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EXPERIMENTAL GLAUCOMA MODEL (ISCHEMIA AND REPERFUSION): HISTOLOGY, MOPHOMETRY, PROTEIN AND GENE EXPRESSION OF APOPTOSIS PATHWAY

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Dissertação apresentada junto ao Programa de Pós-Graduação em Medicina Veterinária para obtenção do título de Mestre.

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SUMÁRIO

ABSTRACT

Purpose: The aims of this study were to better understand the mechanism of cell death by apoptosis in a glaucoma model (ischemia / reperfusion) and evaluate the role of apoptosis in this model and if treatment with Sildenafil helps prevent apoptosis.

Methods: 36 rats, from 4 to 6 months, males, Lewis and weighing ± 350g were divided in 5 groups: control group (6 animals) and for groups with ischemia / reperfusion (7 and 21 days), two groups consisting of ten animals treated with sildenafil and two groups of Five animals treated with placebo. Paracentesis of the anterior chamber with needle 30G coupled to saline (0.9%) was made and maintained for 60 minutes. Intraocular pressure was measured by rebound tonometer (Tonovet®). There was histological, morphometric by hematoxylin and eosin and, immunohistochemical staining and qRT-PCR analysis by Caspase-7, Caspase-6, Caspase-9, Tnf-r2, Fas-l, Bcl-2 and Bax. For statistic analysis we used ANOVA and t-test for morphometric analysis and, for immunohistochemistry and qRT-PCR, Fisher exact test was employed with a statistical significance level of $p < 0.05$

Results: Histology and morphometric analysis, proved more changes in the untreated group compared to the treatment and control group. Analysis of immunohistochemistry and qRT-PCR observed the more significant expression in untreated eyes.

Conclusion: Sildenafil apperead to be protective to ganglion cell apoptosis. Cell survival was evident in histology and morphometry. For immunohistochemistry and RT-PCR was observed protective effect in the apoptosis pathways with similar or below expression compared to the control.

INTRODUCTION

Glaucoma is a leading cause of loss of vision and blindness in the world (QUIGLEY, BROMAN, 2006; SAKATA et al., 2007). It leads to visual impairment in 1-4% of the population over 40 years of age (LESKE, 2007), though in some ethnic groups it may affect 20% or more of the population (DUGGAL et al., 2005), with an estimated 80 million people worldwide by 2020 and, 8.4 million of them will be blind in both eyes (QUIGLEY, BROMAN, 2006). Despite these, glaucoma remains an "unsolved" disease, as evidenced by the growing number of patients, stressing the urgent need to develop new strategies to treat this blinding disease (DUGGAL et al., 2005; QUIGLEY, BROMAN, 2006; SCHMIER, HALPERN, JONES, 2007; BALTMR et al., 2010).

A pathophysiological hallmark of glaucoma is the gradual loss of retinal ganglion cells (RGC) and their axons, the optic nerve (ON) fibers, like other neurons conditions such as Alzheimer's or Parkinson's disease (HAYREH, PEER, ZIMMERMAN, 1999; NICKELLS, 2007; LIN et al., 2014). Generally is accepted that glaucomatous degeneration of the optic nerve and retina is linked to the development of elevated intraocular pressure (IOP) (NICKELLS a, 2007). Because this, for many years it has been accepted that increase in intraocular pressure (IOP) is the cause of the disease (SOMMER, 1989).

There are evidences that in humans, in animals and, in models of experimental glaucoma, high IOP causes ganglion cell loss, and that lowering IOP in affected eyes can attenuate the degenerative process (NICKELLS a, 2007). However, today it is recognized that IOP elevation is the main risk factor in glaucoma, but evidence suggests that local ischemia, leading to reduced ON perfusion, is a key event in the pathogenesis of glaucomatous neuropathy (FLAMMER, MOZAFFARIEH, 2007; QUIGLEY, 2011). Reduced levels of nitric oxide (NO), a potent vasodilative mediator, have been demonstrated in glaucoma patients and animal models of the disease (as well as in other neurodegenerative diseases) and are implicated in the impairment of ocular blood flow, leading to neuronal death and loss of vision (DOGANAY et al., 2002; REICHSTEIN et al., 2007).

Several risk factors have been proposed to contribute to glaucoma progression including elevated intraocular pressure, age, genetic background, thinner corneal thickness and vascular degeneration (NICKELLS a, 2007). The existence of any of these factors might determine an individual risk to develop glaucoma, but they are not necessarily the cause of this condition (ALMASIEH et al., 2012). Research into the pathogenesis of glaucoma has been aided by the development of animal models that undergo apoptotic RGC death (REICHSTEIN et al., 2007).

Sildenafil, an approved drug for the treatment of erectile dysfunction, is an inhibitor of phosphodiesterase (PDE), an enzyme that degrades cGMP, the secondary messenger of NO. Therefore, treatment with sildneafil has been shown to be neuroprotective in numerous models of neurodegenerative diseases, as elevated cGMP levels prolong the effect of NO and lead to vasodilation and improved circulation (JOHNSTON, 2005; OREJANA et al., 2012). Sildenafil treatment has resulted in increased neuronal survival, and even neurogenesis, in models of brain, spinal cord and cerebral injury (SERARSLAN et al., 2010; OZDEGIRMENCI et al., 2011).

We propose that treatment with sildenafil may be similarly protective in glaucoma, and may thus offer a novel approach for therapeutic intervention in the pathogenesis of glaucomatous neuropathy. The present study aims to better understand the mechanism of cell death by apoptosis in glaucoma adding data to the scarce literature.

SCIENTIFIC BACKGROUND

Glaucoma

Historically, glaucoma has been described as a disease caused by increased ocular pressure (IOP). Recently glaucoma has been considered a primary optic neuropathy, but this fact is not widely shared by the public and health-care professionals alike. An informal survey of mainstream dictionary definitions of glaucoma still reveals obsolete phrases such as 'increased intraocular pressure that results in a group of eye diseases characterized by pressure that is too high for the optic nerve to withstand' (WEINREB, 2007; VRABEC, LEVIN, 2007).

However, there is abundant evidence demonstrating that ocular hypertension alone is insufficient, but a necessary factor for the development or progression of glaucoma. The Cochrane Eyes and Vision Group define glaucoma as 'a disease characterized by defects in the visual field, damage to the nerve at the back of the eye, and usually raised pressure inside the eye.' This view of glaucoma as an optic neuropathy with elevated IOP as a modifiable risk factor rather than a causative agent for damage, although it still does not take into account the characteristic morphological and functional aspects of the disease (VRABEC, LEVIN, 2007).

Primary humans, glaucoma is classified into three principal types: primary open angle glaucoma (POAG), primary angle-closure glaucoma and congenital glaucoma. The POAG is the most common type in humans (OFRI et al., 1993). However, for example, although high intraocular pressure is common among open-angle glaucoma patients, only a limited subset of individuals with ocular hypertension will develop this disease (OFRI et al., 1993; FRIEDMAN et al., 2004). Moreover, a significant number of patients presenting with glaucoma continue to lose vision despite responding well to therapies that lower eye pressure (ALMASIEH et al, 2012, FRIEDMAN et al., 2004).

In dogs, the main protagonists of glaucoma in animals, can be classified based on the primary cause, in primary, secondary or congenital. Based on the appearance of gonioscopy drainage angle, open, closed or narrow and, according to the duration or with stage of evolution, chronic or acute. Chronic glaucoma is revealed with the progressive elevation of IOP. In contrast, the acute characterized by a sudden increase in IOP (PLUMMER; REGNIER; GELATT, 2013).

Glaucoma has been investigated for some 50 years in dogs. The highest incidence has been reported to Beagle, Welsh Springer Spaniel, and many other breeds.

