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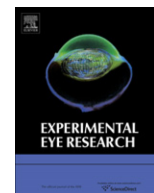

Hypothermia prevents the development of ischemic proliferative retinopathy induced by severe perinatal asphyxia

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A B S T R A C T

Obstetric complications, such as perinatal asphyxia, may cause retinal injuries as retinopathy of prematurity (ROP), a type of ischemic proliferative retinopathy. Up to date there are no appropriate experimental models for studying the long-term sequels of this disease. In the present work, we present an experimental model of perinatal asphyxia which shows structural and ultrastructural retinal alterations at the most inner layers of the retina, such as neurodegeneration, development of neoformed vessels and glial reaction, which are compatible with the histopathological description of ROP. Besides, the application of hypothermia during perinatal asphyxia showed effective results preventing cellular and morphological alterations. This study may contribute to the development of therapies in order to either ameliorate or prevent retinal damage. In this manner, hypothermia may improve life quality and decrease medical, family and social costs of these avoidable causes of blindness.

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1. Introduction

Perinatal asphyxia is the world's most severe problem in Perinatology Services (Cunningham et al., 2005; WHO, 1991). It affects four million newborn babies worldwide each year. According to statistics, a third of them suffer from neurological injuries, including several degrees of ischemic proliferative retinopathy (IPR) (Hill, 1991; Younkin, 1992). In addition, perinatal asphyxia is even a worse problem in underdeveloped countries, because precautions and necessary cares are either not appropriate or badly applied because of their high costs (Costello and Manandhar, 1994). The importance of studying the prevention of the development of ophthalmic pathologies in newborns is easy to understand considering that it is the first cause of blindness in children (Gilbert, 2001; Palmer et al., 1991; Wright et al., 1998).

Normal fetal growth and homeostasis depend on a suitable exchange of gases and metabolic products between the fetus and the mother through the placenta. If this fails, there is insufficient supply, especially of oxygen, which leads the fetus to acute or chronic asphyxia. Ninety percent of perinatal asphyxia occurs during the pre/peripartum period due to abnormalities such as umbilical cord compression, untoward obstetric maneuvers, placental detachment, placenta previa, post-mature placenta, maternal hemorrhage or anemia, gravid toxemia, placental infarct, etc.

Severe perinatal asphyxia originates a global hypoxia–ischemia status, which damages mainly the central nervous system (CNS). The seriousness of the degree and length of perinatal asphyxia with lack of oxygen are decisive for the development of injury sequels such as attention-deficit hyperactivity disorder -ADHD- (American Psychiatric Association, 1987), epilepsy, mental retardation, brain paralysis or spasticity, and visual-hearing alterations (Crofts et al., 1998; Younkin, 1992).

All the CNS is very sensitive to oxygen shortage. Regarding the eyes, the inner layers of the retina (inner nuclear layer, inner plexiform layer, ganglion cell layer, optical nervous fiber layer and inner limiting layer) seem to be particularly sensitive to oxygen

Abbreviations: CNS, Central Nervous System; CTL, Control group; GFAP, Glial fibrillary acidic protein; HYP, hypothermic asphyctic group; IR, Inner retina; IPR, Ischemic proliferative retinopathy; PA, Perinatal asphyctic group; ROP, Retinopathy of prematurity.

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level alterations (Osborne et al., 2004), which induce various degrees of retinopathy, the worst of which is blindness.

Retinopathy of prematurity (ROP), as a result of ischemic proliferative retinopathy (IPR), is an avoidable cause of visual impairment and blindness. Children exposed to hyperoxia following neonatal intensive care are highly susceptible to ROP (Smith, 2003). There are two phases in ROP development: the first one has a characteristic oxidative injury which hurts the endothelium and blocks vessels which have not concluded their formation, generating a non-vascular retinal area. The second phase of ROP results from a decrease in the metabolism at this zone, characterized by angiogenesis and stimulated by the increased synthesis of vascular endothelial growing factor (VEGF) and other angiogenic factors (Hardy et al., 2000; Jankov et al., 2001; Smith, 2003; Wilkinson-Berka et al., 2003). Previous results of our laboratory have shown that the histopathology of the retina of rats subjected to an experimental model of perinatal asphyxia (Rey-Funes et al., 2003) is compatible with the descriptions of IPR, which is observed in several diseases, such as ROP, complicated diabetes mellitus and retinal vein occlusion.

