THE POST-TREATMENT WITH MELATONIN ATTENUATES EXPERIMENTAL OCULAR INFLAMMATION

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Abbreviations: BOB, blood-ocular barrier; COX-2, cyclooxygenase-2; ERG, electroretinogram; EIU, endotoxin-induced uveitis; GCL, ganglion cell layer; GFAP, glial fibrillary acidic protein; IPL, inner plexiform layer; LPS, bacterial lipopolysaccharide; MDA, malondialdehyde bis-dimethyl acetal; NOS, nitric oxide synthase; ONL, outer nuclear layer; OPL, outer plexiform

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layer; PG, prostaglandin; PS, photoreceptor outer and inner segment; TBARS, thiobarbituric acid reactive substances; TNFα, tumor necrosis factor α

SUMMARY

Background and purpose: Uveitis is a prevalent intraocular inflammatory disease and one of the most damaging ocular conditions. Previously, we showed that melatonin prevents ocular inflammation induced by an intravitreal injection of bacterial lipopolysaccharide (LPS) in the Syrian hamster. With this background, the aim of this work was to assess the anti-inflammatory effect of melatonin administered after the onset of ocular inflammation.

Experimental approach: Eyes were intravitreally injected with vehicle or LPS. Melatonin was intraperitoneally supplied every 24 h, starting 12 h or 24 h post-LPS injection. A clinical evaluation (with a score index based on clinical symptoms), the number of infiltrating cells, protein concentration, and prostaglandin (PG) E2 and PGF2α levels in the aqueous humor, as well as retinal nitric oxide synthase (NOS) activity, lipid peroxidation and TNFα levels were assessed. Retinal function was assessed by scotopic electroretinography, and light microscopy and immunohistochemistry were used to evaluate the retinal structure.

Key results: Both post-treatments with melatonin significantly decreased clinical symptoms, reduced the leakage of cells and proteins, and decreased PG levels in aqueous humor from eyes injected with LPS. In addition, melatonin avoided the decrease in scotopic electroretinogram a- and b-wave amplitude, protected the retinal structure, and reduced the increase in NOS activity lipid peroxidation, and TNFα levels induced by LPS.

Conclusions and Implications: These results indicate that a post-treatment with melatonin significantly attenuated ocular inflammation induced by LPS in the Syrian hamster, and support the use of melatonin as a therapeutic resource for uveitis treatment.

INTRODUCTION

Uveitis is an ocular condition characterized by intraocular inflammation caused by several different etiologies, and is a significant cause of visual loss (Dick, 2000; Durrani et al., 2004), with high prevalence worldwide (London et al., 2010). Various chemical mediators play key roles in ocular inflammation, including nuclear factor-κB, pro-inflammatory cytokines (Commodaro et al., 2010), oxidative and nitrosative stress (Wu et al., 1997), and prostaglandins (PGs) (Sand and Krogh, 1991), among others.

Unraveling which are the most critical mechanisms is unlikely to be achieved in studies which are limited to the clinically observable ocular changes that are seen in human uveitis. Far more detailed and invasive studies are required, preferably in a readily available animal model. Endotoxin-induced uveitis (EIU), a widely recognized experimental model of acute ocular inflammation (Rosenbaum et al., 1980) which mimics the central features of human uveitis, is induced by systemic or local injection lipopolysaccharide (LPS), a Gram-negative bacterial cell wall component. LPS induces the breakdown of the blood-ocular barrier (BOB), leukocyte infiltration, and enhances the expression of various inflammatory mediators, such as PGs (Bellot et al., 1996), NO (Bellot et al., 1996; Mandai et al., 1994), tumor necrosis factor-α (TNFα), and interleukin 6 (De Vos et al., 1994), all of which contribute to the development and progression of the disease. Moreover, oxidative biomarkers are shown to be elevated in EIU (Wu et al., 1997), suggesting that inflammation and oxidative stress cooperatively contribute to its pathogenesis. Even though EIU was generally considered as a model of anterior uveitis, various studies show that it also involves the inflammation of the retina (Miyamoto et al., 1996). We have demonstrated that in the Syrian hamster, an intravitreal injection of LPS provokes a significant decrease in the scotopic electroretinogram (ERG) which correlates with focal destruction of photoreceptors, alterations in Müller cells, and retinal infiltration (Sande et al., 2008), supporting that at least in the Syrian hamster, EIU is a panuveitis model.

Present pharmacological treatment for uveitis primarily includes corticosteroids and nonsteroidal anti-inflammatory drugs. However, these drugs do not completely control the disease in many patients, and in long term application, they may result in multiple adverse effects such as cataract, glaucoma, susceptibility to microbial infection, and nephrotoxicity (El Afrit et al., 2007; McGhee et al., 2002; Nguyen et al., 2006). Hence, there is a need for therapeutics with safer modes of action. In that context, much attention has been paid to a variety of candidates as ocular anti-inflammatory agents. Most of these treatments which were administered before or immediately after the injection of LPS were effective in the prevention of the ocular inflammation induced by LPS. In this vein, it was demonstrated that oral supplementation of resveratrol for 5 days until LPS injection (Kubota et al., 2009), ginkgo biloba extract (Ilieva et al., 2004) or fucoxanthin administered immediately after LPS injection (Shiratori et al., 2005), as well as lutein or dexamethasone intravenously administered at 30 min before, at the same time, and at 30 min after LPS treatment (Jin et al., 2006) significantly prevent ocular inflammation. However, the utilization of these treatments in a clinical setting is mostly limited because the onset of uveitis is largely unpredictable. In contrast, therapeutic strategies that are effective when administered after the onset of the inflammatory process may have more translational relevance as therapeutic resources for uveitis treatment.

Melatonin is an endogenous neuromodulator in the retina of vertebrates (Tosini et al., 2012), including Syrian hamsters (Faillace et al., 1995). Several lines of evidence support that melatonin may act as a protective agent in ocular conditions such as photokeratitis, cataract, retinopathy of prematurity, and ischemia/reperfusion injury (Rosenstein et al., 2010; Siu et al., 2006). Moreover, we have recently shown that melatonin prevents retinal glaucomatous (Belforte et al., 2010), and diabetic (Salido et al., 2013) damage. In previous reports, we have demonstrated that melatonin significantly prevents the biochemical, clinical, histological, and functional alterations induced by EIU in Syrian hamsters (Sande et al., 2008) and cats (Del Sole

et al., 2012). The above observations prompted us to evaluate the anti-inflammatory effect of melatonin administered after the onset of ocular inflammation induced by a single intravitreal injection of LPS in Syrian hamsters. The results indicate that a post-treatment with melatonin significantly attenuated ocular inflammation induced by LPS in the Syrian hamster, and support the use of melatonin as a therapeutic resource for uveitis treatment.