Uveitis and neoplasms are the most common eye diseases related to glaucoma. POAG studies in dogs showed that breeds Shiba-Inu and Shih-Tzu are the most affected, however, it is reported that the type of primary angle closure there is the most common in this breeds (PLUMMER; REGNIER; GELATT, 2013, SCOTT et al., 2013).

In humans, loss of vision in glaucoma patients is due to progressive death of RGCs and their axons, which constitute the ON fibers (HAYREH; PEER; ZIMMERMAN, 1999; NICKELLS, 2007). Traditionally this neuronal and axonal death has been attributed to increase in intraocular pressure (IOP), but now recognized a significant portion of glaucoma cases present with normal or low IOP (CRISH, CALKINS, 2011), and it is the main accepted that IOP elevation is one of several risk factors for the disease (QUIGLEY, 2011; CRISH; CALKINS, 2011). Evidence suggests that another, as local ischemia, leading to reduced ON perfusion cause glaucomatous neuropathy is reduced blood flow in the inner retina and ON (FLAMMER et al, 2002).

Current evidence shows that patients suffer from low ocular perfusion pressure, as well as abnormal vascular auto regulation capacity, impairing the ability to adapt to increased IOP or decreased blood pressure (FLAMMER, MOZAFFARIEH, 2007; FLAMMER et al., 2002). The result is RGC death and ON atrophy, as well as reperfusion injury and ON remodeling. Research into the pathogenesis of glaucoma has been aided by the development of animal models that undergo apoptotic RGC death (REICHSTEIN et al., 2007; GRIVICICH, REGNER, DA ROCHA, 2007; LUCHS, PANTALEÃO, 2010).

The implication of these data is that current medical and surgical therapy, aimed at lowering IOP, has limited success in preserving vision in glaucoma patients. Therefore, it is evident that there is a pressing need to develop new therapeutic approaches that can be used, in conjunction with hypotensive drugs, to preserve vision (WEINREB, 2007).

Apoptosis

Apoptosis is a modality of programmed cell death that can be identified by different morphological characteristics and the involvement of specific proteins that regulate it (REGNER, DA ROCHA, 2007; LUCHS, PANTALEÃO, 2010). Cell death also is part of normal development and maturation cycle, and is also component of many response patterns of living tissues to xenobiotic agents (i.e. micro organisms and

chemicals) and to endogenous modulations, such as inflammation and disturbed blood supply (CLAVIEN et al., 2000).

Cell death is a fundamental cellular response that has a crucial role in shaping our bodies during development and in regulating tissue homeostasis by eliminating unwanted cells. The first form of regulated or programmed cell death to be characterized was apoptosis (DEGTEREV, YUAN, 2008). The term 'apoptosis', defined as a controlled type of cell death that can be induced by a variety of physiologic and pharmacological agents, was first described by Kerr, Wyllie, and Currie in 1972 to describe a morphologically distinct form of cell death on the basis of the following main morphologic criteria: cellular shrinkage, condensation and margination of the nuclear chromatin, DNA fragmentation, cytoplasmic vacuolization, membrane blebbing, cell lysis, and the formation of apoptotic bodies (KERR et al., 1972).

However, the apoptosis pathway was described in Caenorhabitis elegans in the early 1990s. Subsequent genetic analysisof mammalian apoptosis presented a more complex, in which individual apoptosis genes from C. elegans have expanded into large multi-protein families. These findings suggest a redundancy, functional specialization and compensatory regulation of mammalian apoptotic signalling and execution might be important features of mammalian apoptosis (DEGTEREV; YUAN, 2008).

In some cases there is the type of stimuli and/or the degree of stimuli that determines if cells die by apoptosis or necrosis. At low doses, a variety of injurious stimuli such as heat, radiation, hypoxia and cytotoxic anticancer drugs can induce apoptosis but these same stimuli can result in necrosis at higher doses. Finally, apoptosis is a coordinated and often energy-dependent process that involves the activation of a group of cysteine proteases called "caspases" and a complex cascade of events that link the initiating stimuli to the final demise of the cell (ELMORE, 2007).

Using conventional histology, it is not always easy to distinguish apoptosis from necrosis, and they can occur simultaneously depending on factors such as the intensity and duration of the stimulus, the extent of ATP depletion and the availability of caspases. Necrosis is an uncontrolled and passive process that usually affects large fields of cells whereas apoptosis is controlled and energy-dependent and can affect individual or clusters of cells. (ZEISS, 2003; ELMORE, 2007).

Necrosis proceses characteristic features, such as organelle swelling, mitochondrial dysfunction, massive oxidative stress and rapid plasma-membrane permeabilization that are thought to be indicative of the catastrophic nature of cell

death, rather than a result of cellular regulation. The general view of the relationship between apoptosis and necrosis is that milder insults to the cell cause apoptosis, whereas more intense insults induce uncontrollable necrosis. It is thought that apparently unregulated, the process accounts for the bulk of cell death events in acute pathologies (ELMORE, 2007, DEGTEREV; YUAN, 2008).

The primary mechanism of RGC damage in glaucoma is not well understood, but there is evidence that neuronal loss in this disease occurs largely by apoptosis. This selfdestructive, genetically driven, death program is activated in all neurons. It is now widely accepted that neurotrophic factors promote neuronal survival by inhibiting default apoptotic pathways. During development of the nervous system, young neurons require trophic factors for their survival, differentiation and the establishment of synaptic connections (DEGTEREV; YUAN, 2008; ALMASIEH et al., 2012).

Neurotrophic factors are produced in limited amounts; therefore only neurons exposed to optimal levels of these molecules survive, whereas less fortunate neurons are eliminated by apoptosis. In rodents, 65% of RGCs die during retinal development. Excess RGCs are eliminated in two successive phases of cell death in the retina: the first phase peaks at embryonic day 6, when RGCs differentiate; and the second phase coincides with the arrival of RGC axons to the brain, when these neurons become dependent on target-derived trophic support (LEWIN et al., 1998; ALMASIEH et al., 2012).

The induction of apoptosis can occur by external or internal stimulus. Two major general pathways of induction of apoptosis: receptor or extrinsic pathway and the mitochondrial or intrinsic pathway (GRIVICICH; REGNER; DA ROCHA, 2007). The extrinsic pathway promotes the activation of caspase 8, which activates caspase 3 or cleaves at pro-apoptotic gene bcl-2 family, by linkingto the Fas receptor and TNFR (PAROLIN; REASON, 2001). The intrinsic pathway is mediated by internal stimuli of intracellular stress, as well as DNA lesion or disruption of the cell cycle or in metabolic pathways (LUCHS; PANTALEÃO, 2010).

Once activated, the majority of the caspases have the ability to catalyze the activation of multiple other members of this family, resulting in amplification of the proteolytic cascade (PAROLIN; REASON, 2001). The caspases is classified in two groups: the initiator caspases (caspase-2, 8, 9 and 10) and executors caspases (caspase-3, 6 and 7). The functional forms of the initiator caspases promote directly or indirectly

the activation of executors caspases (PAROLIN; REASON, 2001; GRIVICICH; REGNER; DA ROCHA, 2007; LUCHS; PANTALEÃO, 2010).

Both extrinsic and intrinsic pathways and at the point of the execution phase, considered the final pathway of apoptosis. It is the activation of the execution caspases that begins this phase of apoptosis. Execution caspases activate cytoplasmic endonuclease, which degrades nuclear material, and proteases that degrade the nuclear and cytoskeletal proteins (ELMORE, 2007).