According to previous studies, decreasing the temperature from 37 °C to 15 °C results in a significant percentage of survival of rats subjected to perinatal asphyxia. Nevertheless, when perinatal asphyxia is induced at low temperature (15 °C), no mortality is observed (Loidl, 1997; Loidl et al., 1998). Hypothermia during perinatal asphyxia has preventive effects against CNS damage when compared to perinatal asphyxia in normothermic conditions (Dorfman et al., 2009; Gisselsson et al., 2005; Katz et al., 2004; Loidl et al., 1998). Hypoxia–ischemia induces neuronal injury, which mechanisms include: excitatory amino acid release (Choi, 1987; Herrera–Marschitz et al., 1993), free radical generation (Bonne et al., 1998; Capani et al., 2001), release of nitric oxide (NO) and other neurotransmitters, lactic acid accumulation, massive calcium entry into the cell, degradation and lipoperoxidation of membrane phospholipids (Farooqui et al., 1994) and alterations in axonal cytoskeleton components (Cebral et al., 2006).

The aim of this work was to investigate structural and ultrastructural changes in the retina induced by severe perinatal asphyxia exposure and to study the use of hypothermia as a therapeutic tool to prevent retinal damage. The goal of this work is the development of a non-invasive experimental model that allows studying ophthalmologic injuries compatible with IPR and ROP.

2. Materials and methods

2.1. Development of retina injury by a perinatal asphyxia

Sprague–Dawley albino rats with genetic quality and sanitary certification from the animal facility of our Institution were used following the international rules and guidelines of the Federation of European Laboratory Animal Science Associations (FELASA). All the procedures concerning the animal manipulation and treatment were performed according to the Guide of Animal laboratory Care (revisited in 1996) by the National Institute of Health Guide for the Care and Use of Laboratory Animals (Publications No. 80-23). The animal model described below has been approved by the Ethical Committee of CICUAL: "Comité Institucional para el Uso y Cuidado de Animales de Laboratorio" (Resolution N° 2079/07), Facultad de Medicina, Universidad de Buenos Aires, Argentina.

Time pregnant rats were anesthetized by intraperitoneal injection of chloral hydrate 28% (w/v, 0.1 ml/100 g of body weight), sacrificed and histerectomized after their first pup was delivered vaginally; these animals formed the control group (CTL), $n = 6$. Uteri containing the remaining fetuses were placed for 20 min in a water bath at either 37 °C (normothermic condition, perinatal asphyctic group (PA), $n = 6$) or 15 °C (hypothermic condition, hypothermic asphictic group (HYP), $n = 6$). Immediately after that, the fetuses were removed from the uterus horns, dried of their fluids and manually stimulated to breathe, being gasping the sign of recovery. Umbilical cords were ligated and animals left to recover for around 1 h before being given to surrogate mothers which had delivered normally 24 h before the experiment, mixing their normal litters with previously marked experimental pups. We considered only experimental animals that adjusted to the following parameters: 1– occipitocaudal length > 41 mm, 2 – weight > 5 g, generating.

2.2. Immunohistochemistry

Sixty-day-old rats were anesthetized by intraperitoneal injection with chloral hydrate 28% (w/v, 0.1 ml/100 g of body weight). Animals were fixed by intracardiac perfusion using a solution of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and finally, enucleated. The anterior segments of the eyes including the crystalline were removed in order to facilitate the post-fixation of the posterior segment, which contains the retinas. After cryoprotection with 30% sucrose in 0.1 M phosphate buffer (pH 7.4), tissues were frozen in powdered dry ice and stored at −80 °C. Sections (20-μm thick) were obtained with a Leitz "Lauda" cryostat and mounted onto gelatin-coated slides (2.5% gelatin, 1% Elmer's glue), air-dried at room temperature, and stored at −80 °C until use. For immunocytochemical staining, sections were pre-incubated with 10% v/v normal bovine serum for 1 h and then incubated for 48 h at 4 °C with IgG anti-GFAP (glial fibrillary acidic protein) monoclonal mouse antibody (1:1000 dilution, Sigma, St. Louis, MO). After that, sections were washed three times in phosphate buffered saline (PBS) and incubated with biotinylated anti-mouse IgG antibody (VECTOR® Lab Inc.) in a wet camera for 1 h at room temperature. Slides were washed two times in PBS and a third layer of the ABC complex was incubated in a wet camera for 1 h 30 min at room temperature. Later, slides were washed two times in PBS and one time in 0.1 M buffer acetate. Staining development was performed with 0.03% 3,3′ diaminobenzidine (DAB), 3% nickel ammonium sulphate and 0.01% hydrogen peroxide diluted in 0.1 M buffer acetate. Slides were dehydrated in graded alcohols and cover-slipped with mounting media DPX (Fluka®). After the immunohistochemical process, some slides were counterstained with Cresyl Violet.

In order to study vessels and microglia, some sections were stained with biotinylated Lycopersicum esculentum (tomato) lectin (Sigma, St. Louis, MO) in a 1:150 dilution and revealed with an ABC kit followed by DAB-Nickel.

Stained sections were observed with an optical Carl Zeiss Axiophot microscope attached to a digital camera (Olympus, Q5) and photographed for analysis.

