% in all brain regions. These results are summarized in Figure 2. Differences in mean fluorescent intensity of the cells were not observed in the 3h-PCO group with respect to either the control or 0h-PCO groups.

In the animals treated with $MgSO_4$, the percentage of S-100 positive cells was similar to control and 0h-PCO groups in all brain regions studied. Thus, in this treated group, the percentage of S-100 positive cells maintained values similar to basal 3 hours after the HI event (Fig. 2). The mean fluorescent intensity values in the $MgSO_4$ group were similar to those of the control, 0h-PCO and 3h-PCO groups (Table 1).

Blood-brain barrier proteins

Transmembrane proteins: occludin and claudin

The immunohistological study revealed that the

Table 1. Evaluation of changes in S-100 immunofluorescence in different brain regions of preterm lambs, by flow cytometry. Values represent mean fluorescence intensity ± SD.

	Healthy	0h-PCO	3h-PCO	$MgSO_4$
Cerebral Cortex	13.0±3.0	12.1±3.2	11.3±2.6	11.1±2.8
Hypothalamus	10.2±3.2	11.2±2.8	09.8±2.4	10.1±2.3
Thalamus	11.0±1.8	10.7±2.6	09.6±2.9	09.8±1.9
Hippocampus	09.6±2.5	08.9±1.8	10.4±2.1	11.2±2.4
Basal Nuclei	08.9±1.9	09.8±2.5	10.4±1.6	10.8±2.1
Cerebellum	12.4±2.4	10.2±3.0	11.8±1.9	10.6±2.2
Pons	09.2±2.1	08.4±3.2	09.8±2.5	10.1±2.5

Table 2. Quantification of changes in the levels of transmembrane proteins following hypoxia-ischemia and magnesium treatment. **A.** Mean intensity of occludin immunofluorescence in different brain regions. **B.** Mean intensity of claudin immunofluorescence in different brain regions of preterm lambs. Values represent mean fluorescence intensity ± SD.

	Healthy	0h-PCO	3h-PCO	$MgSO_4$
(A) Occludin				
Cerebral Cortex	4.2±1.2	6.1±1.0	5.0±0.6	4.8±1.1
Hypothalamus	3.1±0.3	3.8±0.9	3.8±1.5	3.2±0.8
Thalamus	3.8±1.6	4.2±1.3	3.6±1.7	3.5±0.4
Hippocampus	3.9±1.0	3.6±1.2	4.1±0.8	4.0±1.0
Basal Nuclei	4.4±1.2	4.0±1.8	3.9±1.1	4.2±1.1
Cerebellum	4.2±0.8	3.8±0.9	4.1±0.6	3.9±1.0
Pons	3.9±0.3	3.2±1.0	3.6±1.2	4.0±0.8
(B) Claudin				
Cerebral Cortex	10±4.6	7.8±3.8	7.9±1.7	8.1±2.2
Hypothalamus	7.6±2.5	6.9±1.8	7.6±2.0	8.7±2.0
Thalamus	8.2±4.2	6.9±2.1	7.9±0.5	8.1±5.5
Hippocampus	8.8±3.8	6.1±1.9	6.9±2.7	9.1±3.2
Basal Nuclei	6.8±2.3	4.9±3.7	6.5±1.2	8.3±4.3
Cerebellum	6.9±2.5	8.2±1.3	7.9±4.2	8.8±3.8
Pons	7.0±2.1	7.3±1.0	8.2±2.4	7.9±2.6

number of stained cells was similar in all experimental groups studied. According to flow cytometry, the percentage of occludin and claudin positive cells (Fig. 3) in the 0h-PCO and 3h-PCO groups was similar with respect to the control group, in all brain regions, and the mean fluorescent intensity of the cells was also similar in these three groups (Table 2). Results obtained from the animals treated with $MgSO_4$ were essentially the same, without statistical differences with respect to control, 0h-PCO and 3h-PCO groups.

Cytoplasmic accessory proteins: ZO-1 and ZO-2

Also, in both HI animal groups, the percentage of ZO-1 and ZO-2 positive cells in the 0h-PCO and 3h-PCO groups was similar to the control group (Fig. 4). Moreover, the $MgSO_4$ group presented similar values to the control and PCO groups. The values of mean fluorescent intensity (Table 3) did not show differences among all the experimental groups. The

Table 3. Quantification of changes in the levels of cytoplasmic accessory proteins following hypoxia-ischemia and magnesium treatment. **A.** Mean intensity of ZO-1 immunofluorescence in different brain regions. **B.** Mean intensity of ZO-2 immunofluorescence in different brain regions of preterm lambs. Values represent mean fluorescence intensity ± SD.