METHODS

Test systems used

Male Syrian hamsters (average weight 120 ± 20 g), derived from a stock supplied by Charles River Breeding Laboratories (Wilmington, MA, U.S.A.), were kept under a 14 h light: 10 h dark lighting schedule (lights on at 06.00 h), with free access to food and water. A total number of 240 animals were used for the experiments, as follows: 14 animals/ group for assessment of the clinical score, electroretinogram (ERG), and retinal histology; 12 animals/group for the assessment of infiltrated cell number, protein concentration and prostaglandin level in aqueous humor, 4 animals/group for the immunohistochemical study, 10 animals/group for NOS activity assessment, 10 animals/group for lipid peroxidation assessment, and 10 animals/group for TNFα level assessment. The experimental procedures reported here were approved by the Animal Care Committee of the Center for Pharmacological and Botanical Studies, National Research Council, and by the Institutional Committee for the Care and Use of Laboratory Animals (CICUAL) from the School of Medicine, University of Buenos Aires.

Experimental design

Syrian hamsters were anesthetized with ketamine hydrochloride (150 mg/kg) and xylazine hydrochloride (0.5 mg/kg) administered intraperitoneally. A drop of proparacaine (2 %) was administered in each eye for local anaesthesia. With a syringe (Hamilton, Reno, NV, USA) and a

30-gauge needle, 2 µL of lipopolysaccharide (LPS, 1.5 mg/mL in sterile pyrogen-free saline) from *Salmonella typhimurium* were injected into one eye of anesthetized hamsters. Other group of hamsters was intravitreally injected with vehicle (in sterile pyrogen-free saline) into one eye, as previously described (Sande et al., 2008). Injections were applied at 1 mm near the limbus, and the needle was left in the eye for 60 seconds to allow aqueous humor to flow out; this small volume prevented the increase of intraocular pressure and the volume loss. Intravitreal injections of vehicle or LPS were performed at 8 a.m. Melatonin (10 mg/kg) or vehicle (9% ethanol in saline solution) was intraperitoneally administered for 8 days, every 24 h, starting 12 h (Mel 12 h) or 24 h (Mel 24 h) after intravitreal injections. The dose of melatonin was selected on the basis of previous reports (Ortiz et al., 1999; Crespo et al., 1999; Chen et al., 2006). Animals were randomized into 4 experimental groups: Group 1 (control): animals received an intraviteral injection of vehicle (sterile saline solution) and daily intraperitoneal injections of vehicle (9% ethanol in normal saline), starting 12 h after the intravitreal injection; Group 2 (vehicle): animals received an intravitreal injection of LPS and daily intraperitoneal injections of vehicle (9 % ethanol in normal saline), starting 12 h after the intravitreal injection; Group 3 (Mel 12 h): animals received an intravitreal injection of LPS and daily intraperitoneal injections of melatonin, starting 12 h after LPS injection; and Group 4 (Mel 24 h): animals received an intravitreal injection of LPS and daily intraperitoneal injections of melatonin, starting 24 h after LPS injection. For Group 3, melatonin was daily injected at 8 p.m., while for Group 4, melatonin was daily injected at 8 a.m. In the present study, eyes intravitreally injected with vehicle served as the control group because in preliminary studies we found that in comparison with intact animals, the injection of vehicle did not affect retinal function and histology (data not shown).

Measurements made

Clinical score

Different signs were considered when examining hamster eyes after the intravitreal injection of vehicle or LPS, as previously described (Sande et al., 2008). Clinical severity of these signs was graded on a scale from 0 to 14, as follows: degree of conjunctival (0 to 3) and episcleral hyperemia (0 to 3), degree of cornea inflammation (0 to 3), degree of alteration of iris and pupil (vasodilatation, synechia, presence of exudates at the pupil rim, and degree of miosis) (0 to 3). The absence (0) or presence of a cataract was scored 1 or 2 (less or more than 50% of the lens surface, respectively). The animals were examined by a masked observer in a random order at 24, 36, 48 and 72 h post-injection of vehicle or LPS, before the new injection of melatonin.

Integrity of the blood retinal barrier

Animals were euthanized and the aqueous humor was collected immediately from each eye by anterior chamber puncture using a 30-gauge needle under a surgical microscope. Infiltrated cell number and protein concentration in aqueous humor samples obtained at 36 h after the injection of vehicle or LPS were assessed as previously described (Sande et al., 2008). For cell counting, aqueous humor samples were suspended in four amounts of Türk solution, and cells were counted with a haemocytometer under an optical microscope. The number of cells per field was manually counted, and the number of cells per microliter was obtained by averaging the results of four fields from each sample. Protein concentration in the aqueous humor was determined by the method of Lowry et al. (1951), using bovine serum albumin as the standard.

Assessment of PG levels

PGE2 and PGF2α level determination was performed by radioimmunoassay, as previously described (de Zavalía et al., 2010). Briefly, aliquots of aqueous humor (1 μl) were preincubated with a specific antiserum (1: 20 for both anti-PGE2 and anti-PGF2α) for 45 min at 4ºC. Then,

[^{3}H]-PGE2 or [^{3}H]-PGF2α (20,000 dpm) were added and incubated for 1 hour at 4ºC. PG levels were obtained from a standard curve, with an assay limit sensitivity of 1 pg per tube.

Electroretinography

Electroretinograms (ERGs) were recorded as previously described (Sande et al., 2008). Eight days after intravitreal injections, full field ERGs were recorded in anesthetized hamsters following 6-h dark adaptation. Pupils were dilated with 1% tropicamide and 2.5% phenylephrine (Alcon Laboratories, Buenos Aires, Argentina), and the cornea was irrigated with balanced salt solution (Alcon Laboratories, Buenos Aires, Argentina) to prevent keratopathy. Hamsters were placed in a Ganzfeld light stimulator, and recordings were completed within 20 minutes of the induction of anesthesia. A gold ring-shaped electrode was placed in contact with the central cornea; a reference needle electrode was placed through the ear, and a ground electrode was attached to the back of the head. Electrode placement was performed under a 15-W red light, which did not affect dark adaptation and was switched off during the recordings. Both eyes were recorded simultaneously and the response to one flash of unattenuated white light (4 ms) from a photic stimulator set at maximum brightness (5 cd s/m^2 without filter) was amplified (gain set at 100), filtered (1.5-Hz low-pass filter, 1000 Hz high-pass filter), notch activated, and registered with an Akonic BIO-PC device (Akonic, Buenos Aires, Argentina). Each recording started simultaneously with the application of the stimulus. Amplitudes and latencies of a- and b-waves were then measured and analyzed, as previously described (Sande et al., 2008).

Histological examination

Hamsters were euthanized at 8 days after intravitreal injections. Eyes were immediately enucleated and stored in phosphate-buffered saline with 4% formaldehyde for 24 - 48 h. Eyes were then dehydrated, embedded in paraffin, sectioned with a microtome at 5-µm thickness.

Each section was cut along the vertical meridian of the eye near the optic nerve head. The retinal layer thickness (in μm) for each eye was measured in the central and peripheral retina. The ganglion cell layer (GCL) number (cells per 200 μm) was assessed, without distinction of cell types. Measurements were obtained at 0.5 mm dorsal and ventral from the optic disc (central retina), and at 0.5 mm from the *ora serrata* (peripheral retina). Results obtained from five separate sections for each eye were averaged and the mean of five eyes was recorded as the representative value for each group. Microscopic images were digitally captured with a Nikon Eclipse E400 microscope (illumination: 6-V halogen lamp, 20W, equipped with a stabilized light source) via a Sony SSC-DC50 camera. Sections were stained with hematoxylin and eosin and analyzed by a masked observer.