In the extrinsic pathway, an active death receptor recruits the intracellular adaptor protein Fas-associated death domain which in turn recruits procaspase-8 to form a signaling complex. Caspase-8 is cleaved and activated through autoproteolysis leading to subsequent activation of caspase-3 and caspase-6. The expression of both initiator and effector caspases has been investigated in RGCs following acute or chronic optic nerve injury. Active, cleaved caspases- 3, -8 and -9 have been detected after optic nerve transaction or crush, ocular hypertension as well as ischemic injury (HANNINEN et al., 2002; LUCHS; PANTALEÃO, 2010; ALMASIEH et al, 2012). Although caspase-3 was implicated in the primary and secondary waves of RGC apoptosis, it was active for a long period of time and with greater intensity during the primary wave of RGC loss (LEVKOVITCH-VERBIN et al., 2010).

Extrinsic apoptotic signals include an array of death-receptor ligands: Tnf-α, Fas-l, and TNF-related apoptosis-inducing ligands (TRAIL) that bind to their respective receptors to induce cell death. Death receptor activation results in the recruitment of the intracellular adaptor Fas-associated death domain (FADD), which typically recruits the initiator procaspase-8 leading to caspase-8 activation followed by executioner caspase-3 activation and cell death (ALMASIEH et al., 2012).

In the intrinsic pathway, cytochrome C is released from the mitochondria and together with Apaf-1 and procaspase-9 forms the apoptosome, which facilitates caspase-9 activation and downstream cleavage of caspase-3. Cytochrome c, which is released from damaged mitochondria, promotes the formation of a heptameric ‘apoptosome’ megacomplex of APAF1 and caspase-9 (a member of the CED-3-like Cys protease family). This leads to the conformational change and activation of caspase-9. Activated caspase-9 in turn cleaves and activates downstream caspases, including caspase-3, caspase-6 and caspase-7 that carry out the execution phase of apoptosis (DEGTEREV, A., YUAN, 2008; ALMASIEH et al., 2012).

The BAX protein is normally present in the cytoplasm of a cell, but upon activation of the cell death signal, it will translocate and insert into the mitochondrial outer membrane. Several studies suggest that BAX monomers can form a multisubunit pore structure large enough to allow the escape of molecules like cytochrome c. Knock-out mice lacking a functional Bax gene exhibit several supernumerary populations of neurons, including retinal ganglion cells, indicating the importance of Bax in regulating neuronal programmed cell death during development (WEI et al., 2001; KIRKLANDRAet al., 2002).

The central hypothesis of excitotoxic injury is that excess glutamate binds to cell surface ionotropic glutamate receptors, primarily N-Methyl-D-Aspartate (NMDA) receptors (NMDAR), triggering massive Ca influx and activation of pro-apoptotic signaling cascades in neurons. Elevation of endogenous glutamate and activation of glutamate receptors have been shown to contribute to a variety of acute and chronic neurological disorders, including stroke, trauma, seizures, and various forms of dementia and neurodegeneration (KALIA et al., 2008; NING et al., 2013)

In the retina, excess glutamate has been proposed to underlie common neurodegenerative disorders such as retinal artery occlusion and glaucoma. A vast number of studies have now demonstrated that adult RGCs are exquisitely sensitive to exogenously applied NMDA, which triggers rapid death of these neurons, and that inhibitors of NMDAR and/or downstream pathways are neuroprotective in experimental models of retinal ischemia and glaucoma (SEKI et al., 2010; ALMASIEH et al., 2012; NING et al., 2013).

Neuroprotection and sildenafil

Though therapy for glaucoma is focused on reducing IOP, much has been dedicated to the development of drugs that provide neural protection. Substances have been suggested as candidates for neuroprotective therapy based on inhibition mechanisms of degeneration and apoptosis of RGCs, in order to promote their survival (SANDALON et al., 2013). Nitric oxide (NO) is a gaseous molecule labile released from endothelial cells. It induces vasodilation, increased blood flow and decreased vascular resistance. Its inhibition leads to reduced perfusion. It is not therefore surprising that vascular endothelial dysfunction, resulting in decreased levels of NO, is found in glaucoma patients developing increased retinal vascular resistance. Deficient production of NO is therefore a participant in the pathogenesis of glaucoma, while the

increased synthesis and release may prevent the progression of harmful manifestations (DOGANAY et al., 2002; JOHNSTON, 2005; TODA; NAKANISHI-TODA, 2007; OREJANA et al., 2012).

Vasoactive agents were tested, including tadalafil and sildenafil employed in the treatment of erectile dysfunction. Sildenafil is a new vasoactive drug that has been developed for the treatment of erectile dysfunction. It increases intracellular cGMP through inhibition of PDE enzyme as well as by enhancement of NO/cGMPmediated signaling. There is experimental evidence that increasing intracellular cGMP can prevent oxidative stress induction and lipid peroxidation (ABDOLLAHI et al., 2003; MILANI et al., 2005).

Therefore, treatment with sildneafil has been shown to be neuroprotective in numerous models of neurodegenerative diseases, as elevated cGMP levels prolong the effect of NO and lead to vasodilation and improved circulation (JOHNSTON, 2005; OREJANA et al., 2012). Sildenafil treatment has resulted in increased neuronal survival, and even neurogenesis, in models of brain, spinal cord and cerebral injury (SERARSLAN et al., 2010; OZDEGIRMENCI et al., 2011).

It enhances the vasodilator effect of nitric oxide (NO) on the sinusoidal and vessel smooth muscles of the corpus cavernous and increases the arterial blood flow into the sinusoids. Therefore, treatment with sildneafil has been shown to be neuroprotective in numerous models of neurodegenerative diseases, as elevated cGMP levels prolong the effect of NO and lead to vasodilation and improved circulation (JOHNSTON, 2005; OREJANA et al., 2012). Sildenafil treatment has resulted in increased neuronal survival, and even neurogenesis, in models of brain, spinal cord and cerebral injury (SERARSLAN et al., 2010; OZDEGIRMENCI et al., 2011).

The sildenafil has powerful cardioprotective effects and could reduce apoptosis and necrosis in cardiac tissues after ischemia–reperfusion injury (SALLOUM et al., 2008). Ebrahimi, et al. (2009) observed that sildenafil reduced diabetes-induced cardiac cell apoptosis at the end of the first and second weeks, when it reached its peak value. Previous studies have also shown the cardioprotective effects of sildenafil in different experimental models. Fisher et al. (2005) demonstrated that sildenafil attenuated cardiomyocyte apoptosis in a chronic model of doxorubicin cardiotoxicity. Salloum et al. (2008) also showed that acute and prolonged treatment with sildenafil during myocardial infarction (MI) was associated with myocardial salvage from necrosis, reduction of apoptosis, prevention of adverse cardiac remodeling and heart failure, and

improved survival. In the eye the sildenafil can affect ocular blood flow and choroidal volume due to its effects on vascular smooth muscles. It may also have vasodilator effects on choroidal sinusoids and retinal vessels similar to the vasodilation of corpora cavernosa sinusoids (MARMOR, 1999).

This drug is frequently used in older patients with erectile dysfunction and vascular diseases. This population is also at risk of many ophthalmological diseases, such as senile macular degeneration, glaucoma, diabetic retinopathy or ischaemic ocular problems (KOKSAL et al., 2005). In contrast, studies showed that NO causes increased DNA damage, apoptosis, neurotoxicity and inflammation. When the concentration of NO is more than 1 mM, the predominant effects mediated by him include deamidation, oxidation or nitration of DNA via interaction of NO with superoxide or oxygen radicals. When the concentration is less than 1μM, their actions are direct, without interacting with superoxide radicals or oxygen, may regulate physiological activities by different signaling pathways (LEE; CHENG, 2004).

