Methylene blue prevents retinal damage in an experimental model of ischemic proliferative retinopathy


1Laboratorio de Neuropatología Experimental, Instituto de Biología Celular y Neurociencia “Prof. E. De Robertis”, Facultad de Medicina, Universidad de Buenos Aires, CONICET, Paraguay 2155 (C1428ABG), Ciudad Autónoma de Buenos Aires, Argentina.

2Angiogenesis Study Group, Center for Biomedical Research of La Rioja (CIBIR), 26006 Logroño, Spain.

3Primera Cátedra de Farmacología. Facultad de Medicina, Universidad de Buenos Aires, Argentina.

4Centro de Estudios Biomédicos, Biotecnológicos, Ambientales y Diagnóstico (CEBBAD), Universidad Maimónides, Hidalgo 775 (C1405BCK), Ciudad Autónoma de Buenos Aires, Argentina.

5Neurovascular Research Group, Department of Molecular, Cellular and Developmental Neurobiology, Instituto Cajal, CSIC, 28002 Madrid, Spain.

6Laboratorio de Neurociencia, Facultad de Ciencias Médicas, Universidad Católica de Cuyo. San Juan, Argentina.

These authors contributed equally to this work.
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* Corresponding Author: email: ilarrayoz@riojasalud.es (IML)
Abstract

Perinatal asphyxia induces retinal lesions, generating ischemic proliferative retinopathy which may result in blindness. Previously, we showed that the nitrergic system was involved in the physiopathology of perinatal asphyxia. Here we analyze the application of methylene blue, a well-known soluble guanylate cyclase inhibitor, as a therapeutic strategy to prevent retinopathy. Male rats (n=28 per group) were treated in different ways: 1) CTL (control) group comprised born to term animals; 2) PA group comprised rats exposed to perinatal asphyxia (20 min., at 37ºC); and 3) MB-PA group comprised animals born from pregnant rats treated with methylene blue (2 mg/kg) 30 and 5 min before delivery and then the pups were subjected to PA as above. mRNA was obtained at different times after asphyxia for molecular studies and tissue was collected at 30 days for morphological and biochemical analysis. Perinatal asphyxia produced significant gliosis, angiogenesis, and thickening of the inner retina. Methylene blue treatment reduced these parameters. Perinatal asphyxia resulted in a significant elevation of the nitrergic system as shown by NOS activity assays, Western blotting and (immuno)histochemistry for nNOS and NADPH-diaphorase activity. All these parameters were also normalized by the treatment. In addition, methylene blue induced the upregulation of the antiangiogenic peptide, PEDF. Application of methylene blue reduced morphological and biochemical parameters of retinopathy. This finding suggests the use of methylene blue as a new treatment to prevent or decrease retinal damage in the context of ischemic proliferative retinopathy.

KEYWORDS: retina; nitric oxide; ischemic proliferative retinopathy; methylene blue; angiogenesis.
Perinatal asphyxia (PA) is one of the most severe problem in Perinatology Services across the world (9; 15; 23; 27). PA generates a transient global hypoxia-ischemia status which damages the brain, spinal cord, and retina (14; 17; 39; 52). The degree and the length of perinatal asphyxia are decisive for the development of injury sequelae, such as attention-deficit hyperactivity disorder (1), epilepsy, mental retardation, spasticity, and visual or hearing alterations (61). One third of asphyctic neonates develop serious long-term neurological injuries including several degrees of ischemic proliferative retinopathy (IPR) and even blindness (20).

In rats, the retina is particularly sensitive to oxygen alterations (56), and the morphological changes observed in its inner layers are compatible with some alterations observed in human diseases such as retinopathy in diabetes, retinal vein occlusion, and retinopathy of prematurity (ROP), an avoidable cause of visual impairment and blindness in children (20; 61). The changes observed in asphyctic animals include ganglion cell degeneration, neovascularization of the most inner layers of the retina (the internal limiting membrane, the retinal nerve fiber layer, and the ganglion cell layer), and Müller cell hypertrophy in the inner layers of the retina (including the inner nuclear layer, the inner plexiform layer, the ganglion cell layer, the retinal nerve fiber layer, and the internal limiting membrane) (44). Therefore in rodents it makes sense to use the term ischemic proliferative retinopathy to describe these changes.