Immunohistochemical study

Immunodetection of retinal glial cells was performed as previously described (Sande et al., 2008; Dorfman et al., 2013). Briefly, sections underwent heat-induced antigen retrieval by heating at 90ºC for half an hour, and then were preincubated with 2% normal horse serum followed by 0.4% Triton X-100 in 0.01 M PBS for 1 h. Afterwards, sections were incubated overnight at 4ºC with a mouse monoclonal anti- glial fibrillary acidic protein (GFAP) antibody conjugated to Cy3 (1:1200; Sigma Chemical Co., St. Louis, MO, USA). An Olympus BX50 microscope was used for microscopic observations.

Measurement of thiobarbituric acid reactive substances (TBARS) levels

Retinal lipid peroxidation was evaluated by measuring malondialdehyde bis-dimethyl acetal (MDA) levels, as previously described (Moreno et al., 2004). Retinas were homogenized in 300 μl of 15 mM potassium buffer plus 60 mM KCl (pH 7.2). Homogenates were mixed with 75 μl 10% SDS and 1.4 ml 0.8% thiobarbituric acid in 10% acetic acid (pH 3.5), and heated to 100°C for one hour. After cooling, samples were centrifuged at 3,200 *g* for 10 min and the flocculent

precipitate was removed. After addition of 1.0 ml water and 5.0 ml of n-butanol-pyridine mixture (15:1, vol/vol), the mixture was shaken and centrifuged at 2,000 *g* for 15 min. Absorbance of the organic phase was measured using a Jas co FP 770 fluorescence spectrophotometer (Japan Spectroscopic Co. Ltd. Tokyo, Japan), with at an emission λ of 553 nm and an excitation λ of 515 nm. The amount of TBARS was determined according to a standard calibration curve generated from MDA.

NOS activity assessment

Animals were sacrificed by decapitation at 36 h after vehicle or LPS injections, and NOS activity was assessed as previously described (Sáenz et al., 2002). Retinas were homogenized in buffer (100 µl) containing 0.32 mol/L sucrose and 0.1 mmol/L EDTA, (pH 7.4). Reaction mixtures contained 50 µl of the enzyme source and 50 µl of a buffer stock solution (final concentrations: 10 mM HEPES, 3 mM $CaCl_2$, 1 mM NADPH, 5 µM FAD, 1 mM β-mercaptoethanol, L-[^{3}H]-arginine, (5 µCi/ml), and 1µM L-arginine). The reaction was stopped after incubation at 37°C for 30 minutes, by adding buffer containing 50 mM HEPES, 10 mM EDTA, and 10 mM EGTA, pH 5.5, and cooling the tubes for 5 minutes. The solution was mixed with 600 µl of resin Dowex AG50W-X8 (Na^+ form) to remove L-arginine, and centrifuged at 10,000 x g for 5 minutes. L-[^{3}H]-citrulline in the supernatant was quantified by liquid scintillation counting. Nonenzymatic conversion of L-[^{3}H]-arginine to L-[^{3}H]-citrulline was tested by adding buffer instead of the enzyme source.

Assay for TNFα assessment

Retinal TNFα levels were assessed as previously described (Salido et al., 2013). Retinas were homogenized in buffer containing 20 mM imidazole hydrochloride, 100 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 1% Triton, 1 mM sodium molybdynate, 10 mM NaF, 1 mM EDTA, and a cocktail

of protease inhibitors. Homogenates were centrifuged for 10 min at 13,000 rpm, and TNFα levels were assessed in the supernatant using an enzyme-linked immunosorbent assay (ELISA) kit (TNF (Mono/Mono) ELISA Set of BD Biosciences Pharmingen, San Diego, USA), according to the manufacturer's instructions, and measured in an ELISA reader at 450 nm.

Data analysis and statistical procedures

Statistical analysis of results was made by a two-way analysis of variance followed by a Tukey´s test, or by a Mann-Withney U-test, as stated.

Drugs, chemicals reagents and other materials

LPS, melatonin, bovine serum albumin, PGE2, PGF2α, arginine, anti-GFAP antibody, and malondialdehyde bis-dimethyl acetal were obtained from Sigma Chemical Co. (St Louis, MO, USA). [^{3}H]-PGE2, [^{3}H]-PGF2α and L-[^{3}H]-arginine were purchased from New England Nuclear Corp. (Boston, MA, USA), while Dowex was from Bio-Rad Laboratories (Richmond, CA, USA).

RESULTS

Clinical score

The time-course (24 h -72 h) of the clinical severity scores in eyes injected with vehicle or LPS from animals untreated or treated with melatonin is shown in Figure 1. At 36, 48, and 72 (but not 24) h post-injection of LPS, both treatments with melatonin (Mel 12 h and Mel 24 h) significantly decreased the average clinical score induced by LPS, in a similar manner. In eyes intravitreally injected with vehicle from animals untreated with melatonin (control), no significant differences in the clinical score were observed along the study. In vehicle-injected eyes, melatonin did not affect the clinical score (data not shown). Representative images of

Syrian hamster eyes submitted to these treatments are shown in the lower panel of Figure 1. At 36 h after the intravitreal injection of LPS, signs of uveitis, such as episcleral vessel congestion, miosis, iris swelling, and synequiae were observed in animals untreated with melatonin, whereas both treatments with melatonin decreased the occurrence of inflammatory signs.

Integrity of the BOB and PG level assessment

The integrity of the BOB was examined by the assessment of cell count and protein concentration in the aqueous humor at 36 h after the injection of vehicle or LPS. LPS induced a significant increase in these parameters (Figure 2A and 2B, respectively), whereas both treatments with melatonin significantly reduced cell number and protein content in aqueous humor from hamster eyes injected with LPS, with a similar efficacy. Both treatments with melatonin significantly reduced the increase in PGE2 and PGF2α levels in aqueous humor induced by LPS, as shown in Figure 3A and 3B, respectively.

Retinal function

The average amplitudes of scotopic ERG a- and b- waves, as well as representative scotopic ERG traces from Syrian hamster eyes, are shown in Figure 4. At 8 days post-injection, LPS decreased scotopic ERG a- and b-wave amplitude, whereas both treatments with melatonin significantly reversed the effect of LPS. The ERG a- and b-wave latency did not differ among groups, as shown in Table 1.

Retinal histology

Figure 5 shows the histological analysis performed at 8 days after the intravitreal injection of LPS. Retinal cell infiltration, hemorrhage, and structural disorganization were observed in animals untreated with melatonin, as shown in Figure 5, whereas the daily treatments with

melatonin starting 12 h, or 24 h post-injection of LPS, preserved the retinal structure and decreased cell infiltration in a similar extent. In the absence of melatonin, LPS induced a decrease in retinal layer thickness at the center and periphery, whereas both treatments with melatonin (Mel 12 h and Mel 24 h) decreased the effect of LPS on these parameters, as shown in Table 2. Retinal immunoreactivity for GFAP was analyzed at 8 days after the intravitreal injection of vehicle or LPS in animals untreated or treated with melatonin. The injection of LPS induced an increase in retinal GFAP levels which was reduced by both treatments with melatonin, as shown in Figure 6.

