Research Square

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Effect of Trigonelline in a Model of Apoptosis in Rat Retina

Sevinç Arzu Postacı (postaciarzu@gmail.com)
Fırat Üniversitesi Tıp Fakültesi: Firat Universitesi Tip Fakultesi https://orcid.org/0000-0002-3778-6583
Ülkü Çeliker
Fırat Üniversitesi Tıp Fakültesi: Firat Universitesi Tip Fakultesi
Nevin İlhan
Fırat Üniversitesi Tıp Fakültesi: Firat Universitesi Tip Fakultesi
Ferda Dağlı
Fırat Üniversitesi Tıp Fakültesi: Firat Universitesi Tip Fakultesi
Ali Dal
Fırat Üniversitesi Tıp Fakültesi: Firat Universitesi Tip Fakultesi

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Abstract

Purpose: To evaluate the efficacy of drop trigonelline and oral trigonelline (TG) treatment in a model of N-methyl D-aspartate (NMDA)-induced retinal apoptosis in rat retina. To compare with brimonidine tartrate (BT) drops with known retinal neuroprotective activity.

Methods: 42 Wistar Albino male rats were randomly divided into 6 groups of 7 each. No action was applied to Group 1. Group 2 (negative control) was given intravitreal Phosphate Bufferd Saline (PBS) on the first day of the experiment and did not receive any treatment. Groups 3, 4, 5 and 6 were given intravitreal NMDA on the first day of the experiment. Group 3 (positive control) didn't receive post-injection treatment. For 21 days from the second day of the experiment, oral TG was given to group 4, TG drops were given group 5 and BT drops were given group 6. Histopathological and biochemical evaluations were performed in all groups.

Results: Severe retinal degeneration was observed in group 3 compared to group 2 ($p<0.001$).There was no statistically significant difference between group 1 and group 2 ($p>0.05$). TUNEL, Brn3a and caspase3 staining in group 5 and group 6 were similar to group 2 ($p>0.05$). Group 5 and group 6 compared to group 3 were observed significant decrease in iNOS levels($p<0.05$). Decreasing MDA levels and increasing SOD levels were detected in group 4,5,6 compared to group 3 ($p<0.05$).

Conclusion: In our study, it was determined that TG drops showed similar retinal neuroprotective efficacy to BT drops.

Introduction

Glaucoma is the cause of irreversible vision loss with retinal ganglion cell (RGC) damage [1]. Although high intraocular pressure (IOP) is an important risk factor for retinal nerve fiber loss in glaucoma, decreased IOP cannot prevent glaucoma progression in some patients [2]. Fort his reason, neuroprotective treatments have gained importance in the treatment of glaucoma [3].

Glutamate excitotoxicity and oxidative stres are considered to be important pathophysiological causes for glaucomatous neurodegeneration. Glutamate causes retinal damage by affecting NMDA receptors [4]. Glutamate causes an increase in intracellular calcium level and subsequently increases nitric oxide synthetase (NOS) levels. With the increase of reactive oxygen products, lysosomal enzymes are activated [5]. NMDA-induced excitotoxicity is used in animals as a model of RGC degeneration [6].

DNA fragmentation, which is the defining feature of apoptosis, can be demonstrated by the Terminal Deoxynucleotidyl Transferase Mediated UTP Nick End Labeling (TUNEL) staining method [7]. Caspase 3 is involved in the common part of the intrinsic and extrinsic apoptotic pathways. The cells in which apoptosis started can be detected by looking at the caspase 3 level [8]. The cells of immunochemically labeled with Brn3a show viable of RGCs [9].

Oxidative stress is one of the most important mechanisms that stimulate apoptosis [10]. Treatment modalities that plan to reduce apoptosis are expected to relatively reduce oxidative stress markers or increase the effectiveness of antioxidant systems.

TG is a vitamin B6 derivative with 100% water solubility, obtained for the first time from fenugreek seeds [11]. Anti-hyperglycemic and anti-hyperlipidemic, neuroprotective, anti-migraine and memory improvement potentials of TG have been demonstrated [12]. As a result of the literature search, we could not find any study evaluating the efficacy of TG in the eye. In this study; we evaluated the neuroprotective and antioxidant efficacy of TG in systemic and drop forms in the NMDA-induced excitotoxicity model. We compared the effectiveness of TG with Brimonidine Tartrate (BT), whose neuroprotective activity is known.