2.3. High resolution optical and electronic microscopy

In order to study structural and ultrastructural alterations, 60-day-old rats were anesthetized by intraperitoneal injection with chloral hydrate 28% (w/v, 0.1 ml/100 g of body weight), fixed by intracardiac perfusion using a solution of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and finally, enucleated. Retinas were dehydrated in graded alcohols, cleared in xylene and embedded in paraffin. Sections (5–10 μm thick) were obtained using a microtome (Leitz, MGW Lauda) and processed by Hematoxylin-Eosin (H&E) staining. Sections were observed with a Carl Zeiss Axiophot microscope attached to a digital camera (Olympus, Q5).

Toluidine Blue staining procedure was also performed. Sixty-day-old rats were anesthetized by intraperitoneal injection with

chloral hydrate 28% (w/v, 0.1 ml/100 g of body weight). Animals were fixed by intracardiac perfusion using a solution of 4% paraformaldehyde + 0.25% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) and finally, enucleated. The anterior segments of the eyes including the crystalline were removed and retinas post-fixed by immersion in a 4% paraformaldehyde solution + 0.25% glutaraldehyde for 4 h. They were then washed, post-fixed in osmium tetroxide and dehydrated in graded alcohols and propylene oxide and embedded in Durcupan (Fluka®). Sections (1-μm thick) were obtained using a Reichert ultramicrotome and stained with Toluidine Blue for high resolution optical microscopy (O.M.). Sections were observed with an Olympus O.M. and photographed. Then, ultrathin sections (100-nm thick) were mounted in copper grids, counterstained with lead citrate and observed in a transmission electronic microscope (Zeiss E.M. 10C).

2.4. Image analysis and statistics

Five retina sections from six animals of each experimental group were analyzed. Care was taken in selecting anatomically matched areas of retina among animals before assays. Inner retina thickness, relative optical density (R.O.D.), number of vessels at IR per area, and number of ganglion cells with neurodegenerative signs were measured. R.O.D. was calculated using a scale of 255 gray levels. To avoid external variations, all images were taken on the same day and under the same light. The number of vessels was measured at 400-μm long retina segments and the number of ganglion cells at 160-μm long retina segments of. Four segments were measured per retinal section, and two sections per animal were evaluated. A computerized image analysis program (NIH Scion Image) was used to measure the thickness of the inner retina from the ganglion cell layer to the inner limiting layer. R.O.D. of GFAP immunostaining was also evaluated using this software. Statistical analysis of the determinations obtained was carried out with one-way variance (ANOVA) followed by Fisher's test. Results are expressed as the mean ± SD. The criterion for significance was $p < 0.05$.

3. Results

3.1. Perinatal asphyxia induces structural changes at retina

Structural and ultrastructural changes were evidenced in the retina of the perinatal asphyctic group (PA) as compared to control group (CTL). A high development of neovessels was observed mainly in the most internal zone of the retina in PA sections, as compared with the CTL group (Fig. 1). Numerous neoformed retinal vessels were also observed at the inner retina (IR) of the PA group (arrows in Fig. 1c), considering IR as the layer conformed by the ganglion cell layer, the inner limiting layer, and the optic nerve fiber layer. A significant 100% increase in the thickness of IR was observed in the most internal layers of PA retinas as compared to CTL (graphic at Fig. 1d). HYP did not show significant changes as compared to CTL (compare Fig. 1a and b and graph d).

By Toluidine Blue Staining, an increase in the thickness of the Müller cells internal processes (arrows at Fig. 2c) and neoformed vessels (asterisk at Fig. 2b) at IR of PA rats was observed. HYP animals did not show changes respect to CTL. Besides, neurodegeneration was observed at ganglion cells of PA animals (arrows at Fig. 2b), showing 5 ± 1 ganglion cells with chromatin condensation signs of 12 ± 2 total ganglion cells measured over 160 μm retina length (41.6%). Neither CTL nor HYP showed ganglion cells with neurodegeneration signs. In addition, neurodegeneration was also observed in a small number of neurons of the inner nuclear and outer nuclear layers of PA retinas (not shown). All these structural changes were not evidenced in the retina of the HYP (compare Fig. 2a and d).

3.2. Ultrastructural changes induced by perinatal asphyxia

Ganglion cells with clear signs of degenerative processes at retinas of PA, with shrunken and increased electrodensity nuclei and edematous and vacuolated cytoplasm, were observed by electron microscopy (Fig. 3b). In addition, Müller cell processes with an increase in thickness at PA showed accumulation of gliofilaments as compared to CTL (arrows in Fig. 3d). HYP showed retinal images similar to those of the CTL group without ultrastructural alterations (compare Fig. 3a and c).

3.3. Glial changes induced by perinatal asphyxia

Müller cells at the IR of PA animals showed an increased GFAP immunoreactivity when compared to the CTL group (compare Fig. 4a and c; see graph g) and enlarged, tortuous and engrossed internal processes (arrows in Fig. 4c). These cells produced an increased thickness of the internal glial limiting layer (Fig. 4c, asterisk in d, graph fi). Besides, IR PA vessels showed an increased thickness of the astroglial perivascular feet (Fig. 4e). Retinas of HYP animals did not show significant changes as compared to CTL ones (compare Fig. 4a and b; graphs f and g).