Lipid peroxidation, NOS activity and TNFα levels

Retinal lipid peroxidation, NOS activity and TNFα levels were assessed at 36 h after vehicle or LPS injection in animals untreated or treated with melatonin. LPS increased these parameters, whereas both treatments with melatonin significantly abrogated the effect of LPS on retinal TBARS levels (Figure 7A), NOS activity (Figure 7B), and TNFα levels (Figure 7C) .

DISCUSSION

The present results indicate that a treatment with melatonin after the intravitreal injection of LPS attenuated ocular inflammation in Syrian hamsters. Although more studies are needed to establish the optimal dose of melatonin, a daily treatment with 10 mg/kg melatonin, starting 12 h or 24 h after LPS significantly reduced anterior segment (clinical signs, inflammatory cells, protein concentration, and PG levels in aqueous humor), and posterior segment alterations (ERG, retinal structure, Müller cell GFAP levels, retinal lipid peroxidation, NOS activity, and TNFα levels). Future work will be performed in order to analyze the dose-response relationship for the ocular anti-inflammatory effect of melatonin. In agreement with previous reports (Del Sole et al., 2012; Sande et al., 2008), the present results further confirm the role of melatonin as an ocular

anti-inflammatory, in this case, by showing that even when administered after the challenge with LPS, melatonin was effective in counteracting the uveitis-inducing effects of LPS. These findings are entirely consistent with the fact that melatonin is also a potential therapeutic resource for neurological diseases (e.g., Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and ischemic stroke) that are involved into the inflammation pathogenesis (reviewed by Wang, 2009).

Melatonin was administered under two different daily regimens: one starting at an early phase (i.e., at 12 h post-LPS), a time point at which clinical signs of inflammation were incipient, and the other one, starting at a late phase (i.e., at 24 h after LPS), at which a maximal clinical score was already evident. Both treatments with melatonin significantly reduced the clinical signs, and preserved the BOB integrity (as shown by its effect on protein concentration and the number of infiltrated cells in the aqueous humor). PGs are inflammatory mediators that cause the breakdown of the BOB during EIU. In fact, cyclooxygenase-2 (COX-2) inhibitors have been reported to inhibit inflammatory reactions in various animal models of ocular anterior segment inflammation (Barañano et al., 2009; Kulkarni, 1991). The present results suggest that the effect of melatonin on the anterior segment inflammation, and on BOB integrity preservation could be mediated by decreasing the effect LPS on PG aqueous humor content. In agreement, several reports support that melatonin inhibits COX-2 activity and expression (Franchi et al., 1987; Lim et al., 2012). In addition to its effect on the anterior segment inflammatory process, melatonin also protected the retinal function and histology from the deleterious effect of LPS-induced inflammation. Müller cells that do not express GFAP under physiological conditions are known to express GFAP in several pathological situations, such as retinal ischemia and diabetic retinopathy (Dorfman et al., 2013; Salido et al., 2013), among others. The present results indicate that the injection of LPS provoked a significant alteration in retinal Müller cells, as shown by an increase in GFAP immunoreactivity that was reduced by both treatments with melatonin.

Electroretinography is one of the most reliable methods for objectively evaluating retinal function. As shown herein, both treatments with melatonin avoided the decrease in scotopic ERG a- and b-wave amplitude observed at 8 days post-injection of LPS. Since the a-wave of the flash ERG is classically thought to represent photoreceptor activity, whereas the b-wave reflects inner retinal (bipolar and Müller cells) functions, the protection induced by melatonin could be a panretinal phenomenon. In fact, both post-treatments with melatonin preserved the retinal structure, and reduced GFAP up-regulation in Müller cells induced by LPS in the Syrian hamster retina.

Oxidative stress, increased NO and cytokines production induced by LPS are major triggering factors for ocular inflammation and tissue damage during inflammatory processes (Wang et al., 1996; Yadav et al., 2011). In fact, antioxidants (Sasaki et al., 2009), selective iNOS inhibitors (Goureau et al., 1995), and etanercept (a soluble TNFα receptor) (Avunduk et al., 2004) protect the visual function during retinal inflammation.

Melatonin is one of the most powerful natural free radical scavengers (Galano et al., 2011) and stimulates antioxidant enzymes in several tissues including the retina (Rodriguez et al., 2004; Rosenstein et al., 2010). In addition to the potent antioxidant property of melatonin by itself, some of its metabolites are themselves direct free radical scavengers increasing melatonin´s antioxidant capacity (Hardeland et al., 2009). We have previously shown that melatonin significantly decreases retinal NOS activity, and L-arginine uptake in the Syrian hamster (Sáenz et al., 2002), and it is able to directly scavenge NO (Turjanski et al., 2001). Moreover, as shown herein, melatonin decreased the effect of LPS on a key signal involved in inflammatory processes, such as TNFα. Thus, available evidence supports that manipulation of intracellular redox status using antioxidants, decreasing NO levels, inhibiting TNFα effect or preferably the combination of these treatments, may protect the retina against uveitic damage. In that sense, melatonin could be a promissory resource in the management of ocular inflammation, since by

itself, it exhibits antioxidant and antinitridergic properties, and reduced TNFα levels, particularly at retinal level.

As shown herein, delaying initial treatment with melatonin to 24 h after LPS resulted in a similar protection to that obtained when the treatment with melatonin started at 12 h post-injection of LPS. We do not have any clear explanation for these results; however, these observations support that melatonin could be potentially effective in patients with fully established uveitis, since it was capable of suppressing actively ongoing inflammatory responses. In contrast, in spite of the well-known efficacy of steroidal therapy in uveitis patients, it was shown that dexamethasone is not capable of ameliorating the clinical course of EIU in rats when administered 12 h or 16 h after LPS (Mangano et al., 2008).

Like melatonin, various therapeutic agents have been reported in the past to prevent the inflammatory response in EIU. However, prescription-based use of many of these agents, such as guggulsterone (Kalariya et al., 2010), ethyl pyruvate (Kalariya et al., 2011), lutein (Jin et al., 2006), and astaxanthin (Suzuki et al., 2006) in humans for the treatment of uveitis has not materialized for various reasons. Prominent reasons that limit their use in humans are lack of toxicity studies, lack of knowledge on safe dosage range, and potential toxicity on higher therapeutic dosage. Moreover, the conventional treatments of intraocular inflammation may have a wide range of significant side effects. In this vein, a treatment with melatonin could overcome these issues, as it has been extensively studied and found to be safe for human use (Jan et al., 2000; Malow et al., 2012). The efficacy, clinical safety, and low cost of melatonin could make it an ideal candidate as a pharmacologic agent for uveitis treatment.

Statement of conflicts of interest

None

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Author Contributions

P H Sande: intravitreal injections, clinical score, electroretinography, animal manipulation, aqueous humor measurements, contributions to concept/design.

D Dorfman: histological studies, immunohistochemistry, TNFα assessment.

D C Fernandez: histological studies.

M Chianelli: aqueous humor measurements, NOS activity, lipid peroxidation.