Several mechanisms have been involved in PA-induced neuronal damage in the retina. Nitric oxide (NO) is one of the most widespread intercellular messengers in the retina (13). NO synthase (NOS) is the enzyme responsible for catalyzing the oxidation of L-arginine, yielding equimolar amounts of NO and L-citrulline. There are two
constitutive Ca\textsuperscript{2+} dependent isoforms of NOS, endothelial (eNOS) and neuronal (nNOS), and one inducible isoform (iNOS) that is Ca\textsuperscript{2+} independent. nNOS and iNOS expression and activity have been described in the retina, spinal cord and brain of mammals (11; 45; 46; 63). PA results in high levels of NO, accompanied by alterations in NOS expression and activity (11; 28; 45). NOS immunolocalization in the retina has been confirmed in amacrine, horizontal cells, ganglion cells, Müller cells, photoreceptors and nerve fibers in the inner and outer plexiform layers of the retina (45; 59). NO reacts with the free radical superoxide exacerbating neurodegenerative processes through the formation of peroxynitrites, which promote protein nitration (47). In consequence, an increase in NO concentration in the retina induces cytotoxic effects (8) and NO is postulated as a key neurotoxic factor in ROP (38). Therefore, inhibition of NO actions may provide therapeutic opportunities.

Methylene blue (MB) is a soluble guanylyl cyclase inhibitor that was approved by the FDA as an antidote for the treatment of poison-induced methemoglobinemia because of its powerful antioxidant properties (58). Soluble guanylyl cyclase is regarded as the key enzyme in mediating NO-induced effects (22; 35). It was shown that MB inhibits NADPH oxidase and myeloperoxidase enzymes (19), by either competing for oxygen (3) or acting as a free radical scavenger. Co-administration of MB and rotenone was able to prevent changes in mouse visual function and retinal histopathology (48; 62).

One of the common findings in PA specimens is the induction of angiogenesis in the inner layers of the retina. Aberrant regulation of pro- and anti-angiogenic factors under ischemic conditions may be responsible for retinal neovascularization (10). Activation of hypoxia inducible factor (HIF-1) induces NOS transcription and expression of pro-angiogenic factors such as adrenomedullin (AM) and vascular
endothelial growth factor (VEGF). AM is involved in the induction of vasodilatation, regulation of cell proliferation, and angiogenesis (30). Under low oxygen conditions, transactivation of the AM promoter occurs through a HIF-1α mediated mechanism (16). In the brain, AM is secreted by neurons and glia (51), while we have recently described AM expression in the rat retina (43).

Increased levels of VEGF have been identified in models of retinal ischemia by us and others (21; 43). Hypoxia induces significant increases in VEGF expression in cells of the ganglion cell layer of the inner retina, and this increment would promote angiogenesis (43). VEGF is able to induce expression of matrix metalloproteinases type 2 (MMP2) and type 9 (MMP9), which correlates with the progression of neovascular diseases (18). MMPs expression, induced by hypoxia, down-regulates the action of the anti-angiogenic molecule, pigment epithelium–derived factor (PEDF) which is a cleavage substrate for MMPs (37). PEDF is the main anti-angiogenic and neurotrophic protein of the eye (10; 55).

The aim of the present work was to analyze the application of MB as a potential therapeutic strategy to prevent morphological and biochemical consequences of IPR in a rat model.

**Materials and methods**

**Hypoxic-ischemic injury animal model**

Severe perinatal asphyxia was induced using a noninvasive model of hypoxia-ischemia as described (29). Sprague-Dawley albino rats with genetic quality and sanitary certification from the animal facility of our Institution were cared for in accordance with
the guidelines published in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and in the NIH Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 85-23, revised 1985, available from: Office of Science and Health Reports, National Center for Research Resources, Bethesda, MD 20892), and the principles presented in the "Guidelines for the Use of Animals in Neuroscience Research" by the Society for Neuroscience (available from the Society for Neuroscience, Washington, DC; published in Membership Directory of the Society, pp. xxvii-xxviii, 1992). The animal model described below was approved in all its parts by the Ethical Committee of CICUAL: “Comité Institucional para el Uso y Cuidado de Animales de Laboratorio” (Resolution Nº 2079/07), Facultad de Medicina, Universidad de Buenos Aires, Argentina. Appropriate proceedings were performed to minimize the number of animals used and their suffering, pain, and discomfort. Animals were kept under standard laboratory conditions at 24°C, with light/dark cycles of 12/12 h, and food and water were given ad libitum. Fifteen timed-pregnant Sprague-Dawley rats were used. The first group consisted of normally delivered, non-manipulated pups which were used as normal controls (CTL, n=28 pups). In the second group, dams were intraperitoneal injected, 30 and 5 minutes before sacrifice, with saline solution (PA, n=28 pups). The third group was intraperitoneal injected with 2mg/kg methylene blue (Sigma, St. Louis, MO) at the same times (MB-PA, n=28 pups). A fourth group consisted of MB-treated pups that were not subjected to PA (MB-CTL, n=14 pups). Pregnant rats at term were sacrificed by decapitation and immediately hysterectomized. All full-term fetuses, still inside the uterus, were subjected to asphyxia by transient immersion of both uterine horns in a water bath for 20 minutes at 37°C. After asphyxia, the uterine horns were opened, pups were removed, dried of delivery fluids, stimulated to breathe, and their umbilical cords were ligated. The pups were then placed for
recovery under a heating lamp and given to a surrogate mother. Time of asphyxia was measured as the time elapsed from the hysterectomy up to the recovery from the water bath. The overall mortality rate for the PA group was 60%, similar to previous reports (29), whereas for the MB-PA group mortality was only 20%. To avoid the possible influence of hormonal variations due to the female estrous cycle, only male pups were included in this study.