Material Method

Animals and ethics of study:

The rats are provided by Fırat University Experimental Research Center. In this study, 8-10 weeks old Wistar Albino male rats weighing 225-300 g were used. The rats were kept at room temperature at 22-25 °C for 12 hours in light (7.00-19.00) and in the dark for 12 hours (19.00-7.00). Add-libitum water and food were provided to all groups.

Groups:

Group 1 (Control Group); No procedure was applied during the experiment.

Group 2 (Negative Control Group); On the first day of the experiment (FDE), 2 µl of 0.1M PBS was administered intravitreally with a Hamilton injector. No treatment was applied.

Group 3 (Positive Control Group); FDE, 2 µl of 160 nmol/µl NMDA solution was administered intravitreally with a Hamilton injector. No treatment was applied.

Group 4 (Oral TG Group); FDE, 2 µl of 160 nmol/µl NMDA solution was administered intravitreally with a Hamilton injector and 100mg/kg daily dose of TG was administered with oral gavage method for 21 days.

Group 5 (Drop TG Group); FDE, 2 µl of 160 nmol/µl NMDA solution was administered intravitreally with a Hamilton injector. 20 mg/ml (1mg in each drop) one drop of eye drops was applied in the mornings and evenings for 21 days.

Group 6 (Drop BT Group); FDE, 2 µl of 160 nmol/µl NMDA solution was administered intravitreally with a Hamilton injector. BT (Allergan ALPHAGAN-P %0.15 5 ml) one drop of eye drops was applied in the mornings and evenings for 21 days.

Retinal excitotoxicity:

A combination of 50 mg/kg ketamine hydrochloride (Ketalar, Eczacıbaşı, Turkey) and 5 mg/kg xylazine hydrochloride (Rompun, Bayer, Turkey) was used for anesthesia and analgesia. This procedure was administered to all groups except the control group (Group 1). After intramuscular injection, topical 1% proparacaine hydrochloride was instilled into the right and left eyes of all rats. After the application, 0.1 M 2 µl

of PBS was injected at 1 mm behind the limbus with a 30-gauge Hamilton injector into both eyes of the rats in the group 2. 2 μl of 160 nmol/μl NMDA solution was administered intravitreally at 1 mm behind the limbus to both eyes of the rats in groups 3, 4, 5, and 6 with a 30-gauge Hamilton injector. By this means, retinal excitotoxicity was induced in Groups 3, 4, 5 and 6. After the operation, antibiotic drops were applied to all the eyes. Rats were decapitated on the 21st day of the experiment. Enucleation was performed on both eyes of the rats. Before the enucleation procedure, the orientation suture was passed at the 12 o'clock position in the right eyes of the rats. The right eyes of the rats were evaluated in the medical pathology department, and the left eyes were evaluated in the medical biochemistry department.

Histopathological Examination:

Hematoxylin-eosin staining:

The right eyes of the rats were marked with black ink on the temporal side 2mm ahead of the optic disc. Tissue samples were taken from the marked area in vertical sections to include all enucleation tissue. Hematoxylin-eosin staining was performed on 3-4 μm thick sections.

TUNEL Staining:

Sections of 3-4 μm thickness were obtained from formalin-fixed paraffin-embedded tissues. The sections were subjected to dehydration and rehydration. The tissues were incubated with 0.05% proteinase K for 10 minutes. It was incubated with 3% hydrogen peroxide for 5 minutes to inhibit endogenous peroxidase activity. The tissues were washed again with PBS. It was incubated with equilibration buffer for 6 minutes and incubated for 60 minutes with working solution (70 μl ReactionBuffer + 30% TdTEnzyme) in a humid environment at 37ºC. ABP Biosciences TUNEL Chromogenic Apoptosis Detection kit was used.

Tissues kept in Stop/Wash Buffer for 10 minutes were treated with Anti-Digoxigenin-Perosidase for 30 minutes. It was coated with diaminobenzidine (DAB) substrate. Sections that were counterstained with Mayer's hematoxylin were closed with the appropriate closure solution. The preparations were evaluated under an Olympus BX50 light microscope. In the evaluation of TUNEL staining, nuclei stained blue with Mayer hematoxylin were considered normal, cells with brown nuclear staining were considered apoptotic. 100 normal and apoptotic cells were counted in randomly selected areas at 40x magnification. Statistical analyzes were performed by calculating the apoptotic index (AI) by the ratio of apoptotic cells to total cells (Apoptotic Index = Total number of apoptotic cells / 100).