Scientific Article

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Experimental Glaucoma Model (Ischemia and Reperfusion): Histology, mophometry, protein and gene expression of apoptosis pathway

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Purpose: The aim of this study was to better understand the mechanism of cell death by apoptosis in glaucoma model (ischemia / reperfusion) and check the role of apoptosis in this model and also if treatment with Sildenafil helps prevent apoptosis.

Methods: 36 rats lineage Lewis, adults, males and weighing 350g. The animals were divided in control group (6 animals) and two groups with ischemia / reperfusion (7 and 21 days), each group consisting of ten animals treated with sildenafil and five placebo. Made paracentesis of the anterior chamber with needle 30G coupled to saline (0.9%) and maintained for 60 minutes. Intraocular pressure was measured by rebound tonometer (Tonovet®). There was histological, morphometric by hematoxylin and eosin and, immunohistochemical staining and qRT-PCR analysis by Caspase-7, Caspase-6, Caspase-9, Tnf-r2, Fas-l, Bcl-2 and Bax.

Results: Histology and morphometry analysis, the untreated group there was more changes compared to the treatment and control group. Analysis of immunohistochemistry observed the more significant markup trend in untreated eyes.

Conclusion: Sildenafil treatment is neuroprotective to retinal. Cell survival was evident in the retinal ganglion cell layer of animals treated, shown in histology and morphometry. For immunohistochemistry and qRT-PCR was observed protective effect in the apoptosis pathways with similar expression compared to the control.

Key Words: Glaucoma, Ischemia-reperfusion, Retinal ganglion cell, Apoptosis

INTRODUCTION

The glaucoma is a class of optic neuropathies with manifest visual defects, characterized by gradual degeneration of neuronal tissue due to retinal ganglion cell (RGC) loss and their axons, the optic nerve (ON) fibers, with accompanying loss of visual field over time [1, 2, 3, 4, 5]. It is one of the major causes of blindness in the world and, elevated intraocular pressure is a well-known major risk factor for glaucoma. In addition, there is growing evidence that local ischemia, vascular factors and, the reduced perfusion in the ON may play a role in glaucoma pathogenesis [4, 6, 7, 8].

Glaucoma can be viewed as a neurodegenerative disease, which, like other conditions such as Alzheimer's or Parkinson's disease, is ultimately caused by deficits in neuronal function [9, 10].

Several risk factors have been proposed to contribute to glaucoma progression including elevated intraocular pressure, age, genetic background, thinner corneal thickness and vascular deregulation [12, 13]. The existence of any of these factors determines an individual's risk to develop glaucoma, but they are not necessarily the cause of this condition[37]. Research into the pathogenesis of glaucoma has been aided by the development of animal models that undergo apoptotic RGC death[11].

The implication of these data is that current medical and surgical therapy, aimed at lowering IOP, has limited success in preserving vision in glaucoma patients. Therefore, it is evident that there is a pressing need to develop new therapeutic approaches that can be used, in conjunction with hypotensive drugs, to preserve vision [14, 15].

Sildenafil Citrate (SC), a selective cyclic guanosine monophosphate (cGMP)-dependent phosphodiesterase type 5 (PDE-5) inhibitor, induces vasodilatation by enhancing the smooth muscle relaxant effects of nitric oxide. cGMP has been reported to be involved in the regulation of aqueous humor formation and outflow facility, processes that influence aqueous humor dynamics and intraocular pressure [16-18].

Sildenafil could theoretically have a neuroprotective effect in retina and RGC. Hence, we propose that treatment with sildenafil may be protective in glaucoma, and may thus offer a novel approach for therapeutic intervention in the pathogenesis of glaucomatous neuropathy. The present study aims to better understand the mechanism of cell death by apoptosis in induced glaucoma in an animal model, adding data to the

scarce literature, and to verify if the Citrate Sildenafil treatment may help to prevent GCR apoptosis.

METHODS

Animals

Thirty-six rats (*Rattus norvegicus*), SsNHsd2 lineage, over 6 months, males, average weight between 350g to 450g were used. The animals were clinically and ophthalmically evaluated by routine methods and allocated in a ventilated environment, clean, sanitized and appropriate cages, with access to food and water "*ad libitum*", and were kept under 12-hour cycles of light and controlled temperature. The research was performed attending the standards of the Association for Research in Vision and Ophthalmology – ARVO, as well as the approval of the Ethics Committee on Animal Use (CEUA), Faculty of Agricultural and Veterinary Sciences (FCAV) Universidade Estadual Paulista (UNESP), Jaboticabal (Protocol No. 009328-13).

Groups and experimental protocols

The animals were divided in Five groups, control group (CG), consisting of six animals without glaucoma and four groups with induced acute glaucoma, treated for different periods of time: Sildenafil group – 7 days (SG 7); Placebo groups – 7 days (PG 7); Sildenafil group – 21 days (SG 21); Placebo group – 21 days (PG 21). From these groups, ten animals were treated with SC and five treated with placebo (Table 1).

The induction of glaucoma was held by paracentesis of the anterior chamber in aleatory eye. A 30G gauge needle was coupled to one vial of 0.9% saline, positioned at height of 150cm from the eye. The device was kept in the eye for 60 minutes and IOP was evaluated by using the rebound tonometer (Tonovet®) before, at 3, 30, and 57 minutes of induction (Table 2). Topical of proparacaine hydrochloride (0.5%), and the combination of intramuscular ketamine (75 mg / kg) and xylazine (10 mg / kg), were used for anesthesia. After the experiment the animals received dipirona (200mg/Kg) and morphine (1mg/Kg) to analgesia and, SC at a concentration of 1 mg/Kg (Table 1) by gavage. Placebos was given 0.9% saline solution, with equal criteria. All animals were euthanized by intramuscular injection from combination of ketamine (75 mg / Kg) and xylazine (10 mg / Kg) and subsequent intra cardiac injection of sodium pentobarbital (120 mg / kg), 1 hour after the last dose on the last day of treatment.

Collection, storage and preparation of samples

After euthanasia, both eyes were excised with the optic never and fixed in 10% buffered formaldehyde solution (pH 7.4) for 48 hours. The eyes were than washed in running water, maintained in 70% ethanol. The samples were routinely processed for paraffin embedded. The entire eye was embedded in paraffin and the paraffin block was cut transversely in 3μm thick consecutive sections, in an automatic microtome (RM2255), until it reached the center of the optic nerve, including the retina. For each eye a slide was made, and HE stained for morphometry and histopathology evaluation. Charged slides were cut for immunohistochemistry.

Morphometry and histology

The morphometry was made with the assistance of an image analysis program, Leica QWin v3.013 in conventional optical microscope (Leica DMR) equipped with digital camera (Leica, DFC500). Five images (fields) were obtained from each slide in 20x objective, considering the full extent of the retina, from the ora serrata to ora serrata, including the optic nerve in its central portion. From each image, five evaluations were made: total thickness of the retina and from the each layer (ganglionar cell layer; inner plexiform; inner nuclear; external plexiform; external nuclear; ganglionar cell count). The retina ganglionar cells were counted from the same fields. From each treatment group, the mean of each layer and mean number of ganglionar cells were compared with control group.

Hispathological evaluations were done according to Johnson et al. (2000) [19] and Schmid et al (2014)[20], including inflammatory changes (edema, and inflammatory infiltrate), cells (necrosis/apoptosis/chromatin) and tissue changes (atrophy). We used descriptive analysis and, in both analyses were performed blinded.

Immunohistochemistry

We evaluated the tissue distribution and intensity of the Anti Caspase-7, Anti Caspase-6, Anti-caspase-9, anti-Tnf-r2, anti-Fas-l, Anti Bcl-2, and anti-Bax primary antibodies, focusing on RGCs. Briefly, the slides were deparafinized before antigen retrieval, performed in accordance with the protocol established for each primary antibody (Table 3). The slides were cooled at room temperature for 20 minutes, rinsed in deionized water and treated with 8% hydrogen peroxide in methanol, for 20 minutes

for endogenous peroxidase blockage. Subsequently, they were rinsed in TRIS buffer for five minutes. Sections were subjected to protein block with skimmed milk 16% (Molico®) for 60 minutes and rinsed in TRIS buffer, twice for five minutes each.