Morphological study

Thirty day-old CTL, PA, and MB-PA rats (n=4 per group) were deeply anaesthetized by an intraperitoneal injection of 300 mg/Kg ketamine (Imalgene, Merial Laboratorios, Barcelona, Spain) + 30 mg/Kg xylazine (Xilagesic, Proyma Ganadera, Ciudad Real, Spain), and intracardially perfused with 4% paraformaldehyde in PBS. The eyes were postfixed in the same fixative for 24 h at 4°C, cryoprotected with 20% sucrose in PBS, embedded in OCT, and sectioned in a cryostat (Leica Biosystems, Germany). Frozen sections (15 µm thick) were stained with a monoclonal antibody against glial fibrillary acidic protein (GFAP) (1:500, Sigma) overnight at 4°C, exposed to a secondary antibody (1:300, FITC-anti mouse, Vector Laboratories, Burlingame, CA), and counterstained with DAPI (1:1000, Sigma). Images were acquired in a confocal microscope (TCS SP5, Leica). Other sections were stained with biotinylated *Lycopersicum esculentum* (tomato) lectin (1:150, Sigma) and revealed with an ABC kit (Vector Laboratories) followed by diaminobenzidine-nickel. Slides were counterstained with cresyl violet. To quantify the number of blood vessels, 2 anteroposterior sections intersecting the central area of the sagittal plane were chosen for each retina. Tomato
lectin-positive vessels present in the inner layer of the retina were counted in 4/6 areas, each 400 µm in length.

Constitutive NOS activity

Additional 30 day-old animals from the same 3 experimental groups (n=4 per group) were similarly anaesthetized with ketamine/xylazine, sacrificed by decapitation, and enucleated. Anterior segments of the eyes, including the lens, were discarded and the retinas dissected from the posterior segment, frozen and stored at -80°C until used. The biochemical activity of nNOS was determined measuring the conversion of L-(U-\textsuperscript{14}C)arginine into L-(U-\textsuperscript{14}C)citruline as described (41). Briefly, tissues were homogenized (1:3 w/v) at 4°C in HOSF buffer (20 mM HEPES, 0.2 M sucrose, 5 mM DTT, 1 mM ethylenediaminetetraacetic acid (EDTA), 10 µg/ml soybean trypsin, 10 µg/ml leupeptin, 2 µg/ml pepstatin, 0.1 mM PMSF, pH 7.4). Homogenates were sonicated, centrifuged for 30 minutes at 15,000 rpm (4°C) and supernatants collected. Protein concentration was determined by the Bradford method, using bovine serum albumin as standard. Supernatant aliquots were incubated for 20 minutes, at 37°C, with 20 µM L-(U-\textsuperscript{14}C)arginine in incubation buffer (50 mM KH\textsubscript{2}PO\textsubscript{4}, 0.2 mM CaCl\textsubscript{2}, 50 mM L-valine, 1 mM L-citruline, 1.5 mM DTT and 1 mM MgCl\textsubscript{2}, pH 7.4 with KOH 2N, plus 0.75 µg/ml NADPH, 2.15 µg/ml FMN, 3.74 µg/ml FAD and 1.412 µg/ml BH\textsubscript{4}). The reaction was stopped with addition of Dowex-50 WX8-400 ionic interchange resin and the resultant supernatant containing L-(U-\textsuperscript{14}C)citruline was quantified. To determine the activity of nNOS, the difference between the amount of (\textsuperscript{14}C)citruline produced in control samples and that in the samples processed in incubation buffer containing 2 mM ethylene glycol bis(\textbeta-aminoethyl ester)-N,N\textsuperscript{1}-tetra-acetic acid (EGTA) was calculated.
After anesthesia, CTL, PA, and MB-PA rats of 30 days of age (n=4 per group) were intracardially perfused with physiological solution followed by 4% paraformaldehyde in 0.1M pH 7.4 phosphate buffer at 4°C. The posterior segments of the eyes containing the retinas were post-fixed overnight in the same fixative at 4°C. Following cryoprotection with increasing sucrose concentrations (10%, 20% and 30%) in phosphate buffer saline at 4°C overnight, tissues were included in Tissue Tek®, frozen in powdered dry ice, and stored at –80°C. Sections (15 µm thick) were obtained using a Leitz “Lauda” cryostat and mounted onto gelatin coated slides (2.5% gelatine, 1% Elmer’s glue), air dried at room temperature and stored at –80°C until their use. Cryostat sections (thickness 18 µm) were mounted onto gelatin-coated slides and incubated with 0.1% b-NADPH (Sigma) and 0.02% nitroblue tetrazolium (Sigma) diluted in 0.1 M phosphate buffer (PB), pH 7.4, with 1% Triton X-100, for 1 hr at 37°C. As a negative control, b-NADPH was omitted in adjacent sections. NADPH-diaphorase reactivity was detected as a blue precipitate. Then, sections were washed in PB, dehydrated in an alcohol series, quickly cleared with xylol, and coverslipped.

