



Protective role of melatonin on retinal ganglion cell: In vitro and in vivo evidences



Carolina del Valle Bessone^a, Hugo Diaz Fajreldines^b, Gabriela Edit Diaz de Barboza^c,
Nori Graciela Tolosa de Talamoni^c, Daniel Alberto Allemandi^a, Agata Rita Carpentieri^{d,*},
Daniela Alejandra Quinteros^{a,**,1}

^a Unidad de Investigación y Desarrollo en Tecnología Farmacéutica (UNITEFA), CONICET and Departamento de Ciencias Farmacéuticas, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Ciudad Universitaria, 5000 Córdoba, Argentina

^b Laboratorio de Neurofisiología, Instituto de Neurociencias Córdoba, 5000 Córdoba, Argentina

^c Cátedra de Bioquímica y Biología Molecular, Facultad de Ciencias Médicas, INICSA/CONICET-UNC, Argentina

^d Instituto de Investigación en Ciencias de la Salud (INICSA)/CONICET, Universidad Nacional de Córdoba and Cátedra B de Química Biológica, Facultad de Odontología, Universidad Nacional de Córdoba, Ciudad Universitaria, Argentina

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ABSTRACT

Oxidative stress triggers ocular neurodegenerative diseases, such as glaucoma or macular degeneration. The increase of reactive oxygen and nitrogen species in retinal ganglion cells (RGCs) causes damage to the structure and function of the axons that make up the optic nerve, leading to cell death arising from apoptosis, necrosis or autophagy in the RGCs. The use of antioxidants to prevent visual neurodegenerative pathologies is a novel and possibly valuable therapeutic strategy. To investigate in vitro and in vivo neuroprotective efficacy of melatonin (MEL) in RGCs, we used a model of oxidative glutamate (GLUT) toxicity in combination with L-butionin-S, R-sulfoximine (BSO), which induces cell death by apoptosis through cytotoxicity and oxidative stress mechanisms. Histological sectioning and immunohistochemical assays using the TUNEL technique were performed to determine the damage generated in affected cells and to observe the death process of RGCs. Whit BSO-GLUT the results revealed a progressive RGCs death without any significant evidence of a decreased retinal function after 9 days of treatment. In this way, we were able to develop a retinal degeneration model in vivo to carry out treatment with MEL and observed an increase in the survival percentage of RGCs, showing that BSO-GLUT could not exert an oxidant effect on cells to counteract the effect of MEL. These findings reveal that MEL has a neuroprotective and antiapoptotic effect as evidenced by the reduction of oxidative stress damage. MEL demonstrated in this model makes it a promising neuroprotective agent for the treatment of ocular neurodegenerative diseases when administered locally.

1. Introduction

Various ocular pathologies such as glaucoma [1], age-related macular degeneration [2] and genetic retinal dystrophies [3,4] as well as many forms of retinal ischemia, are triggered by oxidative stress.

Generally, these, diseases that affect the back of the eye, are chronic and degenerative with some of these being related to advanced age.

The term “oxidative stress” refers to the situation in which the production of oxygen reactive species (ROS) attains pathological levels, and at this point the antioxidant capacity of the cell is insufficient to

Abbreviations: ROS, Reactive Oxygen Species; RGCs, Retinal Ganglion Cells; BSO, L-butionin-S, R-sulfoximine; GLUT, Glutamate; RDM, Retinal Degeneration Model; NOS, Nitric Oxide Synthase; MEL, Melatonin; ERG, Electroretinography; TEM, Transmission Electron Microscopy; RD, Retinal degeneration; IS þ OS, Inner and Outer Photoreceptor Segment; ONL, Outer Nuclear Layer; OPL, Outer Plexiform Layer; INL, Inner Nuclear Layer; IPL, Inner Plexiform Layer; GCL, Ganglion Cell Layer; SD, Standard Deviation; GCL, Glutamate-Cysteine Ligase; RGL, Retinal Ganglion Layer

* Correspondence to: A.R. Carpentieri, Instituto de Investigación en Ciencias de la Salud (INICSA)/CONICET, Universidad Nacional de Córdoba and Cátedra B de Química Biológica, Facultad de Odontología, Universidad Nacional de Córdoba, Ciudad Universitaria, 5016 Córdoba, Argentina.

** Correspondence to: D.A. Quinteros, Unidad de Investigación y Desarrollo en Tecnología Farmacéutica (UNITEFA), CONICET and Departamento de Ciencias Farmacéuticas, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Ciudad Universitaria, 5000 Córdoba, Argentina.

E-mail addresses: agata.carpentieri@unc.edu.ar (A.R. Carpentieri), danielaq@fcq.unc.edu.ar (D.A. Quinteros).

¹ These authors contributed equally to this work.

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protect the retinal ganglion cells (RGCs) and other neurons in the retina against oxidative damage [5]. Moreover, the presence of high concentrations of ROS can overwhelm the cell's natural defense mechanisms, with possible effects including the production of endogenous antioxidants [6] and the activation of pathways that lead to programmed cell death. However, as this death results from a series of steps, the inhibition of any one of these may stop this process.