The material was incubated with primary antibody (Table 3). Subsequently, the slides were washed in TRIS buffer pH 7.4. Immediately, they were incubated with secondary antibody Histofine (Histofine Simple Stain MAX PO, Nichirei, Tokyo. Japan) or LSAB (LSAB System HRP K0690, Dako, USA), in accordance with the established protocol. Staining was obtained using 3,3 diaminobenzidine tetrachloride (DAB, Liquid DAB Cromogen®, Carpinteria, USA). The sections were counterstained with Harrys hematoxylin and dehydrated in increasing alcohol concentrations and xylene. The slides were mounted with Entellan and coverslips.

For negative controls, primary antibodies were replaced by TRIS buffer and for positive control, tissues from rat that expressed the protein were chosen according to manufacturer for each primary antibody and to the website: www.proteinatlas.com.

We used semi-quantitative index to discriminate the percentage of positive cells in the GCL, in scores: 1 – 1-25% of positive cells, 2- 26-50% of positive cells, 3- 51-75% of positive cells and 4->76% of positive cells and for intensity staining, classified as mild (1), moderate (2) or strong (3) for each antibody. The distribution and intensity of each antibody was compared to the scores in the control group (for example: Bax normal eye score was >3, scores less than 3 were considered protein down expression).

qRT-PCR

We evaluated the *Caspase-7*, *Caspase-6*, *Caspase-9*, *Tnf-r2*, *Fas-l*, *Bcl-2*, and *Bax*. Samples of paraffin blocks were selected for gene expression. The samples were cut transversely in 4μm consecutive sections, in an automatic microtome (RM2255) into sample appropriate RNA extraction from only the conditions to be studied. The process of deparaffinezed, digestion of protease, isolation of nucleic acid, digestion of nuclease and purification of nucleic acid, were developed by using a commercial RecoverAll™ Total Nucleic Acid kit (Ambion, Life Technologies, MA, USA), according to the manufacturer's instructions. RNA concentration was assessed by spectrophotometer (NanoDrop™, ND-8000, Thermo Scientific, MA, USA) while the RNA integrity was evaluated with Bioanalyzer 2100 and Agilent RNA 6000 Nano kit (Agilent Technologies, CA, USA).

Quantitative reverse transcription-PCR: cDNA synthesis was performed using 2 μg of total RNA in 20 μl reaction volume. This was done using an oligo dT primer according to the manufacturer's manual (Affinity Script; Stratagene; Agilent technologies SA, Morges, Switzerland). The equivalent of 12.5 ng of original total RNA was used for quantitative PCR amplification using the 2× brilliant SYBR Green QPCR Master Mix (Stratagene; Agilent technologies SA, Morges, Switzerland). One μM forward and reverse primers, designed to span an intron of the target gene were used in this study. qRT-PCR was performed with the following cycling conditions: 40 cycles of denaturation at 95 °C for 30 s, annealing at 59 °C for 30 s, and extension at 72 °C for 30 s. Samples were amplified in triplicate, and data were normalized with expression of *β-actin*, *18s*, *B2m*, *Gapdh*, *Hprt*.

Statistics

For the values of total retinal thickness and its layers and the total cell count in the ganglion cell layer, the results were evaluated for normality by applying the Kolmogorov-Smirnov test. We used variance analysis (ANOVA and t-test) to compare the mean of each variable between groups. For the immunohistochemistry scores and RT-PCR, Fisher exact test was employed with a statistical significance level of $p < 0.05$. Regarding the presence of histological changes, descriptive analysis was employed.

RESULTS

Morphometry and Histopathology

In both groups, PG and SG 7 and 21 days there was decrease of the total retina thickness and each layer compared to CG, showing a statistically significant difference ($p < 0.0001$). However, there was no statistical difference between the PG and SG 7 days treatment ($p > 0.4824$), but SG retinal total thickness (168 ± 30,55μm) was closer to control group. In 21 days group there was statistical difference between treatment groups ($p < 0.005$), and the PG demonstrated the thinner retina total thickness (149 ± 20μm.) (Figure 1 and Table 4).

The RGC layer, as to its thickness, was statistically significant in PG and SG (7, 21 days) in comparison to controls ($p < 0.0001$) and between PG and SG (7 and 21 days), so the SG 7 and 21 days presented more preserved (10±2,9μm and 9,2±2,5μm respectively) compared to placebo. (Figure 1 and Table 4).

The thickness of the inner plexiform layer showed decrease statistically significant in SG and PG groups (7 and 21 days) when compared to CG, $p < 0.0001$ for both. There was also a significant difference between the PG and SG groups, on days 7 and 21, $p < 0.0002$ and $p < 0.0045$ respectively, with decreased of thicknesses high in animal eyes treated with placebo. (Figure 1 and Table 4).

There was decrease statistically significant in the external nuclear layer of the retina in PG and SG 7 and 21 days compared to the control group, $p < 0.0001$ for both. In addition, there is no significant difference between the PG and SG groups, on 7 and 21 $p > 0.2206$ $p > 0.4152$ respectively, however, there are more decrease of this layer in PG (Figua 1 and Table 4).

The external plexiform layer was thinner in PG, both in 7 and 21 days treatment, when compared to SG and control animals. SG was closer to control animals, showing the protective effect of sildenafil in both 7 and 21 days treatment (Figure 1 and Table 4).

The external nuclear layer of the retina revealed a decrease in the thickness in both PG and SG, 7 and 21 days treatment, statistically significant, when compared to the control group ($p < 0.0001$ for both). We also observed a reduced in this layer in the SG in 7 day treatment when compared to PG ($p < 0.0461$) (Figure 1 and Table 4).

The RGCs counting was statistically significant when compared PG and CG (7 and 21 days) and CG, $p < 0.0045$ and $p < 0.0060$ respectively, and between PG and SG, showing that SG has the RGCs count closer to control animals. (Figure 1 and Table 4).

In the histopathological analysis, most of the changes were cellular. There was a higher prevalence of lesions in the PG, when compared to controls and SG, notably as to RGC death (Figures 2, 3, 4 and Table 5).

Immunohistochemical

Immunohistochemical results are present in Tables 6 and 7. Statistical analysis of immunohistochemistry showed significant difference for Caspase - 7 (7 days treatment group), $p < 0.0170$, distribution score, when comparing SG and PG with control eyes.

Although there was no statically difference in Tnf-r2 expression between PG and SG in both 7 and 21 days treatment groups, there was more animals in SG groups, with lower Tnf-r2 scores, than PG groups, being more similar to CG. (Figure 5; Tables 6 and 7). The same pattern was observed for Fas-l, Bcl-2 and Caspase-9.

qRT-PCR

qRT-PCR results are present in figure 6. Statistical analysis showed significant difference in all genes studied, when comparing SG and PG with control eyes, however, in SG 21 days (*Bax*), SG 21 days (*Caspase-6*), there was not significant difference when compared to control. *Caspase*-9 trasncript was not observed in control group, since it only amplified in late cycles. *Tnf-r2, Fas-l, Bax, Bcl-2, Caspase-9, Caspase-6* and *Caspase-7* transcript levels were higher in PG (7 and 21 days) in comparison to control group and SG. SG transcript levels for these genes were closer to control group, than PG, showing a protective action of SC treatment in induced glaucoma.