A P Domínguez Rubio: TNFα assessment.

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D M Silberman: NOS activity, lipid peroxidation.

R E Rosenstein: contributions to concept/design, discussion of results, drafting of the manuscript

D A Sáenz: clinical score, contributions to concept/design, discussion of results, approval of the article.

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LEGENDS

Figure 1. Upper panel: Average clinical score of hamster eyes injected with vehicle (control) or LPS from animals untreated (vehicle) or treated with melatonin (Mel 12 or Mel 24 h). At 24, 36, 48 and 72 h post-injection, LPS significantly increased the clinical score. Both treatments with melatonin (starting at 12 h or 24 h post-injection) significantly decreased the clinical score at 36, 48, and 72 (but not 24) hr post-LPS. Data are mean ± SE (n = 14 eyes per group), ***P*< 0.01 vs. vehicle- injected eyes; a: *P*< 0.01 vs. LPS-injected eyes, by Mann-Whitney U-test. Lower panel: Representative photographs of clinical signs observed at 36 h post-intravitreal injections, in eyes submitted to the following treatments: control (A); LPS (B); LPS + Mel 12 h (C) and LPS + Mel 24 h (D). Note episcleral congestion (arrow), synechiae, and cataract (*) in one eye injected with LPS from an animal untreated with melatonin. In eyes from animals treated with melatonin (Mel 12 h and Mel 24 h) the occurrence of inflammatory signs was lower. Note the miosis in the animal submitted to Mel 12 or Mel 24 h.

Figure 2. Effect of LPS on cell number and protein concentration in aqueous humor, at 36 h after intravitreal injection of vehicle or LPS. LPS induced and increase in both parameters, whereas both treatments with melatonin significantly reduced the increase in cell number (panel A) and protein concentration (panel B) in the aqueous humor induced by LPS. Data are mean ± SE (n = 12 eyes per group), ***P* < 0.01 vs. vehicle- injected eyes; a: *P* < 0.01 vs LPS-injected eyes, by Tukey's test.

Figure 3. Effect of post-treatment with melatonin on PGE2 and PGF2α levels in the aqueous humor from eyes injected with vehicle or LPS. At 36 h post-injection, LPS induced a significant increase in PG levels, whereas both treatments with melatonin (i.e., Mel 12 h, and Mel 24 h)

significantly decreased PGE2 and PGF2α content. Data are mean ± SE (n = 12 eyes per group), **P < 0.01 vs. vehicle- injected eyes; a: P < 0.01 vs. LPS-injected eyes, by Tukey's test.

Figure 4. Upper panel: ERG a- and b- wave amplitude in hamster eyes injected with vehicle or LPS from animals untreated or treated with melatonin at day 8 after intravitreal injections. LPS induced a significant decrease in the scotopic ERG a- and b-wave amplitude, whereas both treatments with melatonin (starting 12 h or 24 h post-intravitreal injections) abrogated the effect of LPS. Data are mean ± SE (n = 10 eyes per group), **P< 0.01 vs. control, a: P< 0.01 vs. LPS, by Tukey's test. Lower panel: Representative scotopic ERG traces.

Figure 5. Upper panel: Representative photomicrographs of transverse sections of eyes injected with LPS in the absence or presence of melatonin. At 8 days post intravitreal injection, LPS induced vitreal hemorrhage (black arrow), inflammatory cell infiltration (white arrow), and retinal alterations. Middle and lower panel: photomicrographs of central and peripheral retina. LPS induced marked structural alterations at both eccentricities. Note retinal hemorrhage (black arrow), and inflammatory cell infiltration (white arrow). Both treatments with melatonin preserved the retinal structure, and reduced hemorrhage and cell infiltration. H&E staining. Scale bar: 600 µm (upper panel) and 100 µm (middle and lower panels).

Figure 6. Effect of LPS in the absence or presence of melatonin on retinal GFAP immunoreactivity. At 8 days post-injections, LPS induced an increase in GFAP (+) immunoreactivity in Müller cell bodies and processes, whereas both treatments with melatonin reduced GFAP immunoreactivity, showing only few positive Müller cells in the Mel 24 h group. Shown are photomicrographs representative of 4 animals/group. Scale bar :50 µm**.**

Figure 7. Retinal TBARS, NOS activity, and TNFα levels at 36 h after intravitreal injection of vehicle or LPS. LPS significantly increased lipid peroxidation (panel A), NOS activity (panel B), and TNFα levels (panel C) whereas both treatments with melatonin decreased the effect of LPS on these parameters. Data are mean ± SE (n = 10 eyes/group), $^{**}P < 0.01$ and $^{*}P < 0.05$ vs. vehicle-injected eyes, a: $P < 0.01$ and b: $P < 0.05$ vs. LPS-injected eyes, by Tukey's test.

Table 1. Effect of LPS and melatonin on scotopic ERG a-wave and b-wave latency

Average latency (msec)

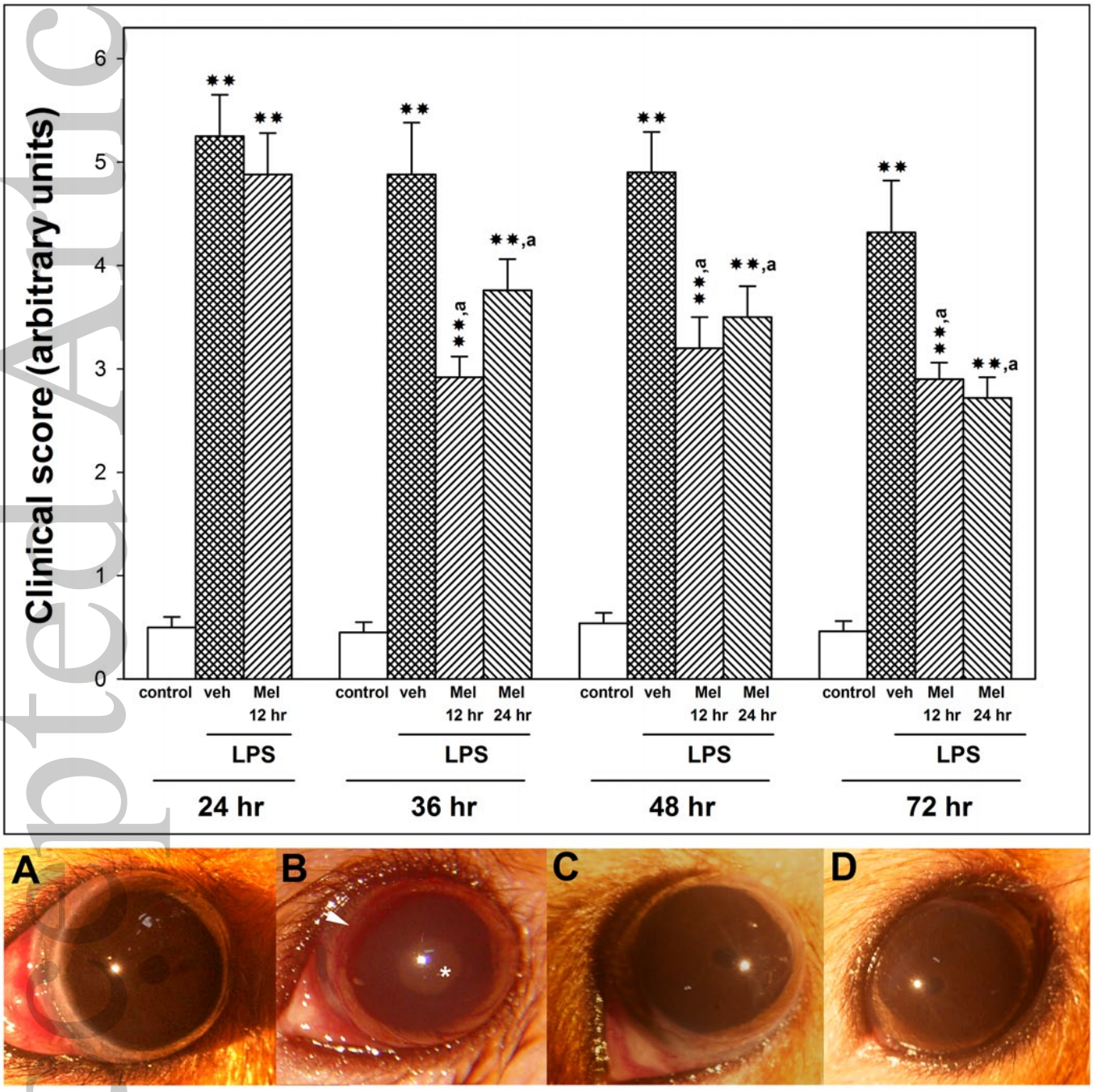
Treatment	a-wave	b-wave
Control	24.4 ± 0.1	54.9 ± 0.5
LPS	23.8 ± 0.2	56.3 ± 0.7
LPS + Mel 12 h	24.8 ± 0.2	52.7 ± 0.3
LPS + Mel 24 h	27.0 ± 0.3	55.1 ± 0.4