	Healthy	0h-PCO	3h-PCO	$MgSO_4$
(A) Zonula Occludens-1				
Cerebral Cortex	4.2±1.2	4.0±1.1	5.0±0.8	4.4±1.2
Hypothalamus	3.8±0.3	4.6±0.6	3.5±1.0	4.1±0.9
Thalamus	3.2±1.6	5.0±0.4	3.6±1.7	4.2±1.3
Hippocampus	3.0±1.0	3.9±1.0	3.1±0.8	3.6±1.2
Basal Nuclei	3.5±1.5	4.8±1.1	3.4±1.1	5.0±0.8
Cerebellum	2.0±0.8	3.5±1.0	3.0±0.6	3.8±0.9
Pons	4.0±0.3	3.0±1.8	3.4±1.2	3.6±1.0
(B) Zonula Occludens -2				
Cerebral Cortex	4.7±1.5	4.6±0.3	3.9±0.4	4.2±2.0
Hypothalamus	4.5±1.7	4.3±0.4	5.2±1.7	3.9±1.1
Thalamus	2.9±0.4	4.3±1.5	3.2±1.7	3.6±0.8
Hippocampus	3.6±0.5	3.2±0.9	5.0±0.4	4.2±1.3
Basal Nuclei	4.4±2.0	4.2±1.2	4.5±1.2	4.8±1.2
Cerebellum	3.5±0.8	3.2±0.6	3.4±1.4	3.6±1.2
Pons	3.1±1.0	3.3±0.8	4.0±0.2	3.4±0.8