DISCUSSION

A number of *in vivo* and *ex vivo* mammalian models have been developed to study retinal ischemia. Clearly the ability to extrapolate data from an animal model to the clinical situation requires a model that closely resembles human retinal ischemia. Small laboratory animals like rat, rabbit and guinea pig are commonly used, but all have a different vascular supply. Even so the rat model presents the most similar to humans [21, 22, 23, 24].

Our method of acute IOP elevation, known as "ischemia-reperfusion", had similar results to the literature concerning the injury by ischemia/reperfusion with emphasis in the loss retinal layer in different stages of apoptosis and a decrease in the count of RGCs [20, 24, 27].

In this study, we examined the possibility of apoptosis involvement in the loss of retinal elements after ischemia-reperfusion insult. In addition to histologic, morphologic and morphmetric evidences of retinal thickness change and lower number of RGCs in acute glaucoma induced animals, most of the histopathological findings were related to necrosis. Pro apoptotic proteins, studied by immunohistochemistry, had a tendency to be higher in PG than in SG, in both 7 and 21 days treatment, although with no statical difference.

The most prevalent histopathological findings were edema, chromatolysis, pyknosis, karyolysis, necrotic cells, loss of RGC and thinning of all neurosensory retinal layers in both groups examined, but the SG 7 and 21 days showed more relationship with control group. This corroborates with the course of glaucoma development [20, 23, 27].

Loss of RGCs occurs in some ophthalmic conditions such as glaucoma and diabetes. RGC death has been reported to occur in many experimental studies using

different methods to induce retinal ischemia [4, 22, 28]. The neurodegeneration in glaucoma results of apoptosis in the RGC and the internal nuclaear layer (INL), but occasionally in the external nuclear layer, mainly generated by activation of the caspase apoptotic cascade, moreover a decrease in plexiform layer [11, 23]. We have observed that after the insult, SC treatment resulting in tissue preservation and lower pro apoptotic proteins expression.

In immunohistochemical analysis the lack of statistical difference could be due to the reduced number of samples, but there was a tendency for the animals treated with SC to have more animals with similar scores to control, than placebo groups. The extrinsic apoptotic pathway is initiated by Fas ligand (Fas-l) or tumor necrosis factor (Tnf) binding to death receptors (Fas, Tnf-r1 and Tnf-r2) located on the cytoplasmic membrane. There is a strong evidence indicating that this pathway is involved in RGC apoptosis in glaucoma, with elevated IOP up-regulation of Fas-l and Tnf-r2 being the cause optic nerve oligodendrocytes and RGCs loss [32-34]. We noticed that SC treatment prevented the higher Tnf-r2 and Fas-l protein and gene expression, with more animals with scores similar to control eyes, which is probably due the sildenafil treatment preventing RGC apoptosis.

There are also many intrinsic factors that can evoke apoptosis without the involvement of death receptors, such as neurotrophin deprivation and oxidative stress. Although the initial steps of the intrinsic pathways are not clear, they all eventually converge to the mitochondria. These insults is caused by Cytochrome C and, regulated by the Bcl-2 family members in the mitochondrial outer membrane. Pro-apoptotic Bcl-2 family members such as Bax, Bid and Bad promote the apoptotic and anti-apoptotic members Bcl-2 and Bcl-xL inhibiting the release of Cytochrome C [22, 32, 34].

In our study, Bax distribution of markings was similar in SG and PG, but there were more animals with intense expression in PG than in SG. Bcl-2, an anti apoptotic protein, had higher protein expression in SG when compared to PG. Control eyes had scores lower than 3, and more animals in PG had the same protein scores as CG. SG had more animals with higher scores, which may be due to a protective effect for apoptosis of RGCs. But, in qRT-PCR *Bax* and *Bcl-2* analysis, we found a very different result, observing the hight expression in PG when compared to SG and CG.

Blocking the pro-apoptotic factors and promoting the anti-apoptotic factors have been proven neuroprotective function to RGC injury [32, 34]. Knock-out mice lacking a functional *Bax* gene exhibit several supernumerary populations of neurons, including

retinal ganglion cells, indicating the importance of *Bax* in neuronal regulation in programmed cell death during development [35]. Besides promoting apoptosis, *Bax* antagonize *Bcl-2*, and we can make this relation in both SG and PG groups, once PG groups had higher *Bax* and *Bcl-2* scores, however, there was a tendency of *Bax* prevalency in this group, and the opposite occurred in SG groups.

The extrinsic and intrinsic pathways promotes the activation of proteases, known as caspases, which play a vital role in cell death process. Once activated, the majority of caspases have the ability to catalyze the activation of multiple other members of this family, resulting in amplification of the proteolytic cascade [36]. Caspases are classified in two groups: the initiator caspases (Caspase-2, 8, 9 and 10) and executors caspases (Caspase-3, 6 and 7). The functional forms of the initiator caspases promote directly or indirectly the activation of executors caspases [22, 23, 32, 36].

Cytochrome c, which is released from damaged mitochondria, promotes the formation of a heptameric 'apoptosome' megacomplex of Apaf1 and Caspase-9 (a member of the CED-3-like Cys protease family). It leads to the conformational change and activation of Caspase-9, which cleaves and activates downstream caspases, including caspase-3, caspase-6 and caspase-7 that carry out the execution phase of apoptosis [37, 38, 39].

In our study, Caspase 9, 6 and 7 showed higher expression in the RGC (PG 7 and 21 days), comproved in qRT-PCR, therefore, the SC treatment was neuroprotective in retina and RGC. Moreover, our results with PG showed apoptosis progress along pathways and, SG presented more survival in retina and RGC, in agreement with the literature [23, 40].

In general gene over expression has been detected in many experimental glaucoma models and they are associated with RGC apoptosis. *Caspase-9* mRNA and procaspase-9 are up-regulated in axotomized rat retina and retinas exposed to elevated intraocular pressure [32, 34, 42]. *Caspase-9* is only detected in damaged retinas [34], which explains that your trasncription was not observed in CG, since it only amplified in late cycles. The extrinsic and the intrinsic pathway are not entirely independent pathways. They cross talk and together amplify and accelerate the apoptotic cascades. As described above, activated *Caspase-9* can activate the downstream extrinsic pathway. Meanwhile, a pro-apoptotic member of the *Bcl-2* family may be activated [34, 37, 42]. Our results showed a dysregulation between pro and antiapoptotic genes resulting in a

proapoptotic signaling cascade in the untreated group. This shown that the SC treatment is related downregulation of the apoptotic pathway.

CONCLUSION

Based on these results, treatment with sildenafil showed to be neuroprotective in the retina in this glaucoma model (ischemia / reperfusion). Apoptosis prevention was evident in the retinal ganglion cell layer of animals treated with SC shown by histology, morphometry, pro and anti apoptotic genes and proteins expression.

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Table 1. Treatment Protocol: discrimination of sildenafil dose and as the treatment period.

Group	Dose	Treatment Period
7 Days	1 mg/Kg	on the day, and 7 day after acute IOP elevation
21 Days	1 mg/Kg	on the day, and 21 days after acute IOP elevation

Table 2. Description from rats used, the selected eye at draw random for the induction of glaucoma, the maintaining time of the elevated IOP, the IOP values evaluated after induction of glaucoma and maintaining of individuals after induction until finish IOP.