One example of ROS mediating a vital cell function is apoptosis, which plays an important role in both cellular homeostasis and a variety of pathologic states [7]. Several studies have shown that apoptosis is the fundamental mechanism in the death of RGCs, and this can be triggered by several processes, with one of the most important being glutamate-induced excitotoxicity. Glutamate is an essential amino acid that plays an important role as the main excitatory neurotransmitter in the retina, and its function is based on mediating the transmission of signals from the photoreceptors to bipolar cells and RGCs. However, if their levels of glutamate are very high, it may produce a toxic effect and lead to neuronal death, with this phenomenon being known as excitotoxicity. Also, high concentrations of glutamate hyperstimulate the NMDA receptors (*N*-methyl-D-aspartate), which in turn open the channels of Ca^{+2} and Na^{+} present in the cells, thereby increasing the concentration of both ions at the intracellular level. Then this intracellular excess of cations causes the formation of free radicals, including nitrous oxide (NO), and the depolarization of the mitochondrial membrane potential, which entails, release of cytochrome C and the subsequent activation of the caspase enzymes involved in apoptosis [8–10].

The existing models of oxidative stress are not only based on the administration of glutamate, there are also studies associated with buthionine sulfoximine (BSO), a selective glutamate-cysteine ligase (GCL) inhibitor [11], which decreases intracellular glutathione. In 1997 Li ZR et al. [12] informed the injection of BSO into newborn rats reduce levels of GSH induces cataracts within 1–2 weeks. Its combination with glutamate on an immortalized RGC line, led to the cells becoming significantly more sensitive to glutamate treatment [13]. However, in the bibliography studies on neuroprotection are defined as a therapy based on preventing, limiting and some cases reversing the degeneration or death of neuronal cells by blocking the mechanisms that can trigger it, regardless of what primary damage was observed. At eye level, the RGCs and the optic nerve are part of the central nervous system, and therefore are susceptible to suffer both processes.

Melatonin can be an effective antioxidant and antiapoptotic compound in the retina, acting as a direct and indirect free radical scavenger [14]. In this way, melatonin (MEL) acts as an autocrine or paracrine neuromodulator that regulates the local circadian physiology [15]. Although it has been shown that MEL may provide neuroprotection in different systems, the full range of actions of melatonin is still not completely known. In that context, experimental evidence supports the actions of it and its metabolites as a direct and indirect antioxidant, scavenging free radicals, stimulating antioxidant enzymes, and enhancing the activities of other antioxidants. Moreover, several lines of evidence suggest that melatonin may act as a protective agent in ocular conditions such as photokeratitis, cataract [16], retinopathy of prematurity, and ischemia/reperfusion injury. We have previously shown the beneficial effect of melatonin against retinal glaucomatous and diabetic damage [17].

The aim of the present study was to evaluate the neuroprotective effect of MEL on RGCs subject to oxidative stress. To carry this out, we used a model of oxidative glutamate toxicity, in combination with BSO. In this context, the main goal was to determine the beneficial effects of MEL on the functional, morphological and histological alterations induced, using to the experimental described below.

2. Materials and methods

2.1. Chemicals

Glutamate, L-butionine-S, R-sulfoximine, melatonin, proteinase K, hydrogen peroxide, methyl green, glutaraldehyde and formaldehyde were purchased from Sigma-Aldrich (St. Louis, MO, USA). For histological evaluation of apoptosis, the ApopTag Plus Peroxidase In situ Apoptosis Detection Kit. S7101 (Chemicon International Invitrogen, Carlsbad, CA, USA) was used Sodium chloride Parafarm®, ketamine hydrochloride solution (Ketamina 50 mg/mL, Holliday Scott S.A., México D.F., México), xylazine (Xilacina 100 mg/mL, Laboratory Richmond S.A., Bs. As., Argentina) and topical propacaine hydrochloride 0.5% (Laboratory Alcon, Texas, EEUU) were utilized in all procedures where anesthesia was necessary. In order to prevent any infection, iodopovidona 5% (Pervinox, Laboratory Elea Phoenix S.A., Bs. As. Argentina) was applied topically.

2.2. Purification and primary culture of RGCs from chicken embryos

RGCs were obtained from retinas of 8-day-old chicken embryos using an immunopanning purification method according to a procedure described in [18]. These were cultured in DMEM medium with the addition of supplements to give a neuron culture.

2.3. Treatment of RGC cultures with oxidant and neuroprotective agents

Cytotoxicity was produced by administration of glutamate (GLUT, 10, 15 or 20 mM), with the oxidative stress being induced by the administration of L-butionine-S, R-sulfoximine (BSO, 0.75 mM). Melatonin 0.5 mM was administered in order to evaluate its capability to prevent the cytotoxic and oxidative effects of this treatment. The experimental set-up consisted of the following three groups of samples: 1) RGC Controls, 2) RGC treated with BSO-GLUT and 3) RGC treated with BSO-GLUT + MEL for 24 and 48 h. In addition, control samples treated with MEL, GLUT or BSO were analyzed in parallel. Cell survival was determined using the crystal violet technique, with the samples being fixed with 1% glutaraldehyde and incubated with 0.1% crystal violet solution. For the immunocytochemistry studies, RGC cultures were fixed with 4% paraformaldehyde solution. The evaluation of apoptosis was performed using by the TUNEL technique, as described below.