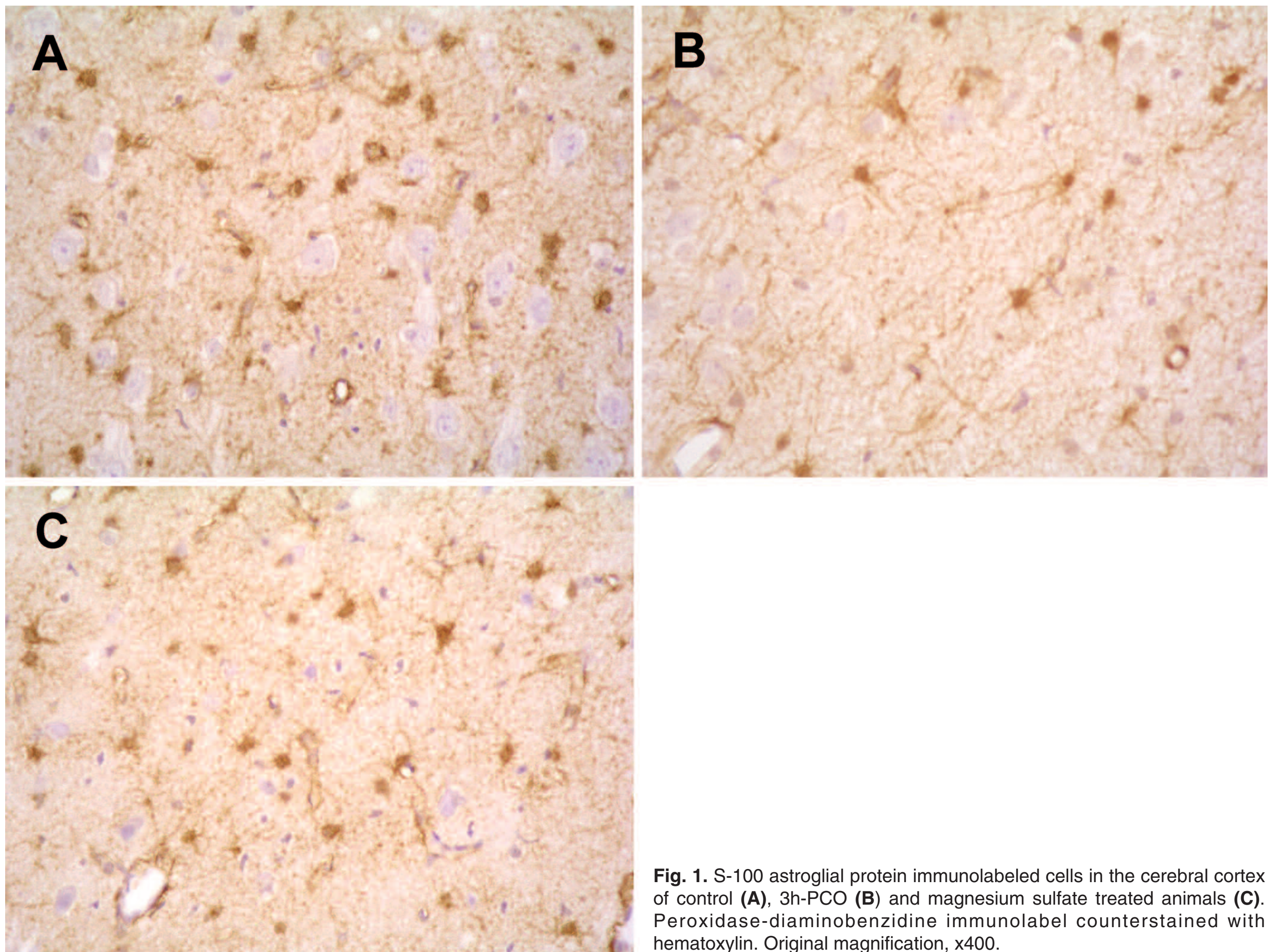

Fig. 1. S-100 astroglial protein immunolabeled cells in the cerebral cortex of control **(A)**, 3h-PCO **(B)** and magnesium sulfate treated animals **(C)**. Peroxidase-diaminobenzidine immunolabel counterstained with hematoxylin. Original magnification, x400.

Table 4. Quantification of changes in the levels of adherens junction proteins following hypoxia-ischemia and magnesium treatment. **A.** Mean intensity of VE-cadherin immunofluorescence in different brain regions. **B.** Mean intensity ofimmunofluorescence in different brain regions of the preterm lambs. Values represent mean fluorescence intensity ± SD.

	Healthy	0h-PCO	3h-PCO	$MgSO_4$
(A) VE-cadherin				
Cerebral Cortex	6.7±1.8	6.8±1.3	4.8±0.9	5.6±0.8
Hypothalamus	4.9±2.1	6.9±1.0	3.5±1.3	6.7±2.1
Thalamus	4.6±0.8	6.0±1.9	8.2±1.2	6.3±2.0
Hippocampus	5.9±2.3	4.7±2.4	5.0±0.7	6.6±1.6
Basal Nuclei	5.8±1.2	6.3±1.7	4.7±1.2	6.9±2.1
Cerebellum	4.4±1.7	5.5±1.3	4.7±1.8	6.4±2.3
Pons	4.8±2.1	5.0±0.8	3.7±2.4	4.1±2.6
(B) ß-catenin				
Cerebral Cortex	4.8±2.4	6.2±2.1	5.1±1.2	3.5±1.9
Hypothalamus	4.3±2.1	2.4±1.6	5.3±1.8	4.0±2.3
Thalamus	5.7±2.2	5.4±1.9	4.3±0.6	6.1±1.9
Hippocampus	6.9±2.5	5.4±1.9	8.1±1.1	7.8±2.2
Basal Nuclei	8.0±1.1	6.1±0.8	6.3±1.4	7.5±1.8
Cerebellum	7.1±1.4	5.4±1.2	6.8±0.7	5.9±1.3
Pons	5.6±0.9	6.2±2.3	5.7±1.8	6.5±1.7

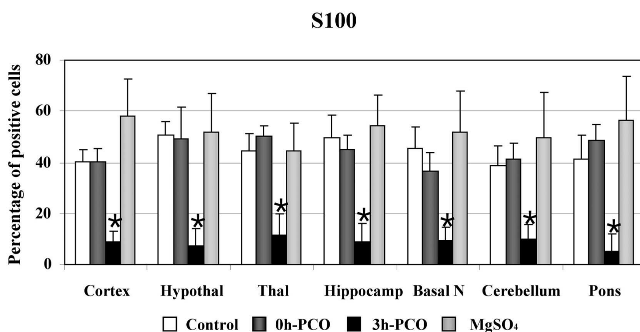

Fig. 2. Percentage of protein S-100 immunopositive cells, expressed as mean ± SD. One-factor analysis of variance was performed between experimental groups at each interval (Control, 0h-PCO, 3h-PCO and MgSO4 groups). (*) p<0.05. (Hypothal: hypothalamus; Thal: thalamus; Hippocam: hippocampus; Basal N.: basal nuclei).

immunohistological study also revealed that the number of stained cells was similar in all experimental groups.