Sildenafil animals					
		Tonovet (mmHg)			
7 Days	**Eye**	**Normal PIO**	**3 min**	**30 min**	**57 min**
1	**L**	5	47	46	52
2	**L**	5	44	45	50
3	**L**	3	43	42	43
7	**R**	8	41	47	50
8	**L**	8	46	42	50
7 – A	**R**	9	34	42	29
8 – A	**R**	10	35	45	35
9 – A	**R**	9	30	39	33
5	**L**	8	45	51	49
1 – A	**R**	9	33	40	30
Placebo animals					
7 Days	**Eye**	**Normal IOP**	**3 min**	**30 min**	**57 min**
1	**R**	9	33	30	42
5	**R**	8	41	43	40
6	**R**	4	33	43	40
7	**R**	8	33	31	34
8	**R**	5	32	31	35
Sildenafil animals					
21 Days	**Eye**	**Normal PIO**	**3 min**	**30 min**	**57 min**
1	**R**	8	38	48	50
2	**L**	6	44	49	48
4	**L**	10	45	52	43
5	**L**	11	37	40	47
6	**L**	8	38	38	48
1-A	**R**	7	35	44	50
2- A	**L**	6	44	38	44
4- A	**L**	9	32	31	36
5 – A	**R**	6	38	35	44
6 – A	**R**	5	28	28	30
Placebo animals					
21 Days	**Eye**	**Normal PIO**	**3 min**	**30 min**	**57 min**
2	**R**	6	35	43	41
4	**L**	6	35	42	39
6	**L**	8	34	37	39
8	**R**	9	23	25	30
9	**R**	10	40	36	38

Table 3. Immunostaining Anti-caspase 7, anti-caspase-9, anti-TNF-R2 anti-FAS-L, anti-Bax, anti-caspase-6, and anti-Bcl-2 antibody in retina.

Primary Antibody	Manufacture	Dilution	Antigen retrieval method	Incubation period	Secondary antibody
Caspase 7	Santa Cruz N-19/SC-492	1:200	Citrate ph6,0/WB	2hours/27°C	LSAB
Caspase 9	Epotomics Ab32539	1:100	Citrate ph6,0/WB	2hours/ 4°C	Histofine
TNF-R2	Santa Cruz SC-1074	1:400	Citrate ph6,0/Pascal	2hours/27°C	LSAB
FAS-L	Santa Cruz (N-20): SC-184	1:2000	Citrate ph6,0/WB	2hours/27°C	Histofine
BAX	Santa Cruz P-19/SC-526	1:300	Citrate ph6,0/ Pascal	2hours/27°C	Histofine
CASPASE-6	Santa Cruz SC-1230	1:100	Citrate ph6,0/ Pascal	2hours/27°C	Histofine
BCL-2	Santa Cruz (C19): SC-649	1:2000	Citrate ph6,0/ Pascal	2hours/27°C	Histofine

Pascal (Dako®); WB: Water Bath

Table 4. Mean values (mean and standard deviation) of the thickness of retinal layers and GCL count.

	7 days treatment				**21 days treatment**			
	Placebo	**Sildenafil**	**Control**	**P**	**Placebo**	**Sildenafil**	**Control**	**p**
Retinal (µm)	163±26,92*	168±30,55*	231±25	<0,05	149±20*°	174±38*°	231±25	<0,05
Ganglionar cell layer (µm)	6,6±1,1*°	10±2,9*°	16±2,0	<0,05	7,0±1,8*°	9,2±2,5*°	16±2,0	<0,05
Inner plexiform (µm)	26±8,6*°	38±12*°	55±6,7	<0,05	33±13*°	43±11*°	55±6,7	<0,05
Inner nuclear (µm)	25±4,6*	26±6,0*	35±5,8	<0,05	23±3,7*	24±5,5*	35±5,8	<0,05
External plexiform (µm)	6,6±1,1*°	9,1±1,5°	9,8±1,6	<0,05	6,7±1,0*°	9,2±2,3°	9,8±1,6	<0,05
External nuclear (µm)	47±11*°	42±8,8*°	63±8,4	<0,05	37±7,2*	40±13*	63±8,4	<0,05
Ganglionar cell count (n°)	123±23*°	197±32°	189±10	<0,05	110±38*°	160±33°	189±10	<0,05

Note: p value is the statistical significance between groups by ANOVA test. * Statistic difference compared to the control (t-test).

° Statistical difference in relation to the treatment groups (t-test).

Table 5. Histopathological changes in the eyes of rats with induced acute glaucoma.

	Description
7 Days Placebo	Discrete reduction of retinal layer, loss of RGC discrete to moderate, chromatolysis discrete to moderate, karyolysis mild to moderate, edema discrete to moderate and, discrete wallerian degeneration in optic nerve.
7 Days Sildenafil	Discrete reduction of retinal layer, loss of RGC discrete, chromatolysis discrete, karyolysis mild, edema discrete and, mild edema in optic nerve.
21 Days Placebo	Moderate reduction of retinal layer layer, loss of RGC moderate, chromatolysis moderate, karyolysis mild to moderate and atrophy moderate to severe of optic nerve.
21 Days Sildenafil	Discrete reduction of retinal layer, loss of RGC discrete to moderate, chromatolysis discrete, karyolysis mild and atrophy discrete of optic nerve

Table 6. Score distribution of markings for immunohistochemistry, by group studied with respect to positive cell for TNF-R2, FAS-L, BAX, BCL-2, Caspase-9, Caspase-6 and Caspase-7 in the retinal ganglion cell layer.

IMMINOLABELING SCORE

GROUPS	TREATMENT	TNF-R2			FAS-L			BAX			BCL-2			CASP – 9			CASP- 6			CASP - 7		
		Glaucoma		P	Glaucoma		P	Glaucoma		P	Glaucoma		P	Glaucoma		p	Glaucoma		P	Glaucoma		P
		≤3	4		≤2	>3		≤2	>3		≤3	4		≤3	4		≤2	>3		≤3	4	
7 days	Placebo	2	3	0,25	1	4	0,08	1	4	1,00	4	1	0,08	1	4	0,13	3	2	0,24	1	4	0,01*
	Sildenafil	8	2		8	2		1	9		2	8		8	4		9	1		9	1	
21 days	Placebo	2	3	0,25	2	3	0,25	1	4	1,00	4	1	0,08	2	3	0,32	1	4	0,08	2	3	0,25
	Sildenafil	8	2		8	2		1	9		2	8		7	3		8	2		8	2	
Control		**6**	**0**		**6**	**0**		**0**	**6**		**6**	**0**		**6**	**0**		**6**	**0**		**6**	**0**	

Legend: 1: <25% of positive cells; 2: 26-50% positive cells; 3: 51-75% positive cells; 4:>76% positive cells

*Statistical difference in relation to the treatment groups

Table 7. Intensity staining score for immunohistochemistry, by group studied for TNF-R2, FAS-L, BAX, BCL-2, Caspase-9, Caspase-6 and Caspase-7 in the retinal ganglion cell layer.

IMMUNOSTAING (intensity of marking)

GROUPS	TREATMENT	TNF - R2			FAS – L			BAX			BCL-2			CASPASE - 9			CASP – 6			CASPASE - 7		
		Glaucoma		P	Glaucoma		P	Glaucoma		P	Glaucoma		P	Glaucoma		p	Glaucoma		p	Glaucoma		p
		D/M	I		D	M/I		D/M	I		D/M	I		D	M/I		D	M/I		D	M/I	
7 days	**Placebo**	3	2	0,24	1	4	0,08	1	4	0,08	4	1	0,60	2	3	0,25	3	2	0,24	2	3	0,07
	Sildenafil	9	1		8	2		8	2		6	4		8	2		9	1		9	1	
21 days	**Placebo**	2	3	0,25	2	3	0,25	1	4	0,11	4	1	0,58	2	3	0,25	4	1	1,00	3	2	0,25
	Sildenafil	8	2		8	2		7	3		5	5		8	2		9	1		8	2	
Control		**6**	**0**		**6**	**0**		**6**	**0**		**6**	**0**		**6**	**0**		**6**	**0**		**6**	**0**	

Legend: Marking intensity immunohistochemistry: D (discrete), M (moderate) and I (severe).