Caspase 3 and Brn3a Immunohistochemical Evaluation:

Sections of 3-4 μm thickness obtained from formalin-fixed paraffin-embedded tissues were taken on polylysine slides. Caspase 3(Fine test) and Brn3a(Bioss) antibodies were applied to these slides with Ventana brand Ultraview Universal DAB Detection Kit and Ultra DAB chromogen protocol in Ventana Bench Mark Ultra model automatic immunohistochemistry device. The preparations were evaluated with an Olympus BX50 light

microscope.
The prevalence of staining with Caspase 3 and Brn3a in 10 randomly selected areas at 40x magnification was scored as follows [13]:

Grade 0: There are no immune positive cells.

Grade 1: The immune positive cell is between 1% and 10%.

Grade 2: The immune positive cell is between 11% and 30%.

Grade 3: The immune positive cell is between 31% and 50%.

Grade 4: More than 51% immune positive cells.

Evaluation of Biochemical Samples:

Eye tissue samples were washed with 0.9% cold (+4ºC) sodium chloride (NaCl) and dried with blotting paper. It was homogenized in a 0.01M PBS (1/10) solution with a homogenizer at 16000 rpm for 4 minutes under appropriate conditions. The homogenate was centrifuged at 5000 xg for 1 hour (+4ºC) and the supernatants were separated and stored at –80°C until analysis. The amount of protein in the supernatants was determined in the Qubit Fluorometer device (Invitrogen, USA) using the Qubit protein measurement kit. SOD level from supernatant samples was determined by Enzyme-Linked Immuno Sorbent Assay (ELISA) method. Protein levels in the supernatants were determined according to the Lowry method. The principle of this method; It is based on the formation of a blue color by the Folin-Phenol reagent of proteins in alkaline environment [14].

Determination of Supernatant SOD Levels by ELISA Method:

Supernatant SOD levels were studied in accordance with the kit procedure using the rat SOD ELISA kit (Andy Gene Biotechnology Co., Ltd.). Absorbances were read spectrophotometrically at 450 nm on EPOCH 2 (BioTek Instrument, Inc, USA) microplate reader. Results were expressed as ng/L. The measuring range of the kit was 10-200 ng/L and the sensitivity was 1.0 ng/L. Intra-Assay CV was <8%; Inter Assay CV was <10%.

Tissue MDA Measurement:

MDA was measured using the method determined by Ohkawa et al. The principle of this method; It is based on the spectrophotometric measurement of the pink complex formed with thiobarbituric acid, at a wavelength of 532 nm, of the MDA released as a result of lipid peroxidation under the effect of temperature of the supernatants obtained from the tissue in an acidic environment (pH: 3.5) and under aerobic conditions. The results are given in nmol/mg protein by dividing the total protein concentrations [15].

Evaluation of Tissue iNOs Protein Levels by Western Blot Method:

Western blot; The transfer of the proteins migrated in the polyacrylamide gel by electrophoresis to the support membrane and the demonstration of the proteins in the membrane by immunological methods. Western blot technique is performed in four steps following electrophoresis. These steps; transferring the proteins in the gel to the nitrocellulose membrane (blotting), covering the nonprotein-bound regions in the nitrocellulose membrane with irrelevant proteins (blocking), reaction with specific antibodies, and imaging of the proteins in the last step.

Statistical Analysis:

Obtained data were determined as mean ± standard deviation. SPSS version 22 program was used for statistical analysis. Inter group evaluation was done with One-way ANOVA and Posthoc Tukey test. $P \leq 0.05$ values were considered statistically significant.

Results

TUNEL Results:

TUNEL, caspase 3 and Brn3a staining levels were detected in the Drop TG and Drop BT groups at a similar rate to group 2 (Tablo 1). TUNEL positive cells were found 9.8 times higher in group 3 than group 2 ($p<0.001$) . No statistically significant difference was observed between group 1 and group 2 ($p>0.05$). TUNEL positivity in the drop TG group was ~62% lower than in group 3 ($p<0.001$). TUNEL positivity was ~66% less in the drop BT group compared to group 3 ($p<0.001$). TUNEL positivity was observed in the drop TG and drop BT groups at a similar level to group 2 ($p>0.05$). There was no statistically significant difference between the oral TG group and group 3 ($p>0.05$) (fig. 1).