Using tomato lectin histochemistry, we observed that the inner plexiform layer of the retina of PA showed reactive microglia cells in the vicinity of vessels of neoformation (Fig. 5c–e) that were not present in the CTL group (Fig. 5a). In addition, a significant increase in the number of vessels at the IR and inner plexiform layer was observed at PA animals (Fig. 5c) as compared to CTL, with a 113 ± 10% increase in total vessels measured over 400-μm long retina segments. Vessels of HYP did not show significant differences with respect to CTL.

4. Discussion

In this work, we show structural and ultrastructural alterations in the retina induced by perinatal asphyxia. The described retinal alterations observed as a consequence of global hypoxia–ischemia are compatible with the histopathology of IPR, including ROP (Schulenburg and Tsanaktsidis, 2004). Therefore, the present experimental model may be a useful tool to study these pathologies. Besides, we applied hypothermia during asphyxia as a therapeutic strategy to protect the CNS against oxygen deprivation, demonstrating for the first time its effective action to inhibit retinal alterations due to perinatal asphyxia.

Previously, several models have been developed to study the effects of perinatal asphyxia, but they are invasive and have been carried out in animals several days after birth (Raju, 1992). Our model has the advantage of being non-invasive (Loidl, 1997; Loidl et al., 1998) and of inducing a global hypoxia–ischemia of the entire organism, including the retina. The goal of the present experimental model of global hypoxia–ischemia is that it offers the possibility of studying perinatal asphyxia long-term effects, because the newborn pups are left to grow with surrogated mothers. Pre-peripartum pathological situations, as well as untoward obstetric maneuvers, are accompanied with different degrees of oxygen deprivation at birth, being IPR a frequent consequence of it. Since the development of the CNS of rats at the moment of delivery is equivalent to that of the CNS of humans at 32 weeks of gestation (Palmer and Vannucci, 1993), the present model offers the possibility of studying and comparing CNS alterations with those produced in humans by prematurity.

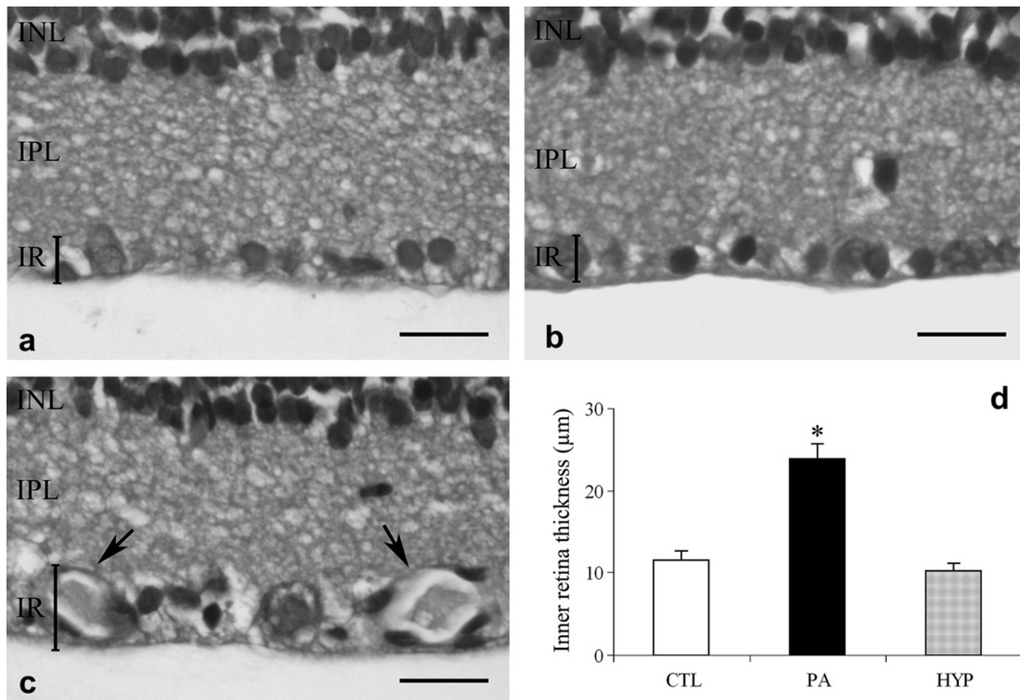

Fig. 1. Structural changes in the inner retina, induced by perinatal asphyxia. H&E staining images of the inner layers of the retina in CTL (a), HYP (b) and PA (c) groups. Scale bar = 20 μm. INL: inner nuclear layer; IPL: inner plexiform layer; IR: inner retina. Note the significant increase in the IR thickness in the PA group (see graph) and the presence of numerous vessels (C, arrows). * shows a significant increase in IR thickness in PA as compared to CTL, $p < 0.05$.