2.4. Animals

Experiments were performed using New Zealand albino rabbits (2.5 ± 0.5) kg, which were kept under controlled conditions in individual cages, with food and water ad libitum, and in a 12/12 h light/dark cycle. The animals were sacrificed after 9 days of treatment, according to protocols. They were anesthetized with phenobarbital and the euthanized using a mixture of 10% O₂ and 90% CO₂ in an acrylic hermetic chamber. Immediately, their eyeballs were quickly enucleated and the retinas were processed as described below for each assay. All experiments were conducted in agreement with the procedures defined in the Association for Research in Vision and Ophthalmology resolution regarding the use of animals in research, the European Communities Council Directive (86/609/EEC), and the Institutional Care and Use Committee (CICUAL) of the School of Medicine Sciences, National University of Cordoba (Res. 44/17). Every effort was made to minimize the number of animals used in each experiment.

2.5. Retinal degeneration model (RDM) in New Zealand rabbits

The experiments related to the RDM model were carried out by means of intravitreal administration in the rabbit eyes of a dose of 200 µM GLUT and 75 µM BSO, dissolved in sodium chloride 0.9% solution. Previously, the corneas were anesthetized topically with

propacaine hydrochloride (0.5%) eye drops. The application (40 μ L) was made to the anterior chamber of the left eye, in the upper temporal quadrant, located 4 mm from the sclerocorneal limbus, using a syringe with a 29-gauge needle (TERUMO). For a comparative analysis (negative control), an equal volume of sodium chloride 0.9% was injected in the right eye. In both cases, immediately after removing the needle, a cotton swab with iodopovidona 5% was applied to prevent bacterial infection. After 9 days of treatment, the animals were sacrificed and their eyeballs were quickly enucleated, according to the protocols. These eyes were evaluated through histological studies, and the evaluation of apoptosis was performed using the TUNEL technique and Transmission Electron Microscopy (TEM). Each sample was assayed in 5 rabbits.

2.5.1. Intravitreal injection of melatonin

Melatonin (1 mg/mL) was dissolved together 200 μ M GLUT + 75 μ M BSO in a 0.9% sodium chloride solution. As above, the animals were locally anesthetized with a drop of propacaine hydrochloride 0.5% (v/w), administered in each eye, by means of a syringe with a 29-gauge needle (TERUMO®). Then 40 μ L of the solution were injected into the left eye of anesthetized rabbit, while an equal volume of 0.9% sodium chloride solution was injected in the right eye. These injections were applied at 4 mm from the sclerocorneal limbus in the upper temporal quadrant and in both cases, immediately after removing the needle a cotton swab with iodopovidona 5% was applied to prevent bacterial infection. After 9 days of treatment, the rabbits were sacrificed and the eyeballs were quickly enucleated. These eyes were evaluated by means of histological studies, and the evaluation of apoptosis was performed as described above (Section 2.5).

2.6. Electroretinography (ERG)

ERG is a non-invasive technique for recording the function of retinal neurons. By subjecting the retina to known intensities of light, an electrical response can be elicited and recorded. The level of activity was assessed for both eyes before injection and at 0, 2, 5 and 9 days after administration of control and test formulations. Before, this examination, the rabbits were sedated by an intramuscular injection containing a combination of xilacina (100 mg/mL, dose 0.25 mL/kg) and ketamina (50 mg/mL, dose 0.75 mL/kg). Subsequently, they were anesthetized locally with a drop of propacaine hydrochloride (0.5%), administered on the surface of each eye. A reference electrode was inserted at approximately 0.5 cm from the posterior commissure of the eye. In addition, other a grounding electrode was inserted in the skull, between the ears, and a silver positive electrode was placed in contact with the central cornea. The electroretinograms (ERGs) were recorded for one eye at a time.

A LED photo stimulator of the flash type was used with settings of 7.85 and 11.78 $\text{cd}\cdot\text{s}/\text{m}^2$ (10 and 15 Lux), and 1 Hz of frequency. The cycle time was set at 300 ms at a distance of 20 cm from the animal and the light intensity of the environment was about 6.28 $\text{cd}\cdot\text{s}/\text{m}^2$ (8 Lux). AKONIC BIO-PC equipment (Akonic BIO-PC, Akonic, Argentina) was used to amplify and record the signal. Which was recorded with a gain of 100 μ V, without attenuation, using high frequency (300 Hz), low frequency (1.5 Hz) and notch (50 Hz) filters. For each study, 100 stimuli were applied, and the average value of the records was registered. In order to avoid diurnal variation in the ERG responses, all ERGs were carried out at the same time of the day. The ERG results were analyzed by measuring the latency and amplitude of the a-waves and b-waves. Control and test samples were compared by using an analysis of variance of the student *t*-test. A *p* value of > 0.01 was considered statistically significant [19].

2.7. Histological studies

At the end of the experiments (9 days), the rabbits were sacrificed

and their eyeballs were immediately enucleated and immersed in a fixative containing 4% paraformaldehyde in PBS (pH = 7) for conservation. Thereafter, they were dehydrated in increasing concentrations of ethanol (70, 80, 95 and 100%), diaphanized in butyl acetate and immersed in paraffin. Eyes were sectioned (5 μ m) along the vertical meridian of the optic nerve, and stained with Hematoxylin–Eosin for visualizing sections by means of light microscopy (Olympus BX41 microscope) [20–22]. The images obtained were captured using an Infinity 1 camera coupled to Infinity Analyze software. The whole of the retina, including the area of the optic nerve, was examined counting of the RGCs was performed with the assistance of Image J software.