Scotopic ERG a- and b- wave latency in hamster eyes injected with vehicle or LPS from animals untreated or treated with melatonin at day 8 after intravitreal injections. No significant differences in these parameters were observed among groups. Data are mean ± SE (n = 10 eyes per group).

Table 2. Histological analysis of retinas from eyes exposed to light in the absence or presence of LPS

		PS	ONL	OPL	INL	IPL	Cell number in GCL/200μm
Control	Center	24.1 ± 0.9	34.6 ± 0.7	11.3 ± 1.4	17.4 ± 2.6	34.5 ± 1.2	15.3 ± 0.3
	Periphery	22.4 ± 1.2	26.5 ± 0.2	9.4 ± 0.6	10.8 ± 1.5	19.0 ± 0.9	9.0 ± 0.4
LPS	Center	14.3 ± 0.6**	26.1 ± 0.9**	6.4 ± 0.9*	9.6 ± 0.5*	36.0 ± 0.6**	8.3 ± 0.5**
	Periphery	7.3 ± 0.8**	15.8 ± 0.5*	6.8 ± 1.7	6.8 ± 0.8	9.2 ± 0.3**	5.3 ± 0.7**
LPS + Mel 12h	Center	21.49 ± 1.3[a]	28.5 ± 1.8**	8.5 ± 0.47	14.9 ± 1.2	32.8 ± 2.6[a]	13.3 ± 1.1[a]
	Periphery	19.7 ± 2.2[a]	25.6 ± 1.1[b]	9.6 ± 0.5	11.8 ± 1.7	22.5 ± 2.7[a]	8.7 ± 0.3[a]
LPS + Mel 24h	Center	22.3 ± 0.9[a]	28.3 ± 0.3**	7.9 ± 0.8	20.1 ± 1.4[a]	34.6 ± 1.2[a]	13.7 ± 0.9[a]
	Periphery	23.2 ± 1.7[a]	25.7 ± 4.22[b]	6.9 ± 0.9	10.3 ± 1.9	22.8 ± 1.9[a]	9.3 ± 0.9[a]

Retinal layer thicknesses (in μm) from eyes injected with vehicle or LPS in the presence or absence of melatonin. At 8 days post-injection, the intraviteral injection of LPS induced a significant decrease in all retinal layer thickness in the central retina, and in most of retinal layer thickness at the periphery, whereas both treatments with melatonin (Mel 12 h and Mel 24 h) significantly reduced the effect of LPS on most of these parameters. A similar profile was observed for GCL cell number. Data are mean ± SE (n = 5 eyes per group). ** P <0.01, *P <0.05, vs. vehicle-injected eyes, a: P <0.01, b: P <0.05 vs. LPS-injected eyes in the absence of melatonin, by Tukey's test. PS, photoreceptor outer and inner segment; OPL, outer plexiform layer; ONL, outer nuclear layer; IPL, inner plexiform layer; INL, inner nuclear layer; GCL, ganglion cell layer.



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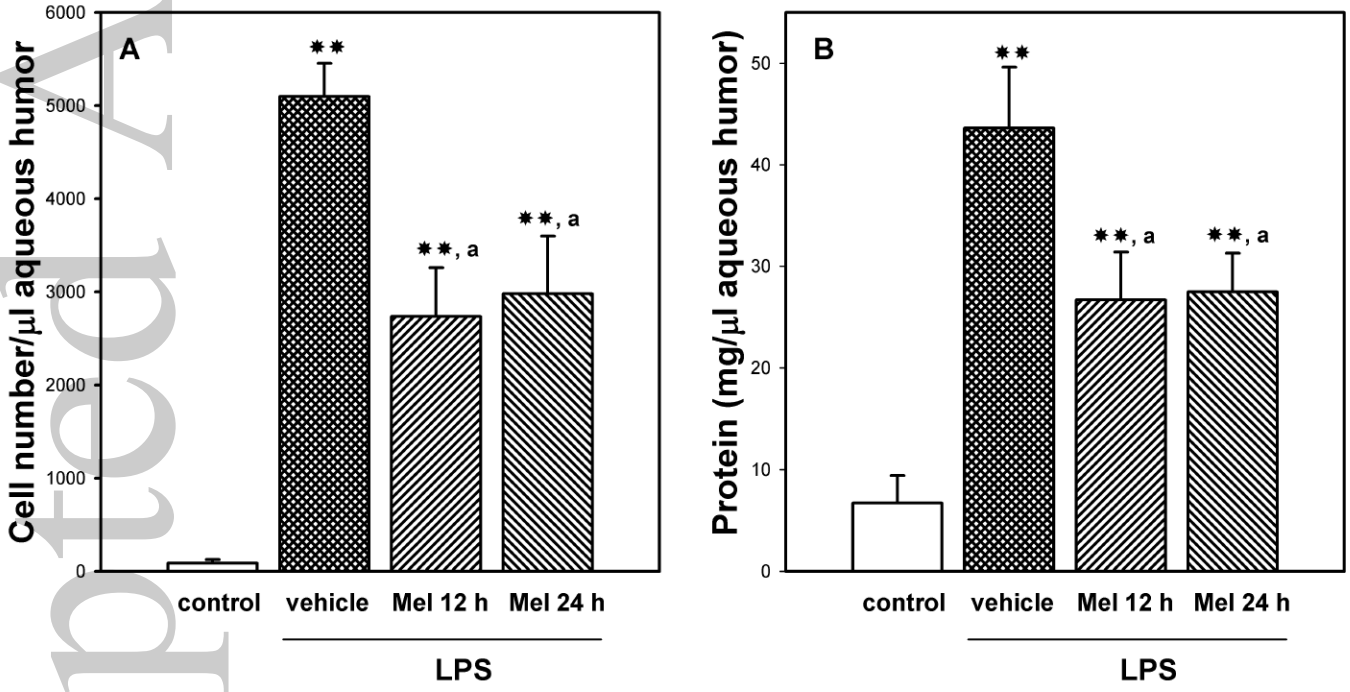