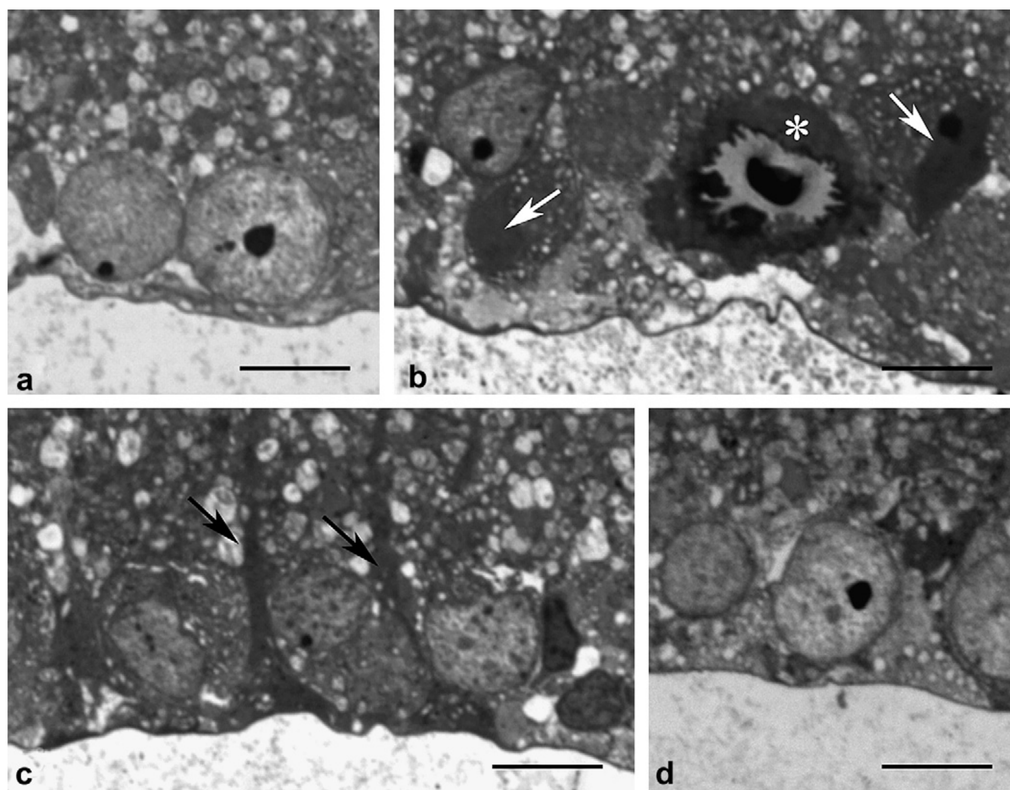

Fig. 2. Neuronal degeneration in the retina of perinatal asphyctic rats. Inner layers of retina of CTL (a), PA (b–c) and HYP (d) groups stained with Toluidine Blue. In the retinas of rats exposed to PA, ganglion cells appeared with different degrees of degeneration with chromatin condensation (white arrows), neoformed vessels (asterisk) and thickened Müller cells inner processes (black arrows) and their feet forming an enhanced inner limiting membrane. Scale bar: 10 μm.