**NOS immunohistochemistry**

For immunohistochemical detection of neuronal NOS (nNOS), slides were obtained as above and endogenous peroxidase activity was blocked with 1 % hydrogen peroxide in PB for 30 minutes. Then, sections were blocked with 10% normal goat serum in PBS, pH 7.4, for 1 h. Slides were incubated overnight (at 4°C) with a rabbit polyclonal anti-
nNOS antibody (1:3,000 dilution) produced at the Cajal Institute (46). The specificity of
the staining was corroborated in adjacent sections by omission of the primary antibody.
Immunoreactivity was visualized with biotinylated goat anti-rabbit IgG (1:100; Sigma),
developed with the ABC kit (Vector Laboratories) and 0.03% 3,3’-diaminobenzidine
(Sigma), 3% nickel ammonium sulphate, and 0.01% hydrogen peroxide diluted in 0.1
M buffer acetate, yielding a black product. Sections were dehydrated, cleared, and
mounted.

**SDS-PAGE and Western-blotting**

Retinas of 30 day-old rats of the 3 groups were dissected out (n=4 per experimental
group). Tissues were homogenized (1:3, w/v) in lysis buffer (see above) at 4°C.
Homogenates were centrifuged for 30 minutes at 15,000 x g and the supernatants
collected. Protein concentration was determined by the Bradford method, with bovine
serum albumin as standard, using a NanoDrop spectrophotometer (ND100). Then, 25
µg of each sample were mixed 1:1 with sample buffer (10 ml Tris–HCl 0.5 M, pH 6.8,
16 ml sodium dodecylsulphate (SDS) 10% (w/v), 8 ml glycerol, 2 ml 2-mercaptoethanol
and 0.2 ml bromophenol blue 0.1% (w/v)) and heated for 3 minutes at 95°C. Samples
were run on SDS–polyacrylamide gels (10% running gel with 3.5% stacking gel), with
0.25 M Tris–glycine, pH 8.3, as the electrolyte buffer, in a Bio-Rad Mini-Protein II
(Bio-Rad, Madrid, Spain). Kaleidoscope Prestained Standards (Bio-Rad) were used as
molecular weight markers. For Western blot analysis, proteins were transferred at 1.5
mA/cm² for 1 h onto 0.2-µm polyvinylidene difluoride (PVDF) membranes
(Immobilon-P, Millipore, Bedford, MA) by semi-dry transfer methods (Bio-Rad). For
protein identification, membranes were incubated overnight at 4°C with primary
antibodies (rabbit anti-nNOS at 1:1,000; rabbit anti-MMP2 from Abbiotec at 1:500;
mouse anti-MMP9 from Millipore at 1:500; or rabbit anti-PEDF from Bioproducts MD at 1:500). To standardize the results, a monoclonal IgG anti-β-actin antibody (Abcam) was used at a dilution 1:5,000 in the same membranes. To visualize immunoreactivity, membranes were incubated with anti-rabbit or anti mouse peroxidase- labeled IgGs, developed with a chemoluminiscence kit (GE Biosciences, Miami, U.S.A.), and exposed to X-ray blue films (CEA, Strängnäs, Sweden). Developed films were scanned with a computer-assisted densitometer (GS-800, Bio-Rad) and optical density quantified by NIH Scion Image software.