Caspase 3 Results:

Caspase 3 positivity was found to be ~12.5 times higher in group 3 compared to group 2 ($p<0.001$). No statistically significant difference was observed between group 1 and group 2 ($p>0.05$). Caspase 3 positivity was ~71% less in the drop TG group compared to group 3 ($p<0.001$). In the drop BT group, caspase 3 positivity was lower by ~67% compared to group 3 ($p<0.001$). Caspase 3 positivity was found to be similar to group 2 in the TG and BT drops groups ($p>0.05$). There was no statistically significant difference between the oral TG group and group 3 ($p>0.05$) (fig. 2).

Brn3a Results:

Brn3a positivity in group 3 was ~25% less than group 2 ($p<0.001$). No statistically significant difference was observed between group 1 and group 2 ($p>0.05$). It was observed that BRn3a staining was ~52% higher in the drop TG group compared to group 3 ($p<0.001$). In the drop BT group, ~64% more staining was detected compared to group 3 ($p<0.001$). Brn3a positivity was detected in the drop TG and drop BT groups at a similar

rate to group 2 ($p>0.05$). There was no statistically significant difference between the oral TG group and group 3 ($p>0.05$) (fig. 3).

Tissue MDA Levels:

MDA levels were found to be higher in group 3 compared to group 2 by ~38% ($p<0.001$) (fig. 5) . Compared to group 3, MDA levels decreased by ~31% in the oral TG group, ~30% in the drop TG group, and ~22% in the drop BT group ($p<0.05$). MDA levels in all treatment groups were similar to group 2 ($p>0.05$).

Tissue SOD Levels:

SOD level was ~61% lower in group 3 compared to group 2 ($p<0.001$). SOD level in the drop TG group was ~2.6 times compared to group 3; It was found to be ~2.3 times higher in the oral TG group and ~2.4 times higher in the drop BT group ($p<0.001$). Oral TG, drop TG, and drop BT groups had similar SOD levels with group 2 ($p>0.05$).

Tissue iNOs Protein Expression Levels:

When the tissue iNOs protein expression levels were considered in the control group (100%) and calculated; It was determined that iNOs expression level in group 3 increased by ~32% compared to the control group ($p<0.005$). Compared to group 3, reductions in iNOs protein expression levels were observed in oral TG (~15%), drop TG (~21%) and drop BT (~20%) groups. While these decreases were statistically significant in the TG drop and BT drop groups ($p<0.05$), they were not statistically significant in the oral TG group.

Discussion

Trigonelline is a substance that has been shown to elongate axons and dendrites, and to improve memory [16]. In the Parkinson's disease model created in rats to evaluate the antiapoptotic and neuroprotective efficacy of TG; It was observed that antioxidant system enzymes such as SOD, glutathione, and catalase increased, while MDA decreased [17]. In rats with learning and memory disorder models, TG has been found to decrease hippocampal MDA and acetylcholine esterase levels, as well as increase SOD, glutathione, and catalase levels [18]. Although it is known that systemic TG administration has neuroprotective effects in neurological diseases, we did not find a study evaluating the effectiveness of TG in the field of eye diseases in our literature review. Firstly, we created a model of NMDA-induced apoptosis in rats. Oral TG dose; As in Alzheimer's and Parkinson's experimental models, we administered 100mg/kg per day for three weeks [17,19]. Although no dose studies were performed on topical TG, we used a higher dose (20mg/ml) compared to the topical BT group (1.5 mg/ml), since no toxic effects were observed in high-dose systemic intakes of TG. We compared the results with BT which has been shown to have antioxidant and antiapoptotic properties in previous studies [20,21]. As a result of this study; in topical TG and BT groups; TUNEL and caspase 3 positivity, which are indicators of apoptosis, were statistically significant reduced compared to the positive control groups. This result shows immunohistochemically that drop TG protects from apoptosis just like drop BT. Likewise, the

significant decrease in MDA and NOS levels compared to the positive control group also supports the antiapoptotic activity of TG. On the other hand, statistically significant difference in Brn3a staining and SOD levels suggested that the antiapoptotic effect mechanism was via free oxygen radicals, consistent with the literature.