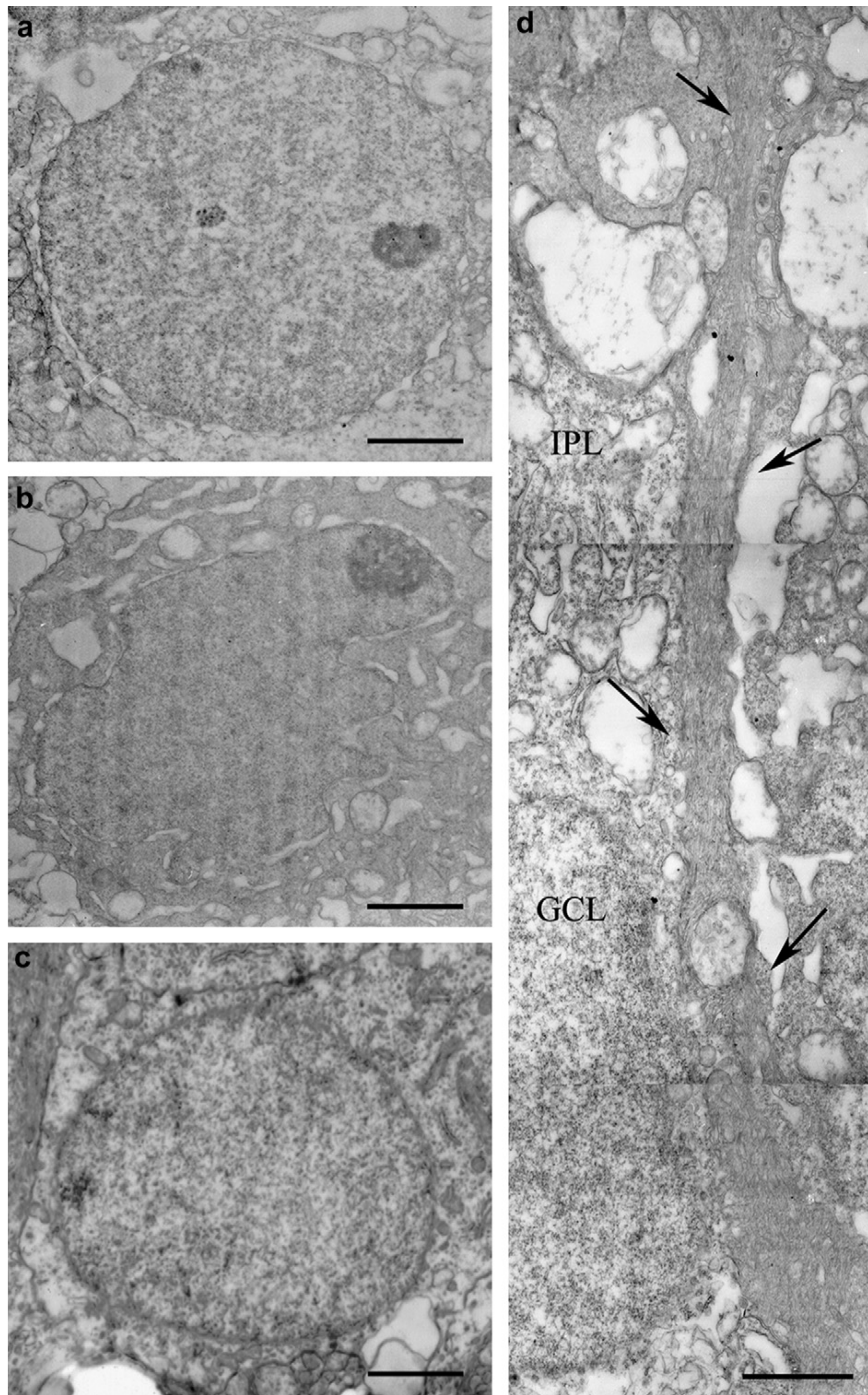

Fig. 3. Ultrastructural changes in the retina, induced by perinatal asphyxia. Images of electron microscope showing ganglion cell nuclei of CTL (a), PA (b) and HYP (c) groups. Note the degenerative edematous cytoplasm in PA accompanied with an increased electrondense nuclei with a shrunk pattern. d shows a photomontage image of the inner cell process of a Müller cell of the PA group that is tortuous because of its high accumulation of gliofilaments (arrows). Scale bar: 2 μm. IPL: inner plexiform layer; GCL: ganglion cell layer.

In our study, we observed marked structural and ultrastructural alterations in the retina, mainly affecting the ganglion cell layer, the optic nervous fiber layer and the inner limiting layer, together with a severe increase in IR thickness, astrogliosis and neovascularization development. Retinal degeneration in the layer of the photoreceptors and bipolar cell bodies of six-week-old rats subjected to moderate hypoxia-ischemia at birth (15 min of perinatal asphyxia) has been recently reported (Kiss et al., 2009). Previously, we have shown that animals subjected to 15 min of perinatal asphyxia show a survival of 100% (Loidl et al., 2000), whereas, in the present work, animals subjected to 20 min of perinatal asphyxia showed 25% survival, representing a severe status of hypoxia–ischemia (Loidl, 1997; Loidl et al., 2000). The present scheme leads to severe signs of retinopathy and is