2.8. In situ detection of apoptosis by the TUNEL assay

Apoptotic cells were visualized using the terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) assay with the In Situ Cell Death Detection Kit, POD (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's instructions. Briefly, sections of paraffin-embedded tissue were dewaxed in xylene and rehydrated by washing with a graded series of ethanol and double distilled water. After rinsing with phosphate buffered saline (PBS) solution for 5 min, the tissue samples and RGC culture were incubated with 20 μ g/mL proteinase K for 15 min. Afterwards, these were rinsed with distilled water and incubated with a solution of 3% hydrogen peroxide (H_2O_2) in PBS for 5 min at room temperature, in order to prevent enzyme activity. After rinsing the slides twice with PBS for 5 min, the samples were placed inside a humidified chamber in darkness and incubated with the TUNEL reaction mix, at 37 °C for 60 min, to allow the end-labeling reactions to occur. A colour reaction was activated by the addition of diaminobenzidine (DAB) and allowed to elapse for 3–6 min. The samples were counterstained with 0.5% methyl green solution, prior to being analyzed by light microscopy (Olympus BX41). All the sections were examined under Infinity Analyze software, with tissue sections of mammary glands with extensive apoptosis extracted from rats being used as positive control. Under light microscopy, the apoptotic nuclei were colored brown, as positive, whereas non-apoptotic cells were blue. The TUNEL-positive cells were quantified in relation to total number of cells in the RGL and expressed as the percentage of apoptotic index [23]. This was evaluated at a 20 \times magnification with the assistance of image j software.

2.9. Transmission Electron Microscopy (TEM) examination

The retinas were washed for 2 h with 1 mL of PBS (pH = 7.0) solution and fixed with 2% of glutaraldehyde and 4% formaldehyde in 0.1 M cacodylate buffer. Then, they were post-fixed with osmium tetroxide at 1% in the same buffer, dehydrated and embedded in Araldite. Thin sections were cut with a diamond knife on an ultramicrotome (JEOL JUM-7) and mounted on glass slides, after which, they were examined by means of a transmission electron microscope (Zeiss LEO 906E) [24–26].

3. Results

3.1. Effect of BSO-GLUT on RGCs

Cell cultures were noticeably affected by the deleterious effect of BSO/GLUT cytotoxicity, with, the RGC survival decreasing considerably as a consequence of the oxidative stress caused by glutathione depletion produced by the administration of BSO. Using the crystal violet technique, which measures cell survival, the optimal dose was determined for producing the desired oxidative and cytotoxic effects, and corresponded to a concentration of 0.75 mM BSO + 20 mM GLUT because it is significantly different with respect to control. These effects were proportional to the concentration to the BSO/GLUT concentration as it evidenced in Fig. 1A. Fig. 1B displaying the quantitative

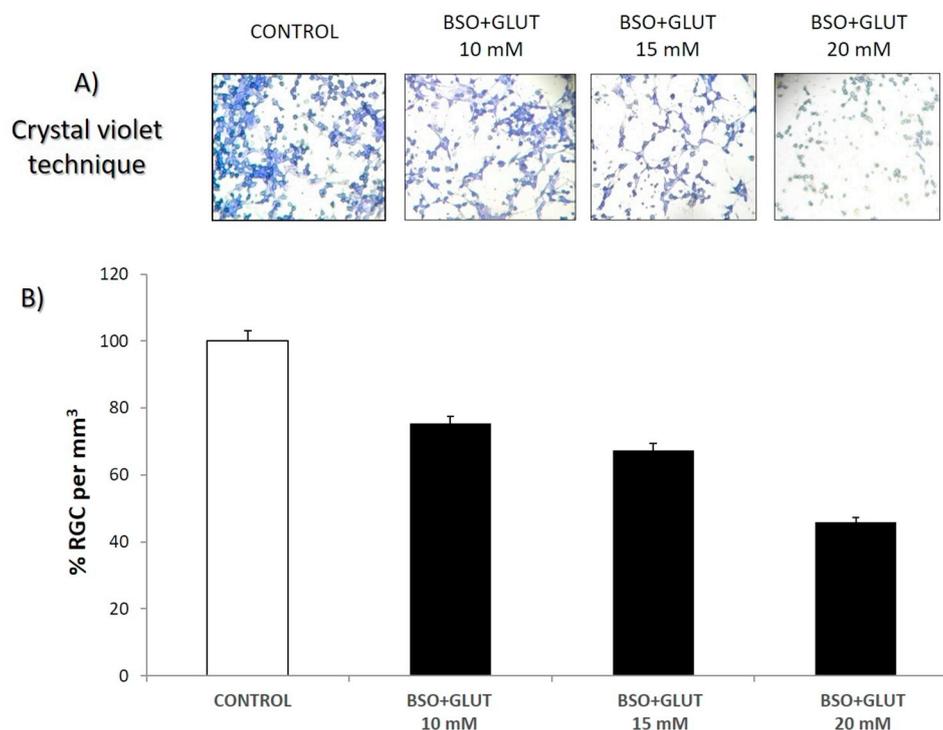


Fig. 1. A) Light microscopy analysis of test samples processed according to the crystal violet technique. B) Quantitative analysis of survival percentage of RGCs (**p* < 0.001). Bars represent means \pm SD.

comparison of three different concentrations and a control sample.