Brn3a family, it has been shown to play important roles in differentiation, survival and axonal elongation during the development of RGCs. Brn3a is known to stain RGCs specifically [9]. Caspase-3 is an important parameter used as an indicator of apoptosis. An increase in caspase 3 level indicates increased apoptosis and caspase-3 inhibitors are being tried as a new treatment option to increase RGC survival [22]. In our study, Brn3a staining and caspase 3 level in the drop TG group was found to be similar level to the negative control and drop BT groups, proving that retinal survival was increased with drop TG treatment.

NOS levels were found to be statistically significant increased in aqueous humor compared to controls on glaucoma patients [23]. In our study, in which we triggered the induction of iNOS by using NMDA, it was seen that drop TG could provide a statistically significant decrease in iNOS level and this decrease was similar to drop BT. SOD and MDA values were significantly different in the oral TG group, similar to the drop TG group, compared to the positive control group. However, there was no significant difference in caspase 3, Brn3a, TUNEL and iNOS values compared to the positive control group, unlike the drop TG group. We think that this difference is due to the fact that drop TG reaches a higher concentration in the vitreous than oral TG when used at the specified doses.

MDA initiates and drives lipid peroxidation, damages membranes, denatures DNA; suggests that the ongoing damage in glaucoma may be partially related to MDA [24]. In a study, the MDA level in aqueous humor samples of glaucoma patients was examined and it was found that there was a correlation between severe visual field loss and MDA enzyme level [25]. Activating antioxidant systems is very important in glaucoma patients, as it is promising in the treatment of many diseases. In the case of increased oxidants, the body initially responds by increasing the antioxidant systems, but in long-term pathological conditions, the antioxidant systems may be damaged [26]. In a study, it was determined that serum SOD, glutathione peroxidase and catalase levels in glaucoma patients were found to be statistically significant decreased compared to control groups, and the effectiveness of oxidative stress in glaucoma patients was demonstrated [27]. SOD is produced mostly in the retina among the eye tissues and is an important antioxidant marker in the retina [28]. In our study, while both drop TG and oral TG groups increased antioxidant-effective SOD levels, they decreased oxidant-effective MDA levels. It may also be beneficial in the treatment of eye diseases in which free oxygen radicals play a role in its pathophysiology. More studies are needed to determine the place of TG in the treatment of glaucoma.

In our study, similar efficacy was found for SOD and MDA levels in the groups treated with oral TG, drop TG and drop BT. In retinal histological evaluations, drop TG and drop BT were found to be effective in reducing apoptosis, while oral TG was found to be ineffective. While oral TG caused effective changes in many biochemical parameters, it did not provide an improvement in iNOS levels. Drop TG and drop BT similarly showed antiapoptotic activity by reducing iNOS at a statistically significant level. We think that TG eye drops, which have neuroprotective effects similar to those of BT, whose neuroprotective efficacy is known in clinical use, may be effective in the treatment of glaucoma.

Declarations

- None of the authors has a conflict of interest with or source of funding for the submission.
- This submission was supported by Fırat University Scientific Research Project Automation.
- This manuscript is not sent to another journal.
- The authors have no financial or proprietary interests in any material discussed in this article
- This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Fırat University.
- The materials used in the publication are preserved and can be re-evaluated if necessary

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Tables

Table 1

TUNEL, caspase 3 and Brn3a staining levels

	Group 1	Group 2	Group 3	Oral TG	Drop TG	Drop BT
TUNEL(Apoptotic Index %)	1,21	1,27[b,c]	12,52[a]	10,13[a,d]	4,67[b,c]	4,26[b,c]
Caspase 3 Score	0,11	0,12[b,c]	1,5[a]	1,17[a,d]	0,43[b,c]	0,5[b,c]
Brn3a Score	3,33	3[b,c]	2,25[a]	2,75[a,d]	3,43[b,c]	3,71[b,c]

a: $p<0.05$ compared to the group 1; b: $p>0.05$ compared to the group 1; c: $p<0.05$ compared with group 3; d: $p>0.05$ compared with group 3