Zymograms

Retinas from 24 h-old animals of the CTL, PA, and MB-PA groups (n=4 per group) were homogenized in lysis buffer and total protein contents were quantified as above. Equal amounts of protein (50 μg) were loaded on each well of a 10% gelatine zymogram gel (Invitrogen, Carlsbad, CA, USA). After electrophoresis, the gel was incubated for 30 min in renaturing buffer and then in developing buffer for another 30 min, both at room temperature. Gels were incubated in developing buffer overnight at 37°C. Following several washes, the gels were stained with Simply Blue (Invitrogen), destained, and scanned with Odyssey (Li-cor, Lincoln, Nebraska, USA). Gelatinolytic activity for each enzyme (MMP2, 72 kDa; MMP9, 92 kDa) was quantified by image analysis (Image-J, NIH, Bethesda, MD, USA).

Image analysis

Six random retinal sections from 4 animals of each experimental group were analyzed. Care was taken on selecting anatomically matched areas of retina among animals before
assays. Slides were analyzed using an Olympus BH2 microscope (Olympus Optical Corporation, Tokyo, Japan) attached to a video camera (CCD Sony-XC77) coupled to a computer equipped with a video card (Data Translation). The central area of the sagital plane was chosen for each retina. Inner retina thickness, relative optical density (R.O.D.), immunoreactive cellular area (I.C.A.), and number of immunoreactive cells per surface unit were evaluated using the Scion Image software (developed by Wayne Rasband, 1995, NIH, Research Services Branch, NIMH, Bethesda, MD). Only those cells that had a grey level darker than a defined “threshold” (defined as the optic density 3-fold higher than the mean background density) were considered specific immunoreactively stained cells. The mean background density was measured in a region devoid of immunoreactive cells, immediately adjacent to the analyzed region. R.O.D. was calculated using a grey scale of 255 grey levels. To avoid external variations, all images were taken the same day and under the same light conditions. The number of cells was measured in retina segments of exactly 160 µm in length. Four segments were measured in each retinal section. Since immunohistochemistry and Western-blotting are semiquantitative techniques, R.O.D. values were expressed as % of change with respect to the CTL group considering CTL’s R.O.D. as 100%. Co-localization was studied by immunofluorescence using a Nikon C1 Plus laser microscope (Nikon Inverted Research Microscope Eclipse Ti, Nikon Corp., Tokyo, Japan) and images analyzed with the EZ-C1 software (EZ-C1 Software v3.9, Nikon Ltd.). Adobe Photoshop software (Adobe Photoshop CS5, Adobe Systems Inc., Ottawa, Ontario, Canada) was used for digital manipulation of only brightness and contrast when preparing the shown images.

RNA isolation and quantitative real time PCR (qRT-PCR)
At different times (2, 6, 12, 24, and 48h) after recovery from asphyxia, pups of the 3 experimental groups (CTL, PA, and MB-PA) (n=4 per group) were deeply anaesthetized with ketamine/xylazine, decapitated, and enucleated. The posterior segments of the eyes were frozen and stored at -80°C until used. Tissues were homogenized with TRIzol (Invitrogen, Madrid, Spain) and RNA was isolated with RNeasy Mini kit (Qiagen, Germantown, MD). Three µg of total RNA was treated with 0.5 µl DNaseI (Invitrogen) and reverse-transcribed into first-strand cDNA using random primers and the SuperScript III kit (Invitrogen). Reverse transcriptase was omitted in control reactions, where the absence of PCR-amplified DNA confirmed lack of contamination from genomic DNA. Resulting cDNA was mixed with SYBR Green PCR Master Mix (Invitrogen) for quantitative real time polymerase chain reaction (qRT-PCR) using 0.3 µM forward and reverse oligonucleotide primers (Table 1). Quantitative measures were performed using a 7300 Real Time PCR System (Applied Biosystems, Carlsbad, CA). Cycling conditions were an initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. At the end, a dissociation curve was implemented from 60 to 95°C to validate amplicon specificity. Gene expression was calculated using absolute quantification by interpolation into a standard curve. All values were divided by the expression of the house keeping gene 18S.

Statistical analysis

Values are expressed as mean ± standard deviation. At least two experimental repeats were evaluated in all cases. Twelve sections of each animal were analyzed. Results were evaluated using one-way analysis of variance (ANOVA) and comparisons between
groups were made by Fisher, Scheffe, and Bonferroni-Dunn tests, using the GraphPad software (GraphPad Software, San Diego, CA). Differences were considered significant when p<0.05.