3.2. Detection of apoptotic RGCs with or without administration of MEL

The antiapoptotic effect of Melatonin was evaluated by observing cell survival by means of the crystal violet technique. To carry this out, two different test samples (RGC treated with BSO-GLUT and RGC treated with BSO-GLUT + MEL) and one control sample (RGC culture) were studied. From this assay, a noticeable decrease in cell survival was observed in the samples treated with BSO-GLUT, with respect to the control groups, 48 h after treatment. However this effect was prevented when melatonin (0.5 mM) was administered together with BSO-GLUT. In addition, the number of TUNEL (+) cells was increased in the case of samples treated with BSO-GLUT, with respect to controls or samples treated with BSO-GLUT + MEL (Fig. 2).

3.3. Retinal degeneration model (RDM) in New Zealand rabbits

At concentrations of 75 μ M BSO and 200 μ M GLUT, the histological analyses after nine days of treatment showed a normal configuration in all layers of the untreated rabbit retina. However, sporadic ruptures in the layers of photoreceptor cells and irregularities with intercellular spaces in the outer nuclear layer (ONL) were detected in retinas treated with BSO-GLUT. Moreover, the loss of integrity of the inner nuclear layer (INL) and a decrease in the RGCs were observed. From the results shown in Fig. 3, it is evident that the intravitreal injection of melatonin in rabbit eyes was able to reduce the histological alterations provoked by BSO-GLUT. Fig. 3II also shows clear evidence of the protective effect of MEL (1 mg/mL), which appeared to be capable of protecting the cells from the deleterious effect of BSO-GLUT, resulting in a survival percentage similar to that of control cells.

3.4. In situ detection of apoptosis by the TUNEL assay

To be able to infer the amount or percentage of RGCs in the apoptotic state an in vivo test was performed using the TUNEL technique

(Section 2.8). The intravitreal administration of BSO-GLUT induced a significant increase in the percentage of the apoptotic index of the RGCs ($63.48 \pm 3.65\%$) in comparison to the control sample ($37.95 \pm 6.71\%$). Thus, we can deduce that oxidative stress and cytotoxicity of BSO-GLUT led to apoptosis of RGC. However, when MEL was administered together with the BSO-GLUT solution, no statistically significant changes were observed to the control eye (Fig. 4) with the apoptotic index of RGCs in the treated eyes and untreated eyes being ($37.7 \pm 6.22\%$) and ($40.49 \pm 8.2\%$), respectively.

3.5. Electroretinography (ERG)

As a first approach, we investigated the threshold variation between control vs treated eyes in albino rabbits. Alterations in the conditions of application of the stimulus may define which types of retinal cells suffer some form of aggression. Here we observed that a single intravitreal injection of BSO-GLUT or BSO-GLUT + MEL at a concentration of 200 μ M and 75 μ M + 1 mg/mL, respectively did not have any negative effects on retinal function after 9 days of treatment. On comparing the eyes injected with each dose, their latencies remained constant and the a- and b-wave amplitudes in the ERG measurements were unchanged. Thus, no retinal functional toxicity according to the ERG responses was found after nine days of treatment.

3.6. Ultra-structural changes in the retinal ganglion cell layer of eyes treated with BSO-GLUT

An electron microscopy analysis revealed normal structure in the retinal ganglion layer (RGL) of control eyes at the end of the experiments (9 days). Therein, all layers had normal localized cisternae of the endoplasmic reticulum, ribosome and mitochondria. A normal distribution of lipofuscin granules and vacuole-like structures were also observed (Fig. 5A). In contrast, disordered organelles, the rupture of the nuclear and plasma membranes and swollen degenerated mitochondria were observed in the RGL of the eyes treated with BSO-GLUT. An irregular endoplasmic reticulum cisternae around the nucleus, with an