Table 2

MDA and SOD levels

	Group 1	Group 2	Group 3	Oral TG	Drop TG	Drop BT
MDA(nmol/mg)	2,31	$2,34^{b,c}$	$3,23^{a}$	$2,24^{b,c}$	$2,27^{b,c}$	$2,54^{b,c}$
SOD(ng/L)	31,51	$37,36^{b,c}$	$14,6^{a}$	$34,73^{b,c}$	$38,46^{b,c}$	$35,98^{b,c}$
a: $p<0.05$ compared to the group 1; b: $p>0.05$ compared to the group 1; c: $p<0.05$ compared with group 3; d: $p>0.05$ compared with group 3						

Figures

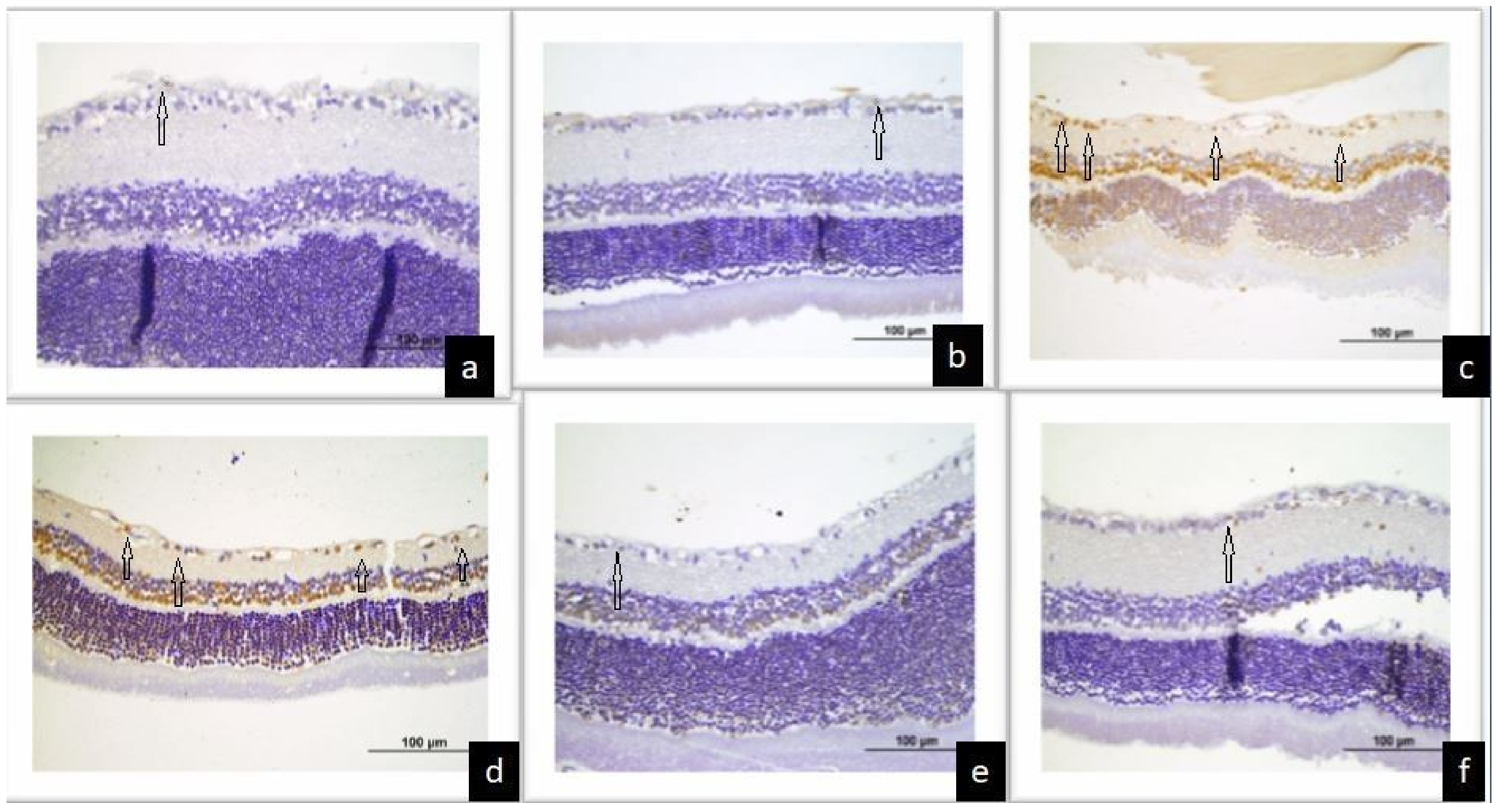

Figure 1

TUNEL staining. Group I (a) and Group II (b): few apoptotic cells (arrow); Group III (c) and Group IV (d): increased apoptotic cells (arrows); Group V (e) and Group VI (f) after treatment decreased apoptotic cell (arrow)