Results

MB reduces asphyxia-induced inner retinal thickness, gliosis, and angiogenesis

Perinatal asphyxia induced an increase in inner retinal thickness (Fig. 1A,B,G), gliosis as revealed by GFAP staining (Fig. 1A,B), and retinal angiogenesis as shown by tomato lectin staining (Fig. 1D,E,H) when compared with non-asphyxiated control animals. Application of MB significantly reduced the increase in all these parameters (Fig. 1C,F,G,H).

nNOS isoform expression and activity is modulated by MB in adult rats

First, we investigated whether MB had any influence on regulating asphyxia-induced increased NOS activity. As previously shown (43), the retinas of animals subjected to perinatal asphyxia had higher constitutive NOS enzymatic activity than control animals (Fig. 2A). Similar results were obtained when studying nNOS protein expression in Western blots. The retina of asphyctic animals had higher levels of nNOS than control
rats (p<0.01) but the treatment with MB reduced constitutive NOS activity and nNOS expression even below control levels (Fig. 2A,B).

A morphometrical analysis was carried out studying both nNOS immunoreactivity and NADPH-diaphorase reactivity (an enzymatic activity of the NOS enzymes). In both cases, some ganglion cells and some amacrine cells stained positive (Figs. 3, 4). The number of nNOS immunoreactive cells was significantly higher in the PA group for both cell types, and MB prevented this increase in all cases (Fig. 3). The same pattern was observed with the NADPH-diaphorase staining (Fig. 4), which is quantified in Table 2.

Expression of angiogenesis regulating factors

The expression of several key regulators was studied over time. These included nNOS, iNOS, GFAP, the proangiogenic factors VEGF and AM, MMP2, MMP9, and the antiangiogenic factor PEDF. No changes were found in the expression of nNOS, iNOS, or GFAP, suggesting that the regulation of these gene products is post-transcriptional. Contrary to expectations, PA did not induce overexpression of VEGF or AM at any of the times tested (not shown). At the transcriptional level, there was a significant increase in MMP2 expression elicited by MB treatment at 6 h post asphyxia (Fig. 5A) but MMP2 gelatinolytic activity significantly decreased 2 h after asphyxia (Fig. 5B). MMP9 mRNA expression showed a marked increase in asphyctic animals at 6h and this was significantly attenuated by MB (Fig. 5C). No changes were observed in gelatinolytic activity for MMP9 at 2 h (Fig. 5D). When MMP expression was investigated by Western blotting, no significant changes were observed for either
MMP2 or MMP9 (Fig. 6A,B). Interestingly, the anti-angiogenic factor PEDF did not change by asphyxia but had a significant upregulation following MB treatment (Fig. 5E). This result was further confirmed by Western blotting (Fig. 6C).

Discussion

This work demonstrates that MB is an effective agent against a number of the deleterious effects of perinatal asphyxia in the retina; these benefits were accompanied by changes in NO, MMPs, and PEDF. Therefore, MB could be useful in reducing IPR development. Previously, we showed that NO increases in the retina 21 days after injury (45). Here we determined that application of MB inhibited NOS enzymatic activity, thus blocking the NO dependent neurotoxic cascade.

The most sensitive region of the eye to oxygen shortage corresponds to the most inner layers of the retina (38). In this region, we identified enhanced expression of nNOS in amacrine and ganglion cells following experimental asphyxia (45). Excessive production of NO could be highly toxic, combining with other reactive oxygen species to form peroxynitrite and destroying vital cell components (47). Here we have shown that MB can stop the whole cascade of events by preventing excessive NO formation.

Experimental therapeutic strategies against ROP described in the literature include the use of antioxidants (42), D-penicillamine (40), allopurinol (50),
indomethacin (36), dexamethasone (49), rofecoxib (57), and angiotensin-system modulators (12), among others. However, the most effective treatment to prevent retinopathy, so far, is hypothermia. A therapeutic trial employing hypothermia showed a significant decrease in brain tissue injury, and improved survival and neurological outcomes, at up to 18 months of age (4). Recently, we have reported that hypothermia is able to prevent gliosis and angiogenesis development in an experimental model of IPR (43). While both hypothermia and MB are effective in protecting the retina, the latter agent acts directly on the nitrergic system, competing with NO for binding to iron in soluble guanylate (34), preventing the increase of cyclic GMP and its negative effects as shown in cardiac and pulmonary tissues (2; 32; 60). NO is also related to angiogenesis by increasing survival of endothelial cells (64) and by being downstream of the VEGF signaling pathway (54). In addition, NO regulates MMP9 expression (31).