Adherens junction proteins: VE-cadcatenin and ß-catenin

As occurred with the other BBB proteins, VE-cadherin and ß-catenin immunofluorescence was unaltered according to flow cytometry measurements and immunohistological studies at 0h and 3h after partial cord occlusion. Both the percentage (Fig. 5) and the mean fluorescent intensity (Table 4) of positive cells were similar in PCO and $MgSO_4$ groups, without statistical differences with respect to control.

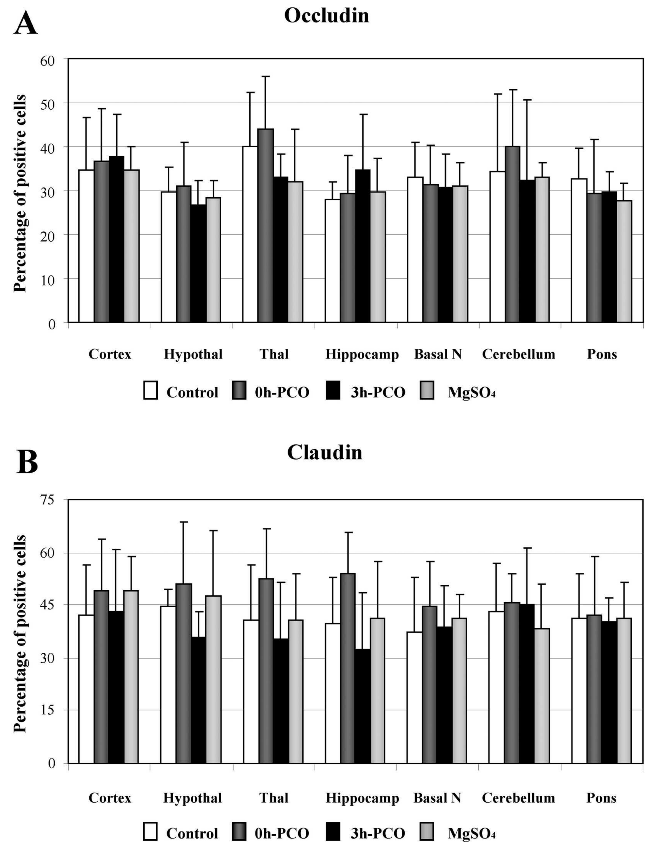

Fig. 3. Percentage of cells with cytoplasmic accessory protein immunoreactivity following partial umbilical cord occlusion and magnesium sulfate administration. **A.** Occludin, **B.** Claudin. Each value represents the mean ± SD. (Hypothal: hypothalamus; Thal: thalamus; Hippocam: hippocampus; Basal N.: basal nuclei).

Discussion

Our results suggest that the $MgSO_4$ treatment preserved the ischemia-induced reduction in expression S-100 protein. We speculate that $MgSO_4$ treatment confers neuroprotection by restoration of blood brain permeability in hypoxia-ischemia.

Over the last decade, several biochemical markers of early abnormal function of the BBB and thus of brain damage have been found (Hagberg et al., 1993; Blennow et al., 1995; Martín-Ancel et al., 1997; Al Naqeeb et al., 1999; Kapural et al., 2002). The astroglial protein S-100 constitutes a major component of the cytosol of astrocytes and Schwann cells. This protein may play an important role in the differentiation, growth and shape of cells, and is a well characterized biochemical marker of early cerebral injury in adult, as well as in term newborn infants with HI encephalopathy (Gazzolo et al., 1999; Blennow et al., 2001; Böttiger et al., 2001; Nagdyman et al., 2001; Thorngren-Jerneck et al., 2004). The presence of S-100 in serum or in cerebrospinal fluid (CSF) may

be due to combined leakage from damaged or necrotic astrocytes and passage through an impaired BBB (Büttner et al., 1997; Gerlach et al., 2006), indicating severe ischemic cellular injury (Kapural et al., 2002).