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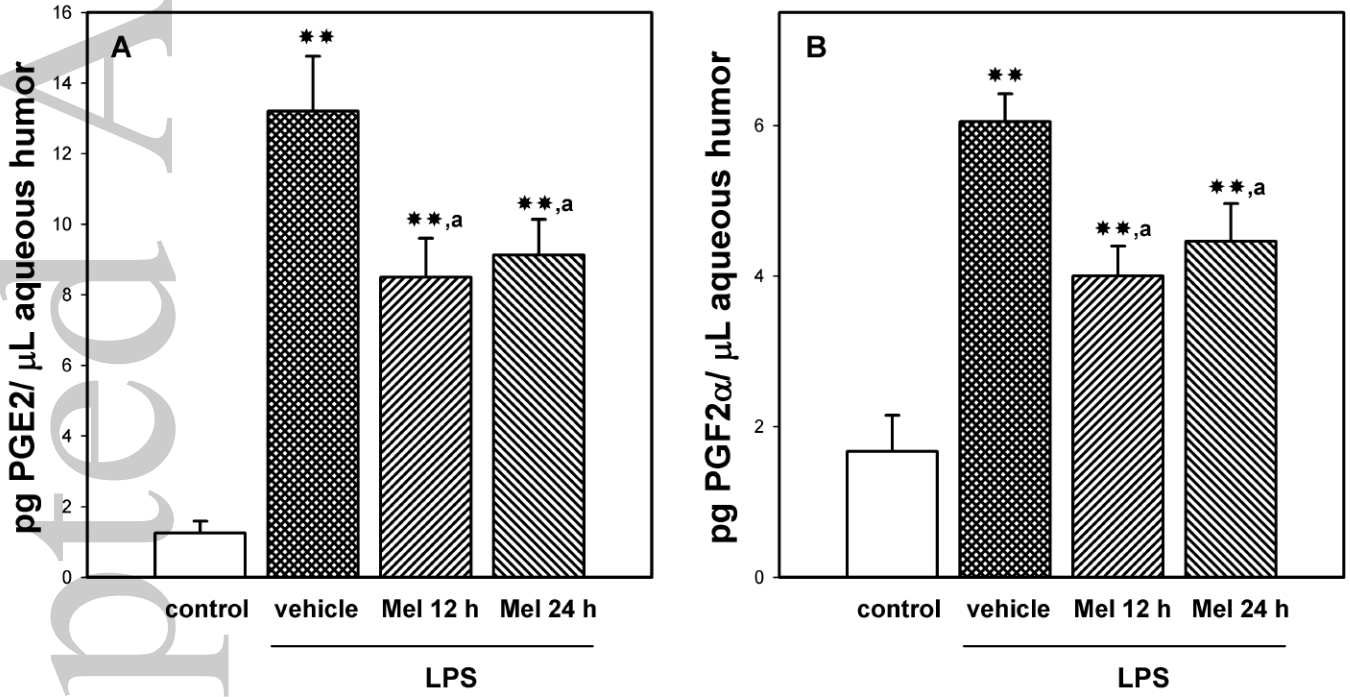

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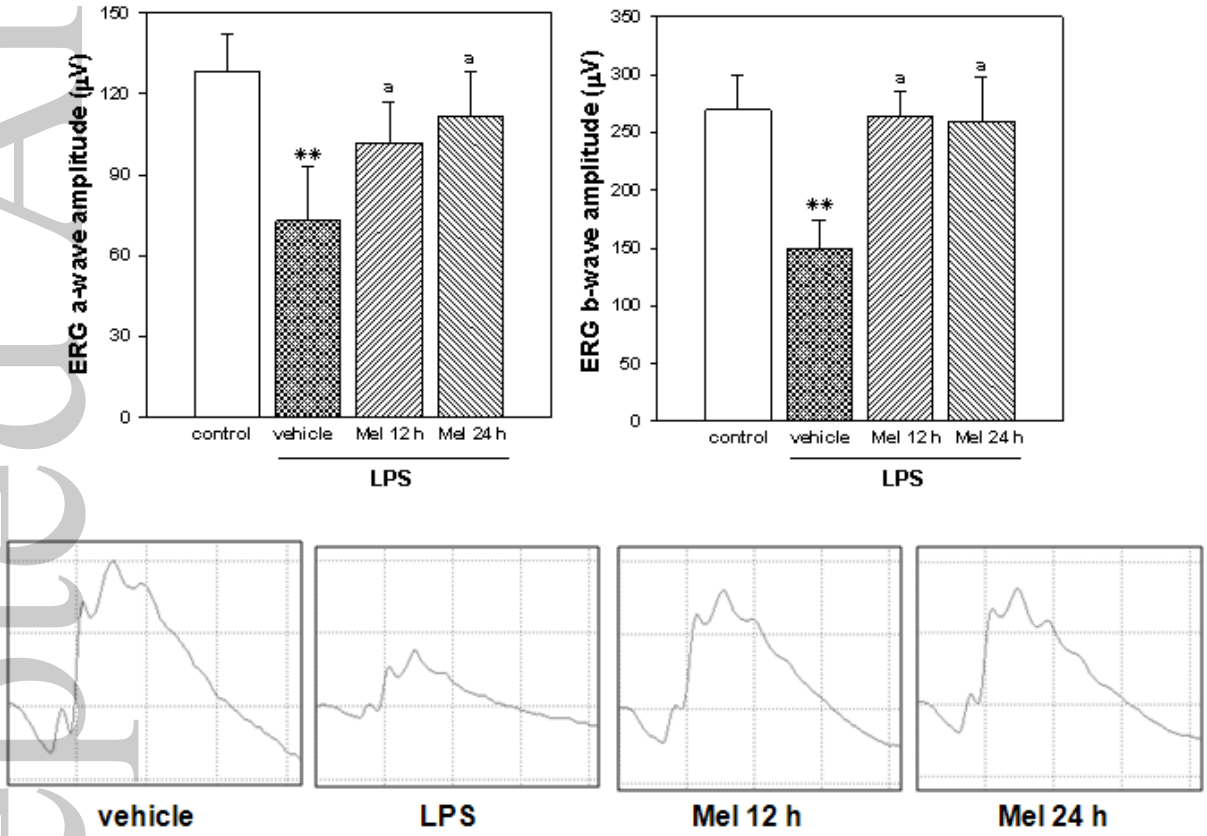