In our qRT-PCR data we found no changes in the expression of nNOS, iNOS or GFAP despite the fact that physiological changes were observed through NOS activity measurements or by immunostaining for GFAP. These apparent discrepancies can be explained by the time at which the mRNA was collected (from 2 to 48 h) and the time at which activity and immunohistochemistry were measured (30 days). A surprising observation was that the proangiogenic factors VEGF and AM did not show any upregulation in the PA group, despite the fact that both molecules are regulated by hypoxia and HIF-1α (16; 25). This may indicate that these factors do not play a relevant role in regulating PA-induced angiogenesis at these times. In previous studies, AM was elevated 7 days post hypoxia and VEGF 15 days post-hypoxia (43). In this context, the molecules that may also be regulating angiogenesis are the MMPs and PEDF. MMP2 was not affected by PA but we observed an initial downregulation of its gelatinolytic activity by MB at 2 h, followed by a mRNA upregulation at 6 h. For MMP9 we saw no
change at 2 h but a clear mRNA overexpression in the PA group at 6 h, which was significantly reduced in the MB group. There were no significant changes in MMP protein expression by Western blotting but the regulation of these proteins is complex and involves several other proteins such as TIMPs, among others. Thus we believe that the most relevant results are those obtained by zymography since they represent the quantitative gelatinolytic effects of these proteins. Both gelatinases are involved in angiogenesis (24) and the diminution of MMP2 gelatinolytic activity by MB treatment identifies this gelatinase as one of the main targets of MB in the retina. MMP2 could be responsible for the inhibition of fibrosis and angiogenesis in the inner retina of animals treated with MB. Another interesting behavior is the one protagonized by PEDF. The expression of this antiangiogenic peptide (37) did not change under asphyctic conditions but it was significantly upregulated by MB, even in the absence of asphyxia. The activation of PEDF may explain the antiangiogenic properties of MB since this peptide is the main angiogenesis inhibitor in the eye (7) and an MMP target (32).

MB is used in the clinic for several applications, including norepinephrine-refractory hypotension (53) and the surgical management of hyperparathyroidism (5). We have to take into consideration the difficulty of applying a preventative measure at the exact time of injury in a clinical setting. Nevertheless, although formal randomized clinical trials have not been performed on this drug, its safety profile is high (5; 26), so we can envision a scenario where MB is used as a therapeutic option in cases with a high risk of developing perinatal asphyxia (6; 33).

**Conclusion**
MB treatment reduced retinal damage originated by perinatal asphyxia. Since this compound has been previously used in the clinic with low to none side effects, clinical trials are warranted in cases with a high risk of developing perinatal asphyxia.

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Figure 1. Representative micrographs of the inner retina in rats of the experimental groups CTL (A,D), PA (B,E), and MB (C,F) immunostained with an antibody against GFAP (green, A-C) or with tomato lectin (D-F). Using these sections, the thickness of the inner retina (G) and the number of blood vessels per microscopic field (H) were quantified. INL: Inner nuclear layer; IPL: Inner plexiform layer; IR: Inner retina. Size bar = 50 μm. Bars represent the mean ± SD of all measurements (n=4 animals, 6
sections per animal). The asterisks represent statistically significant differences with the
CTL group. *: p<0.05; **: p<0.01.

**Figure 2.** Enzymatic activity for constitutive NOS (A) and Western blot analysis for
nNOS (B) in the retina of control animals (CTL), rats subjected to perinatal asphyxia
(PA), and animals treated with methylene blue before the asphyxia (MB). Western blots
were quantified by densitometry, and the nNOS values were normalized by β-actin.
Each bar represents the mean ± SD of all measurements (n=4). The asterisks represent
statistically significant differences with the CTL group. *: p<0.05; **: p<0.01.

**Figure 3.** Immunohistochemical staining for nNOS in the retina of control animals
(CTL), rats subjected to perinatal asphyxia (PA), and animals treated with methylene
blue before the asphyxia (MB). Amacrine (arrows) and ganglion (arrowheads) cells are
immunoreactive for nNOS. INL: Inner nuclear layer; IPL: Inner plexiform layer; IR:
Inner retina. Size bar = 25 µm. The number of nNOS-positive amacrine cells (AC) and
ganglion cells (GC) per field was quantified and is represented in a histogram. Each bar
represents the mean ± SD of all measurements (n=4 animals, 6 sections per animal).
The asterisks represent statistically significant differences with the CTL group. *:
p<0.05.