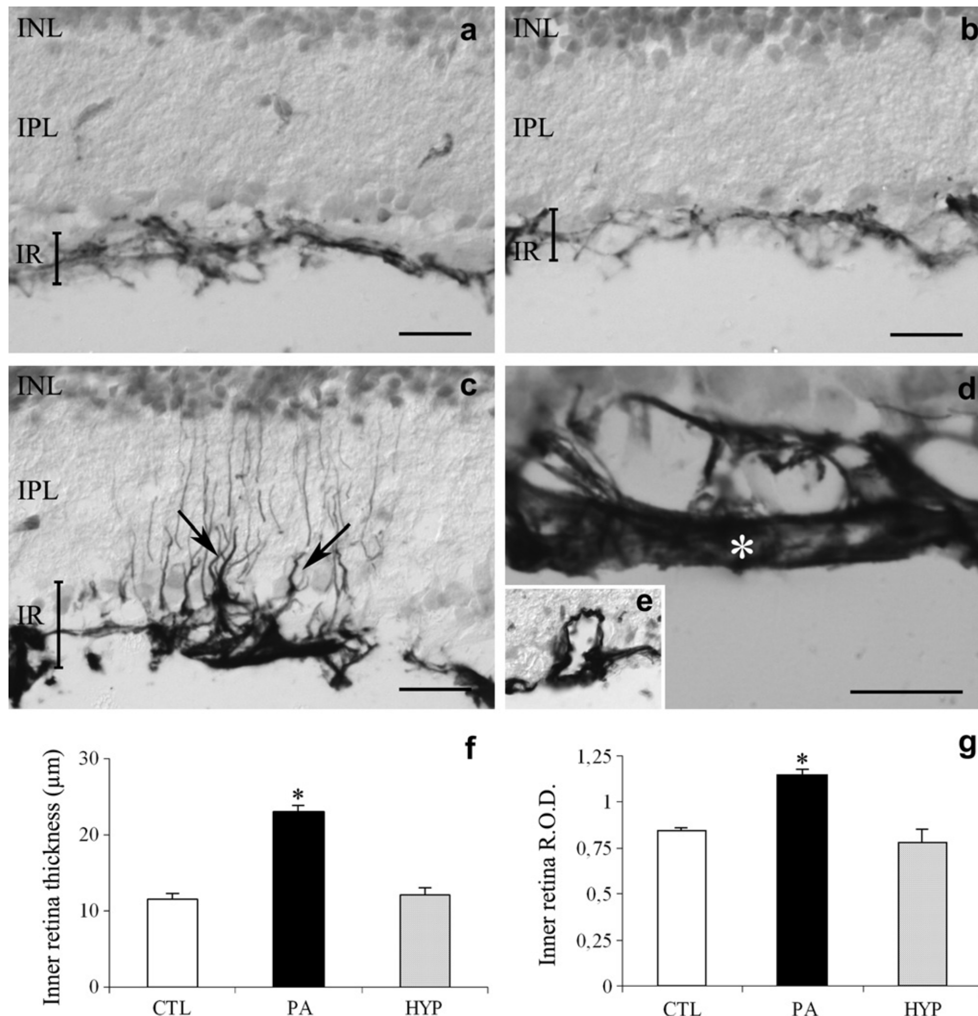

Fig. 4. Müller cells and astroglial alterations in the retina, induced by perinatal asphyxia GFAP immunostaining of the inner retina of CTL (a), HYP (b) and PA (c–e). Note the inner processes of the Müller cells (arrows) in the PA group (c). d, magnification of the inner retina showing an increased inner glial limiting layer (asterisk). e shows vessels with engrossed perivascular astroglial feet. f and g show graphs of inner retina thickness and relative optical density (R.O.D.), respectively, and of GFAP immunoreactivity in the retina of the CTL, PA and HYP groups. Significant IR thickness and R.O.D. was observed in PA as compared to CTL, while no significant changes were observed in the HYP group. $^{*}p < 0.05$ PA vs. CTL. Scale bars: 25 µm. INL: inner nuclear layer; IPL: inner plexiform layer; IR: inner retina.

compatible with pathological descriptions of the second phase of ROP.

In order to prevent retinal lesions, several investigations have attempted to slow down the evolution of IPR and the development of ROP by using therapeutic agents like antioxidants, such as vitamin E (Raju et al., 1997), D-penicillamine (Phelps et al., 2000) and allopurinol (Russell and Cooke, 1995), as well as exposure to early light reduction (Phelps and Watts, 1997) or oxygen supplement (STOP ROP Multicenter Trial, 2000). The application of indomethacin (Nandgaunkar et al., 1999), dexamethasone (Rotschild et al., 1999), rofecoxib (Wilkinson-Berka et al., 2003) or bucillamine (a D-penicillin-like anti-inflammatory drug) has also been suggested to prevent neovascularization. However, results have not been very successful. Early corticoid administration and inositol supplement application in preterm children have shown only a decrease in ROP severity (Howlett, 2003). Unfortunately, these therapeutic advances are distant from being very effective treatments. In contrast, using our experimental model, we observed that decreasing the environmental temperature during perinatal asphyxia leads to a significant increase in survival (Loidl et al., 2000). Hypothermia has proved to be protective against the development of long-term cerebral alterations (Dorfman et al., 2009; Loidl et al., 1998, 2000), and the present work extends these observations to the retina, being a useful therapeutic tool in ROP prevention. In this work, induction of perinatal asphyxia under hypothermic conditions also prevented astrogliosis reaction and development of neovascularization in the inner retina. This protective effect may be related to a reduction in energy demand (Nedegaard et al., 1991; Young et al., 1983).