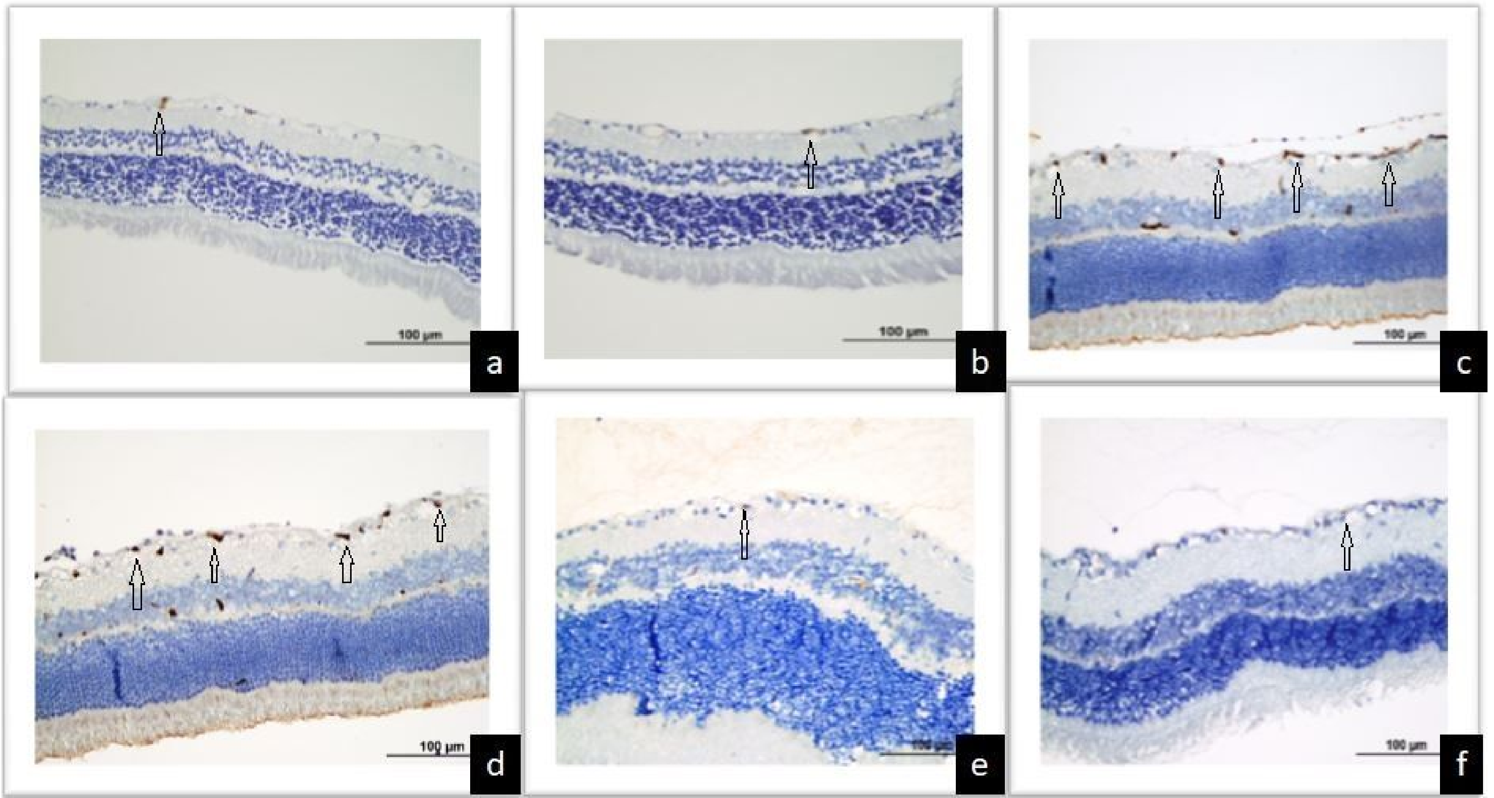

Figure 2

Group I (a) and Group II (b): few caspase 3 positive cells (arrow); Group III (c) and Group IV (d): increased numbers of caspase 3 positive cells (arrows); Group V (e) and Group VI (f) decreased numbers of caspase 3 positive cells after treatment (arrow)