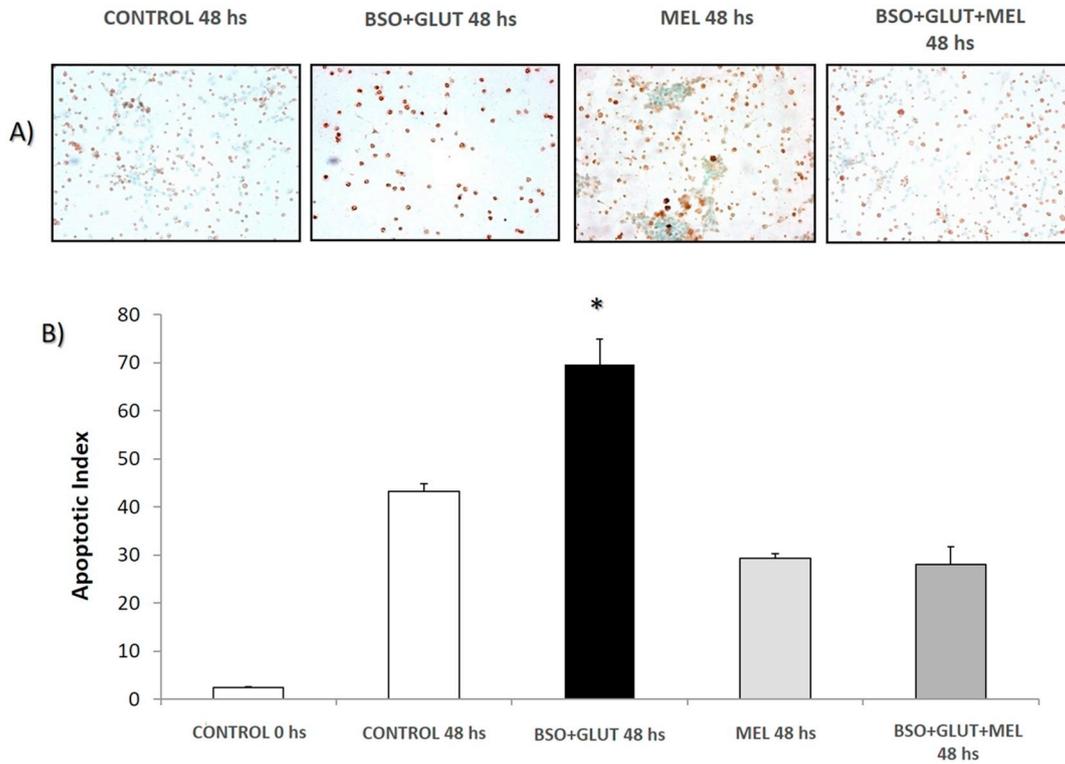


Fig. 2. Antiapoptotic effect of MEL on RGC culture in Retinal degeneration (RD) model: TUNEL assay. A) Images of TUNEL assay on GRC cultures (48 h after treatment). B) Quantitative analysis of proportions of TUNEL-positive cells in RGC cultures (* $p < 0.001$). Bars represent means \pm SD.

increased number of phagosomes and vacuoles in an enlarged cytoplasm were also detected (Fig. 5B), along with a loss of continuity in the basal membrane and an increased inter-lamellar space of the RGL [27–29].

When BSO-GLUT plus MEL were administered, we only observed a slight alteration of the RGC, with the presence of some phagosomes and vacuoles in the cytoplasm, and an increased inter-lamellar space in the RGL. Thus, we can assume that the process of cell death caused by oxidizing agents was minimized for by action of MEL (Fig. 5C).

When BSO-GLUT plus MEL were administered, we only observed a

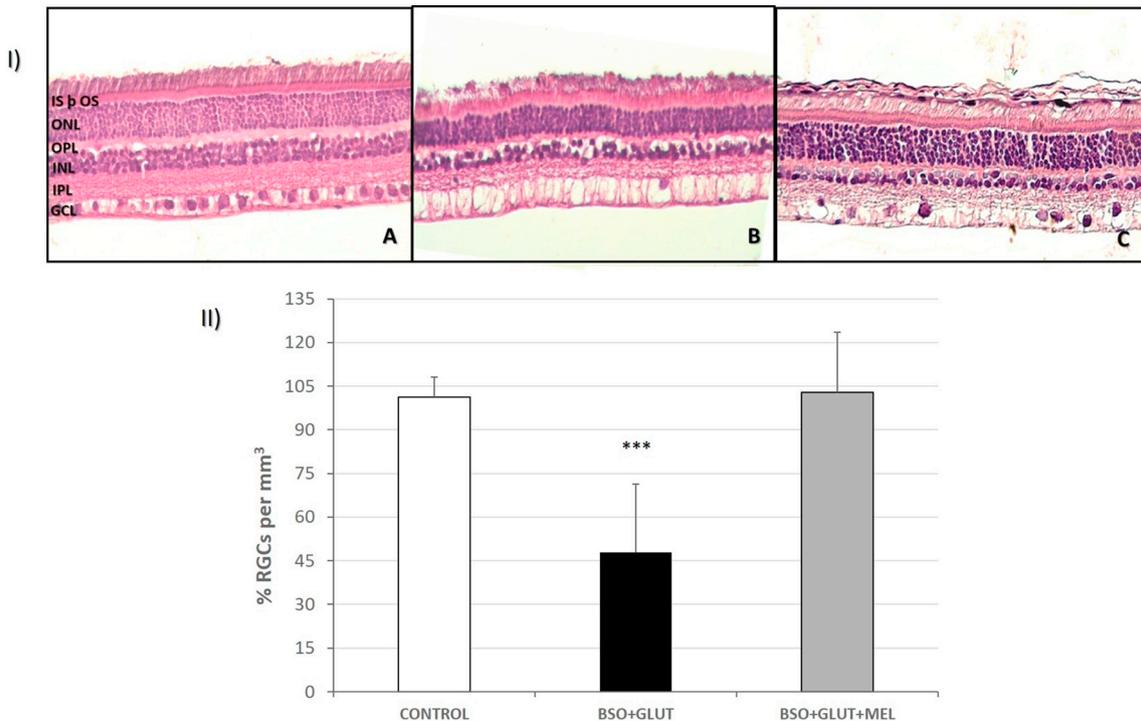


Fig. 3. Retinal degeneration (RD) model in rabbit with melatonin: histological studies. I) Light microscopy analysis of untreated retinas of rabbits (control) (A) BSO-GLUT treatment (B) and BSO-GLUT + MEL (C). II) Quantitative analysis of RGC (***) $p < 0.001$). Bars represent means \pm SD.