Our results suggest that HI brain injury (Goñi de Cerio et al., 2007) is associated with a decrease in the percentage of S-100 positive cells in cerebral tissue just 3 hours after HI injury to preterm lambs. Moreover, this decrease in the number of cells expressing S-100 protein was not accompanied by an increase in the intensity of its expression, because the mean fluorescent intensity of positive cells was similar for control and 3h-PCO groups, suggesting a depletion of the S-100 protein present in the astrocytes. This depletion coincides with elevated serum concentrations of S-100 protein observed by other authors (Nagdyman et al., 2001) as early as 2 hours after a HI event lasting 6 hours. Thus, the early appearance of this protein in plasma may be the result of a dysfunction in the BBB. Because the failure of BBB integrity also gives rise to neuronal damage in many conditions (Büttner et al., 1997; Banks, 1999; Grant et al., 1999; Strelow et al., 2001), early detection could in principle permit intervention to prevent more extensive brain damage.

BBB disruption by protein dislocation or denaturalization with subsequence expression is not the only route to BBB permeabilization (Fischer et al., 2002; Huber et al., 2002; Witt et al., 2003). Thus, in the present study, the levels of S-100 protein decreased but the analysis of the proteins of the BBB did not show differences either in the percentage or in the mean intensity of positive cells. These data suggest that, at least in the early stages of HI trauma, BBB permeabilization may be more a consequence of a loss of interaction or dislocation between the different BBB proteins rather than due to denaturalization and BBB disruption. This would indicate the existence of a therapeutic window for neuroprotection during which the BBB is permeable but its proteins are still not irreversibly damaged. As is known, the therapeutic window is the period of time during which an intervention can be successful in reducing the severity of

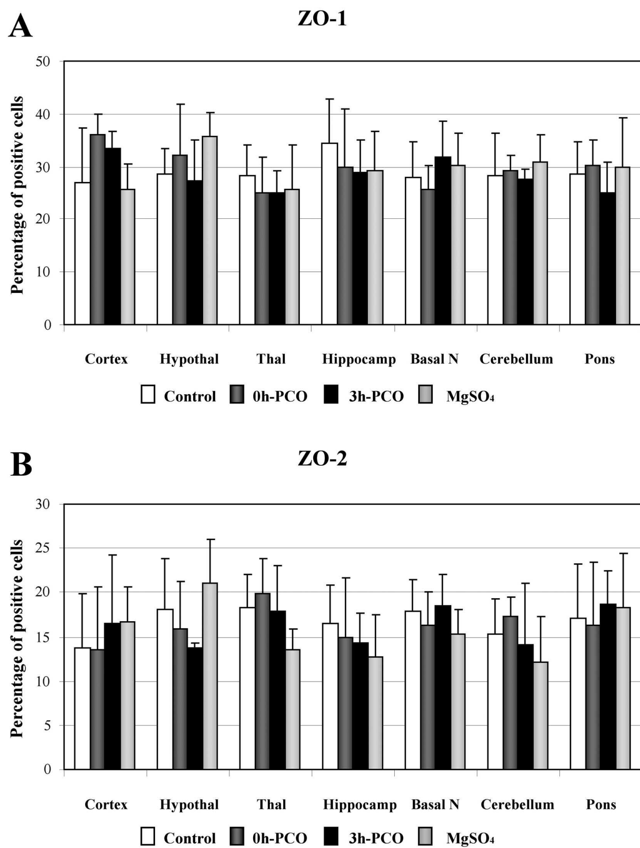

Fig. 4. Percentage of cells exhibiting transmembrane protein immunoreactivity following partial umbilical cord occlusion and magnesium sulfate administration. **A.** ZO-1. **B.** ZO-2. Each value represents the mean ± SD. (Hypothal: hypothalamus; Thal: thalamus; Hippocam: hippocampus; Basal N.: basal nuclei).