**Figure 4.** Histochemical staining for NADPH-diaphorase (arrows) in the retina of
control animals (CTL), rats subjected to perinatal asphyxia (PA), and animals treated
with methylene blue before the asphyxia (MB). INL: Inner nuclear layer; IPL: Inner plexiform layer. Size bar = 25 µm. Quantification of these data appears in Table 2.

**Figure 5.** Quantitative real time PCR analysis of the expression of MMP2 (A), MMP9 (C), and PEDF (E) and zymograms for MMP2 (B) and MMP9 (D) in the 3 experimental groups. All gene data were referenced to the value of the house keeping gene 18S in the same sample. Primer sequences can be found in Table 1. Each bar represents the mean ± SD of all measurements (n=4). The asterisks represent statistically significant differences as indicated. *: p<0.05; **: p<0.01.

**Figure 6.** Western blotting for MMP2 (A), MMP9 (B), and PEDF (C) was performed in retinas of the 4 experimental groups at 6 h after treatments. Each bar represents the mean ± SD of all measurements (n=6). The asterisks represent statistically significant differences as indicated. *: p<0.05.
Table 1. Primers used for qRT-PCR in this study

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>nNOS</td>
<td>GACAACGTTCCTGTGGTCTCT</td>
<td>TCCAGTGTGCTTCAGGTG</td>
</tr>
<tr>
<td>iNOS</td>
<td>AGGCCACCTCGGATATCTCTCT</td>
<td>GCTTGCTCTGGTGGTCTCTG</td>
</tr>
<tr>
<td>MMP2</td>
<td>ACCGTCGCCCATCATCAA</td>
<td>CCTTCAGCACAAGAGGGTTGC</td>
</tr>
<tr>
<td>MMP9</td>
<td>TGTCCAGACAAAGGTACAGC</td>
<td>GAAGAATGATCTAAGCCCAGCG</td>
</tr>
<tr>
<td>VEGF</td>
<td>GCCAGCACATAGGAGAGATGAGC</td>
<td>CAAGGCTCACAGTGATTTCAG</td>
</tr>
<tr>
<td>PEDF</td>
<td>ACCCTCGCATAGACCTTCAG</td>
<td>GGCATTCTCCCTGTAGACCG</td>
</tr>
<tr>
<td>AM</td>
<td>CAAGCAGAGCAGCTCTAGCA</td>
<td>GGTGAGCCAGTTTCTGCAAT</td>
</tr>
<tr>
<td>GFAP</td>
<td>GAAGAAAACCGCATCACCAT</td>
<td>GGCACACCTACATCACATC</td>
</tr>
<tr>
<td>18S</td>
<td>ATGCTTTAGCTGAGTGTCCCG</td>
<td>ATTCCTAGCTGCGGTATCCAG</td>
</tr>
</tbody>
</table>

Annealing temperature was 60°C for all primers. 18S was used as a house keeping gene.
Table 2. Morphometric parameters of NADPH-diaphorase positive amacrine cells.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of cells per field</th>
<th>Cellular area (µ²)</th>
<th>Cellular area (ROD)</th>
<th>Immunoreactive cellular area (µ²)</th>
<th>Immunoreactive cellular area (ROD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL</td>
<td>2.00 ± 0.20</td>
<td>84.52 ± 2.02</td>
<td>0.45 ± 0.16</td>
<td>54.01 ± 2.35</td>
<td>0.48 ± 0.07</td>
</tr>
<tr>
<td>PA</td>
<td>3.07 ± 0.18 (*)</td>
<td>95.14 ± 1.52 (*)</td>
<td>0.60 ± 0.05 (*)</td>
<td>68.68 ± 1.89 (*)</td>
<td>0.63 ± 0.04 (*)</td>
</tr>
<tr>
<td>MB</td>
<td>1.49 ± 0.16 (*)</td>
<td>89.14 ± 2.71</td>
<td>0.48 ± 0.13</td>
<td>55.22 ± 3.48</td>
<td>0.49 ± 0.36</td>
</tr>
</tbody>
</table>

ROD: Relative optical density. Asterisks represent statistically significant differences with CTL. *: p<0.05.
A, B, C: Comparative images of inner retina layers (INL, IPL, IR) under different conditions (CTL, PA, MB-PA).

D, E, F: Microscopic sections demonstrating cellular structures in the inner retina.

G: Bar graph showing inner retina thickness: CTL, PA, MB-PA.

H: Bar graph showing vessels per field: CTL, PA, MB-PA.