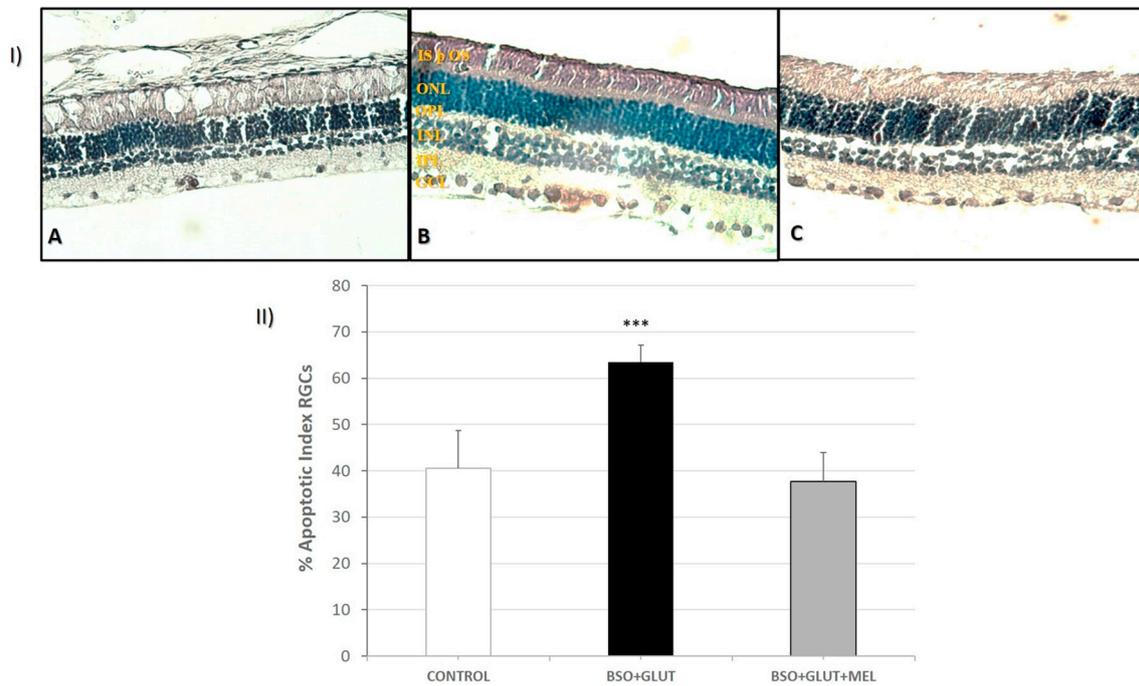


Fig. 4. Retinal degeneration (RD) model with melatonin using TUNEL assay. I) The images of TUNEL assay on untreated retinas of rabbits (control) (A) BSO-GLUT treatment (B) and BSO-GLUT + MEL (C). II) Quantitative analysis of proportions of TUNEL-positive cells in RGCs (***p* < 0,001). Bars represent means ± SD.

4. Discussion

A convenient strategy often used at the first stage of research in the field of neuroprotection for different ocular pathologies, such as glaucoma, is the design of an experimental set-up able to generate reliable that can be interpreted and related to events that occur in vivo. Related to this, the development of neuroprotective approaches capable of preserving the cells after the aggression and the damage generated by oxidizing and cytotoxic compounds, is an important advance in the development of long-term treatments for optical neuropathies.

To date, several eye cells have been successfully cultured, and this permits a cost-effective alternative to animal models for the study of the effects of cell death by apoptosis and the investigation of neuroprotection mechanisms. Cell cultures have the advantage of allowing the rapid screening of potential therapies and the observation of their direct effects, under strict environmental control. They also provide a greater understanding of how compounds work at the cellular level.

The present study mainly focuses on evaluating the synergistic cytotoxic effects of the combination of the BSO-GLUT oxidizing agents as well as the neuroprotective effect of melatonin on the RGCs. In the former case, the main mechanism by which BSO-GLUT caused toxicity in cultures of RGCs was due to oxidative stress, a process in which there

is an imbalance between the generation and elimination of reactive oxygen and nitrogen species. It is known that an excess of extracellular GLUT can be toxic to neurons by causing oxidative stress or excitotoxicity. However, since the viability of RGCs is not affected by GLUT alone [30], it is likely that the reduced viability caused by the BSO-GLUT combination was due mainly to the oxidative stress process. It is also known that BSO causes a reduction in the biosynthesis of glutathione (GSH), and that extracellular glutamate can potentiate this action by reducing the availability of cysteine as a precursor of GSH synthesis [31]. The availability of cysteine is, therefore a limiting factor on the speed for of the synthesis of glutathione, which is an important antioxidant. The increase in ROS caused by BSO-GLUT resulted in reduced RGC survival, with the death process appearing to be DNA destruction, which is in agreement with the apoptosis observed using TUNEL procedure. In order to corroborate the oxidative and cytotoxic effects of the selected oxidizing agents, we used the cellular damage mechanism to evaluate the neuroprotective effect of MEL. This is a hormone that acts as an effective maternal-fetal circadian synchronization agent and in dissociated circadian rhythms, induced by a short light-dark cycle, insomnia and jet lag [17,32,33]. In addition, MEL has been proven to be an antioxidant and anti-inflammatory molecule and is capable of mitigating cell damage associated with oxidative stress