In conclusion, this animal model of perinatal asphyxia seems to be useful for the study of IPR development and of the second phase of ROP, where an increased angiogenesis has been described (Wilkinson-Berka, 2004). The structural and ultrastructural changes observed are in accordance with the current histopathological reports of this affection. Therefore, this model of perinatal asphyxia may contribute with valuable information to unraveling the physiopathological mechanisms of these diseases. This experimental model may allow studying the application of possible therapeutic strategies to prevent IPR. In this manner, the hypothermic treatment applied during the temporal window while asphyxia is induced seems to be an effective therapy, and could be translated into the clinics as an efficient tool to prevent the development of retinal neural damage and angiogenesis due to global hypoxia–ischemia.

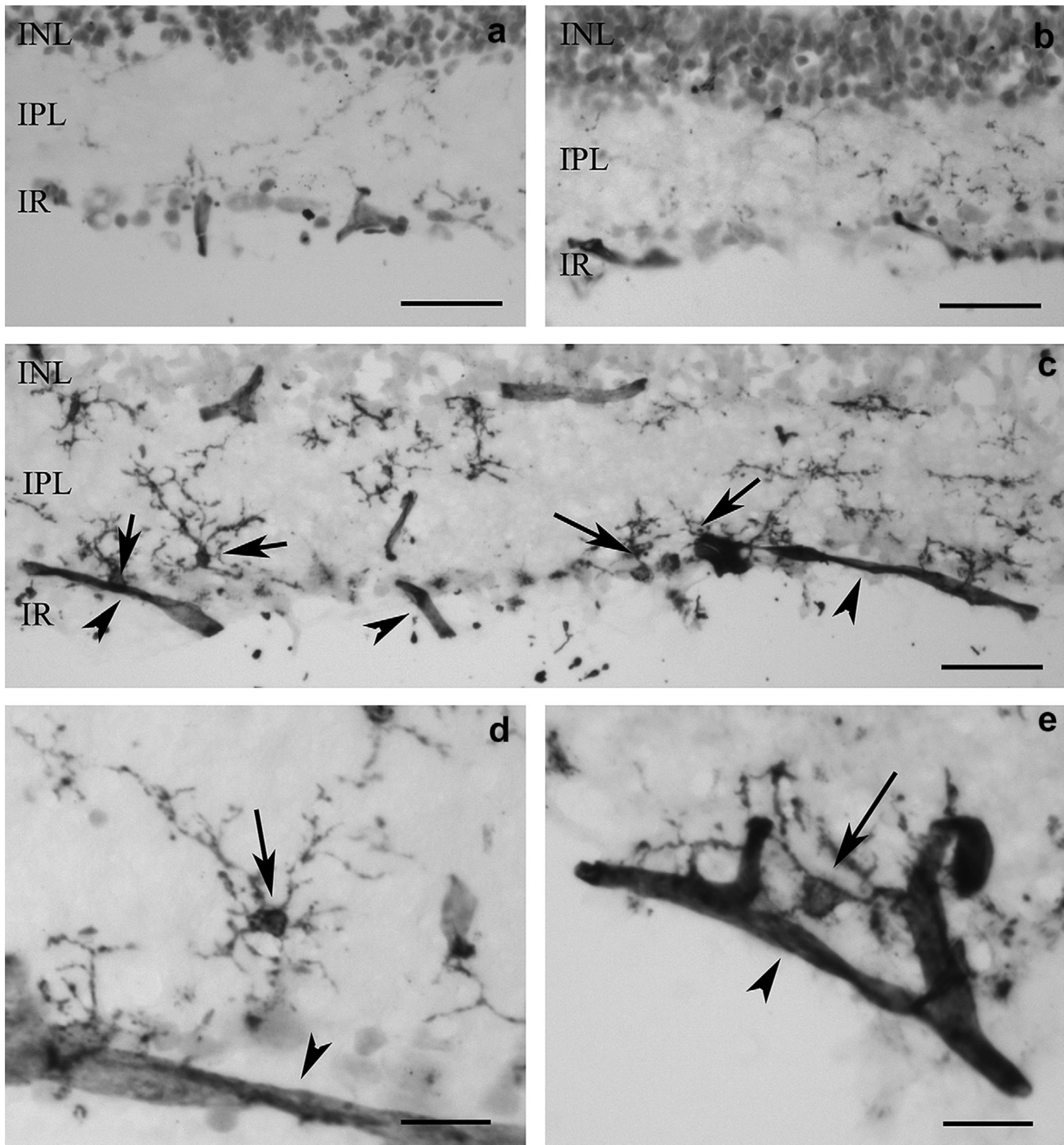

Fig. 5. Microglial alterations in the retina, induced by perinatal asphyxia. Tomato lectin staining of the inner layers of the retina of CTL (a), HYP (b) and PA (c–e). Note in the PA group the numerous reactive microglia cells (arrows) in the vicinity of vessels (head arrows), mainly distributed in the IR of PA retinas (c). Scale bars: a–c: 30 μm, d–e: 10 μm. INL: inner nuclear layer; IPL: inner plexiform layer; IR: inner retina.

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This work is dedicated to the memory of Dr. Cleto E.M. Rey (1932–2007).

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