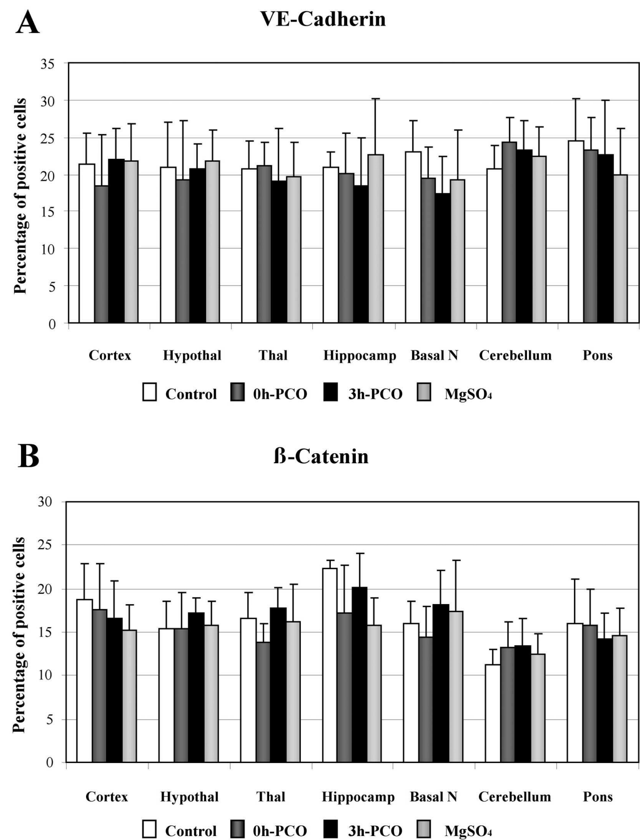

Fig. 5. Percentage of cells exhibiting adherens junction protein immunoreactivity following partial umbilical cord occlusion and magnesium sulfate administration. **A.** VE-cadherin. **B.** Each value represents the mean ± SD. (Hypothal: hypothalamus; Thal: thalamus; Hippocam: hippocampus; Basal N.: basal nuclei).

brain injury. In humans, this time-window is considered to be from 2 to 6 hours after birth asphyxia (Nagdyman et al., 2001).

Here, we have used MgSO4 as a possible neuroprotector during the initial moments after the HI event. Magnesium is the fourth most abundant cation in the body and the second most abundant in the cytosol, and it is essential for many cell functions. Changes in magnesium status before, during and after a brain insult are likely to have a profound effect on neurological outcome (Meloni et al., 2006). Indeed, clinical and experimental studies have shown that subjects with low levels of magnesium in CSF or in serum have worsened neurological outcomes following ischemia and traumatic brain injury (Lampl et al., 1998; McIntosh et al., 1998). There is also evidence demonstrating marked changes in intracellular and extracellular brain magnesium concentrations after HI events (Meloni et al., 2006). Consequently, we decided to administer magnesium as a candidate neuroprotective agent following ischemic and traumatic insults, with a view to restoring magnesium homeostasis in the brain (Muir et al., 1999; Muir, 2001; Van den Bergh., 2002).

There is currently no general consensus about the value of magnesium as a neuroprotective agent. Authors such as Zhu et al. (2004) have suggested that magnesium sulphate administration does not prevent the adverse effects of energetic depletion after a transient HI event, and other studies have pointed to the absence of a therapeutic effect (de Haan et al., 1997; Greenwood et al., 2000). Indeed, magnesium administration has even been considered to be harmful for the fetus (Reynolds et al., 1996), although this opinion is not unanimously upheld (De Haan et al., 1997; Greenwood et al., 2000). These paradoxical perspectives regarding the neuroprotective effect of the magnesium sulphate administration could be the consequence of the considerable variability in study design, making it difficult to directly compare outcomes.

The effect of $MgSO_4$ as a neuroprotector could be very dose sensitive. Thus, administration of doses higher than 250 mg/kg could have a neuroprotective effect, whereas doses higher than 750 mg/kg can produce hypotension and hypoventilation (Roth et al., 1992; Gathwala et al., 2006). Also, the effects of magnesium sulphate administration may depend on the chosen experimental model. Recently, studies with rat models and different magnesium doses presented divergent results, including neuroprotection (Sirin et al., 1998; Miles et al., 2001) and its absence (Milani et al., 1999; Westermaier et al., 2005).

In this study, we have observed that magnesium sulphate administered at a dose of 400 mg/kg over 20 min just after HI injury to preterm lambs does not have any detrimental effects on a variety of physiological parameters, including fetal blood pressure, P_{O2}, P_{CO2} and ventilation. In contrast, it reduced the depletion of S-100 protein, suggesting that BBB permeabilization is avoided, as has been reported recently in a rat model with hypertension (Euser et al., 2008). These findings suggest that magnesium sulphate may have neuroprotective effects leading to reduced brain injury when administered following hypoxic-ischemic events, such as perinatal asphyxia

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