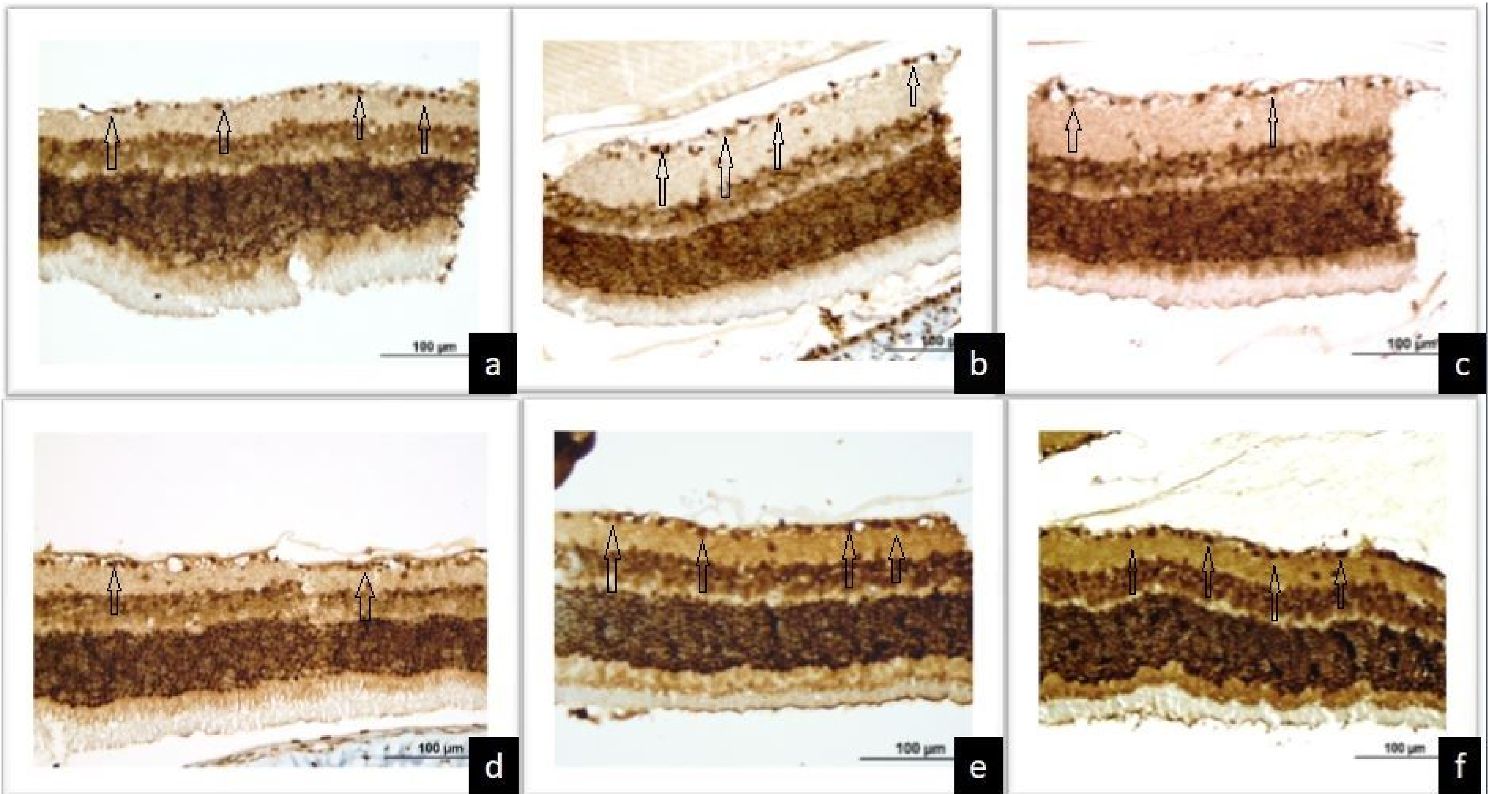

Figure 3

Group I (a) and Group II (b): increased ganglion cell staining (arrows); Group III (c) and Group IV (d): decreased ganglion cell staining (arrow); Increased number of ganglion cell staining after treatment in Group V (e) and Group VI (f) (arrows)