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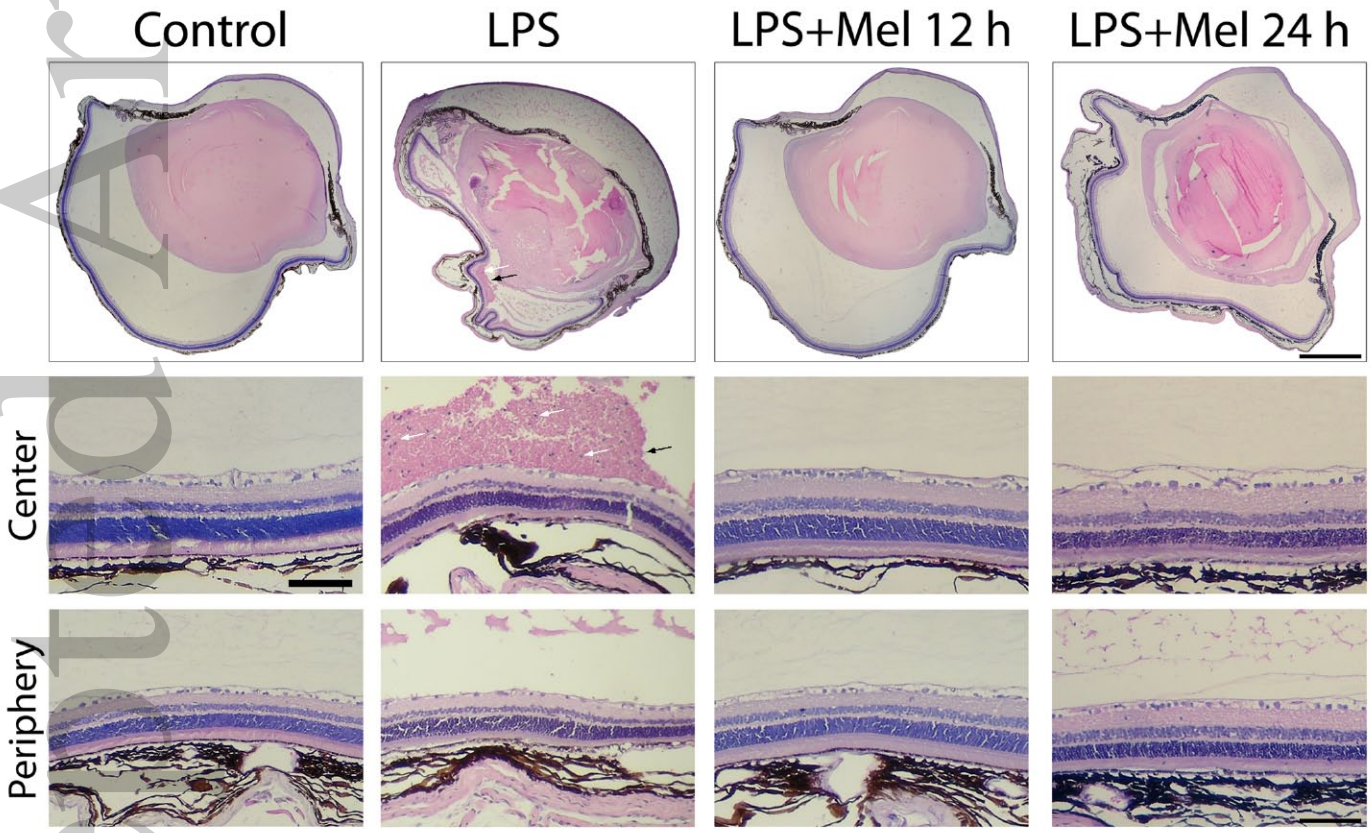

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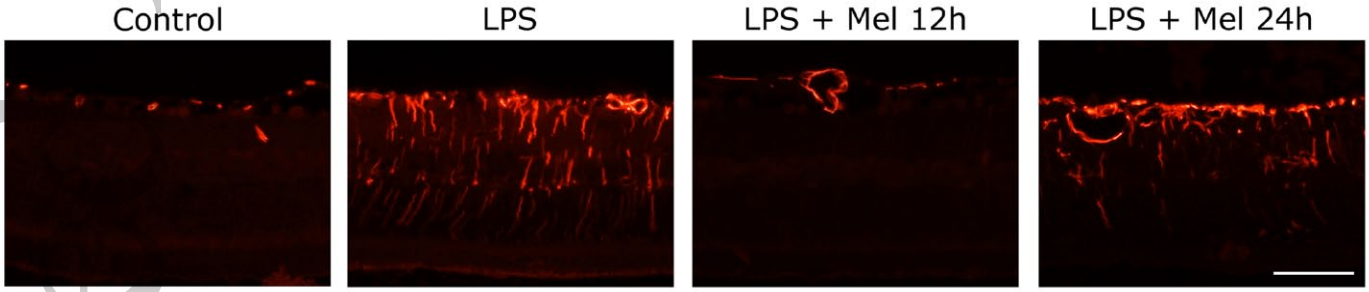

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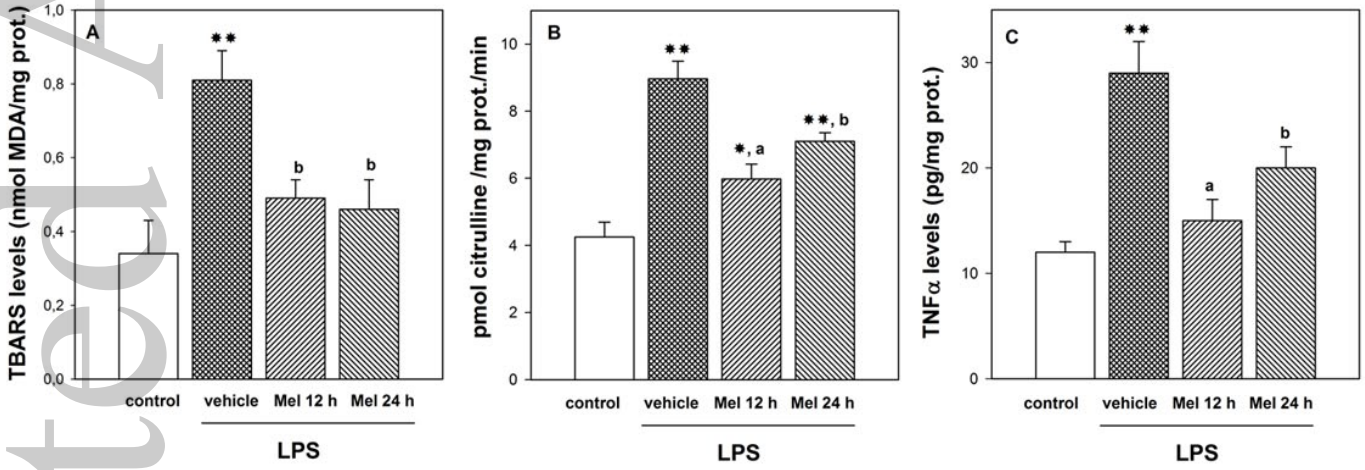

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