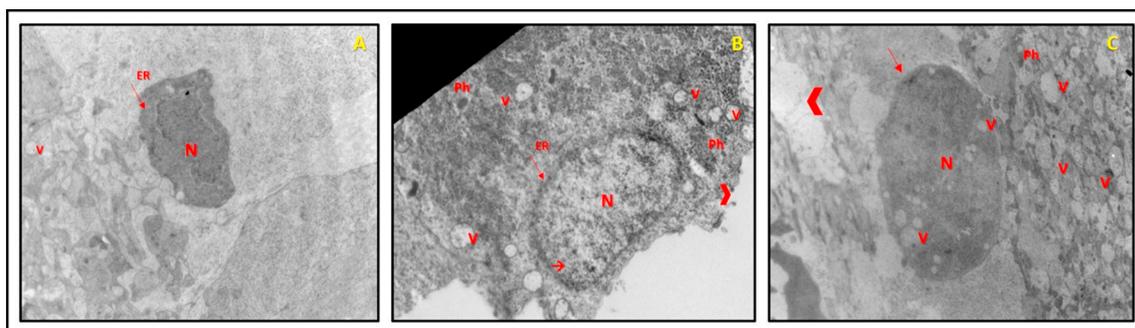


Fig. 5. Ultrastructure of RGCs determined by electron microscopy. Retina of rabbits obtained from control, X6000 (A) BSO-GLUT treatment X10000 (B) and BSO-GLUT + MEL X6000 (C). Dissolved membrane discs; V: Vacuole; N: nucleus; Ph: phagosome; → Cells in apoptosis; → endoplasmic reticulum.

and the processes of inflammation that underlie neurodegenerative disorders. In the present study, when MEL was administered together with oxidizing agents, it produced an inhibition or slowing down of cell damage and also of the process of death by apoptosis. MEL and its metabolites act as antioxidant by neutralizing toxic oxidative species. MEL also stimulates anti-oxidant enzymes such as superoxide dismutase, glutathione peroxidase, catalase and glutathione reductase [34–37] and suppresses pro-oxidant enzymes such as nitric oxide synthase [38,39]. It is possible that MEL be able to activate the enzyme responsible for GSH synthesis, making more effective the antioxidant process. Besides, others processes involved in such mechanism could be also induced, counteracting in both cases the oxidant effect of BSO. In addition, MEL has been able to protect mitochondria against oxidative threats and to enhance respiratory function and consequently reducing electron leakage and increasing ATP generation, both in vivo and in vitro [40,41]. This effect could explain MEL anti-apoptotic action observed in the RGC both in vitro and in vivo.

Next, we evaluated the response of isolated RGCs, maintained in an artificial environment, as well as assessing their behavior in vivo. The results obtained from ERG studies allowed us to infer that there were no significant changes for any of the formulations assayed, demonstrating an invariance in the functionality of the retina against each treatment. Nevertheless, according to the histological and immune-histochemical analysis carried out using TUNEL method, the administration of BSO-GLUT, provoked a significant decrease in the survival of RGC and a significant increase in cell death due to RGC apoptosis.

The functional effects of BSO/GLUT treatment on the photoreceptors were examined by means of flash electroretinography recording. Other studies have reported a dysfunction of these cells in the retinas of rabbits, with a rapid increase in the intraocular pressure of above 60 mm Hg [42]. However, Gerstle et al. in 1973, showed in ERG that the amplitude of the b-wave decreases only if the IOP exceeds a critical level of 30 mm Hg [43]. In our experimental model, the cellular damage induced on RGC did not produce any increase in IOP above normal values during the 9 days of treatment.

In our study, the in vitro test were, based on the phenomena of oxidative stress, and involve endogenous production of ROS by exposing the cells to high concentrations of glutamate in combination with BSO an inhibitor of GSH biosynthesis. This combination resulted in a depletion of GSH and, consequently, a gradual increase in ROS. Thus, it is predictable that an oxidative process can be observed during the development of the illness of a patient suffering from glaucoma and, consequently, a protective effect of MEL as observed in vitro, would be expected for the pharmacologic treatment of this kind of patient. This was also demonstrated by the TEM technique, where cell damage on the RGL generated by the oxidizing agents was observed morphologically. In order to examine the effect of MEL on the cellular alterations induced by the administration of BSO-GLUT, it was concomitantly administered intravitreously. This treatment produced an increase in the survival of RGCs as well as a remarkable reduction of the rate of the apoptotic process.

Summing up, the neuroprotective effect of MEL revealed in tissues or cells subjected to a potent oxidative process makes this compound a very promising tool for the treatment of pathologies that involve serious deleterious effects on the affected organ (i.e. glaucoma).

5. Conclusions

A model of oxidative GLUT toxicity in combination with BSO (which inhibits glutamate cysteine ligase, the rate-limiting enzyme in GSH biosynthesis) was used in order to evaluate the neuroprotective effect of MEL on RGCs, when subject to oxidative stress. Under these conditions, the cells became significantly more sensitive to glutamate treatment. This model appeared to be useful for obtaining a better understanding of the molecular basis of oxidative-stress-induced RGC death. In this study, the experimental set-up was used with the aimed to

evaluating the protective effect of MEL on RGCs damaged by oxidative BSO-GLUT solution. In the framework of this work, MEL was demonstrated to be a very efficient neuroprotector, as could be concluded from RGC viability studies, the antiapoptotic effect and the ultra-structural analysis of the retina.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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