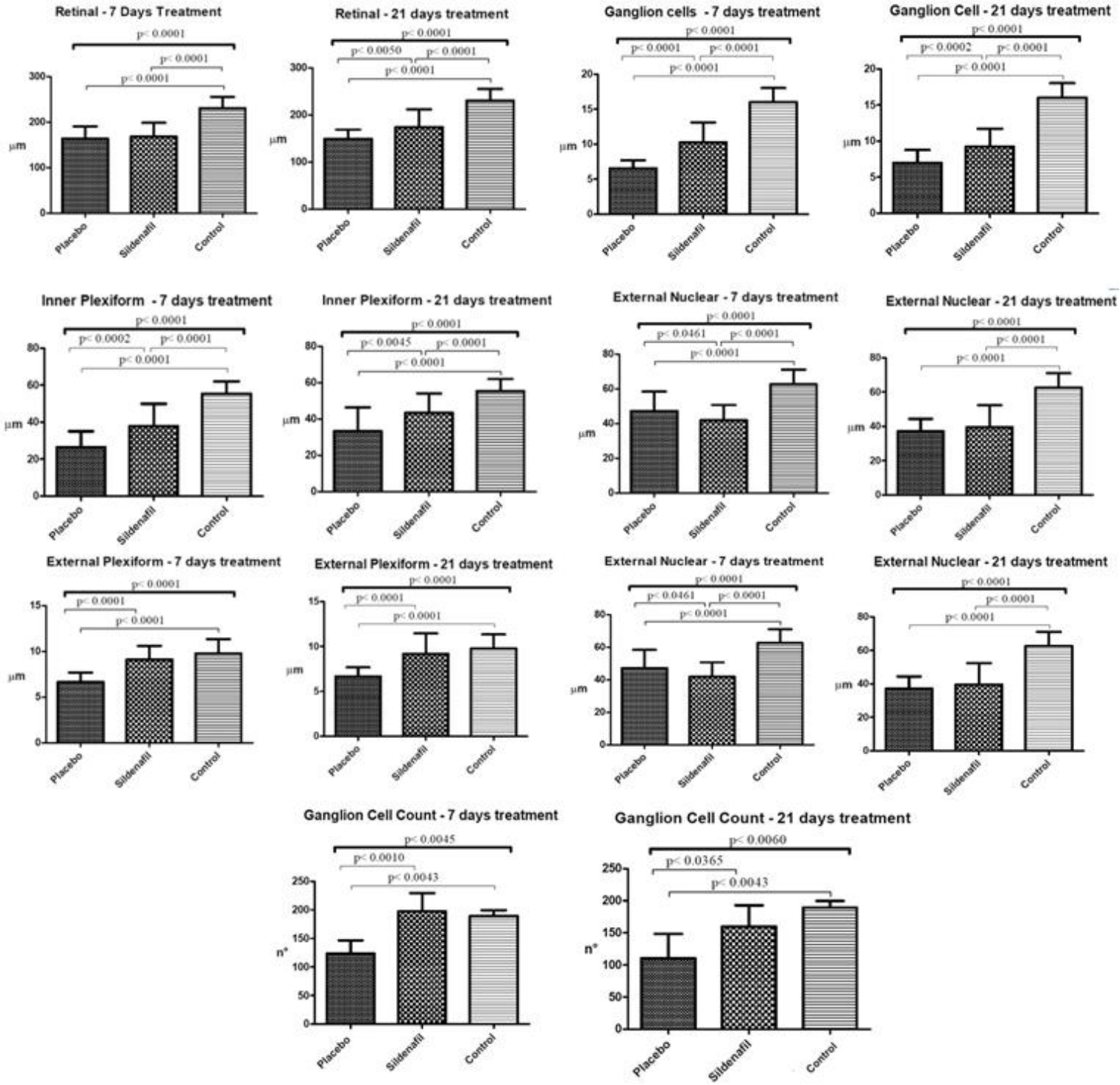


Figure 1. Mean and standard deviation of retinal total thickness and from each retinal layer from rats with induced acute glaucoma treated with Sildenafil from 7 days, and 21 days.

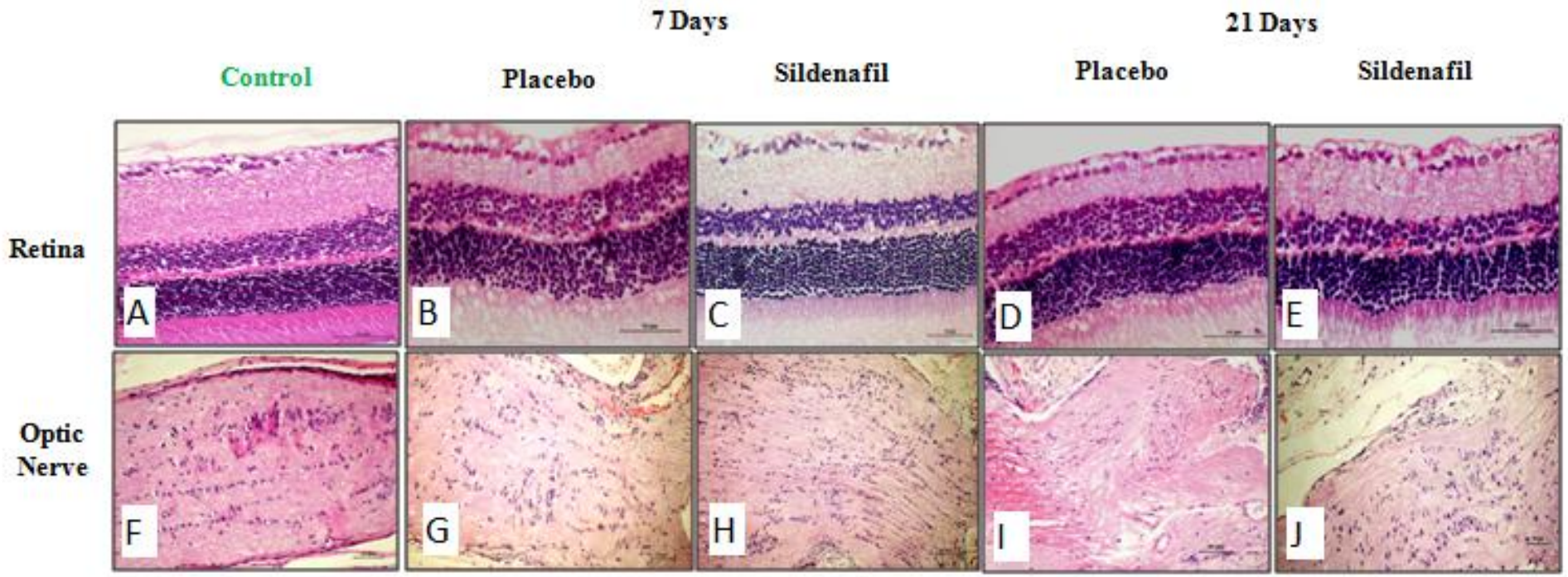


Figure 2. Retina from rats with induced acute glaucoma, illustrating the histopathological changes. A and F represent control group; Retinal atrophy with loss RGC and atrophy of some layers (internal plexiform layer, internal nuclear layer) (B); Mild retinal atrophy with some loss internal plexiform layer and GCL (C); Moderate retinal atrophy with loss RGC and moderate atrophy of internal plexiform layer (D); Discrete retinal atrophy with loss RGC mild and mild atrophy of internal plexiform layer and INL (E); Discrete wallerian degeneration (G); Mild edema (H); Excavation and marked atrophy in the optic nerve (I); Mild atrofy in the optic nerve (Retina: HE, 40x; Optic nerve: HE, 20x).

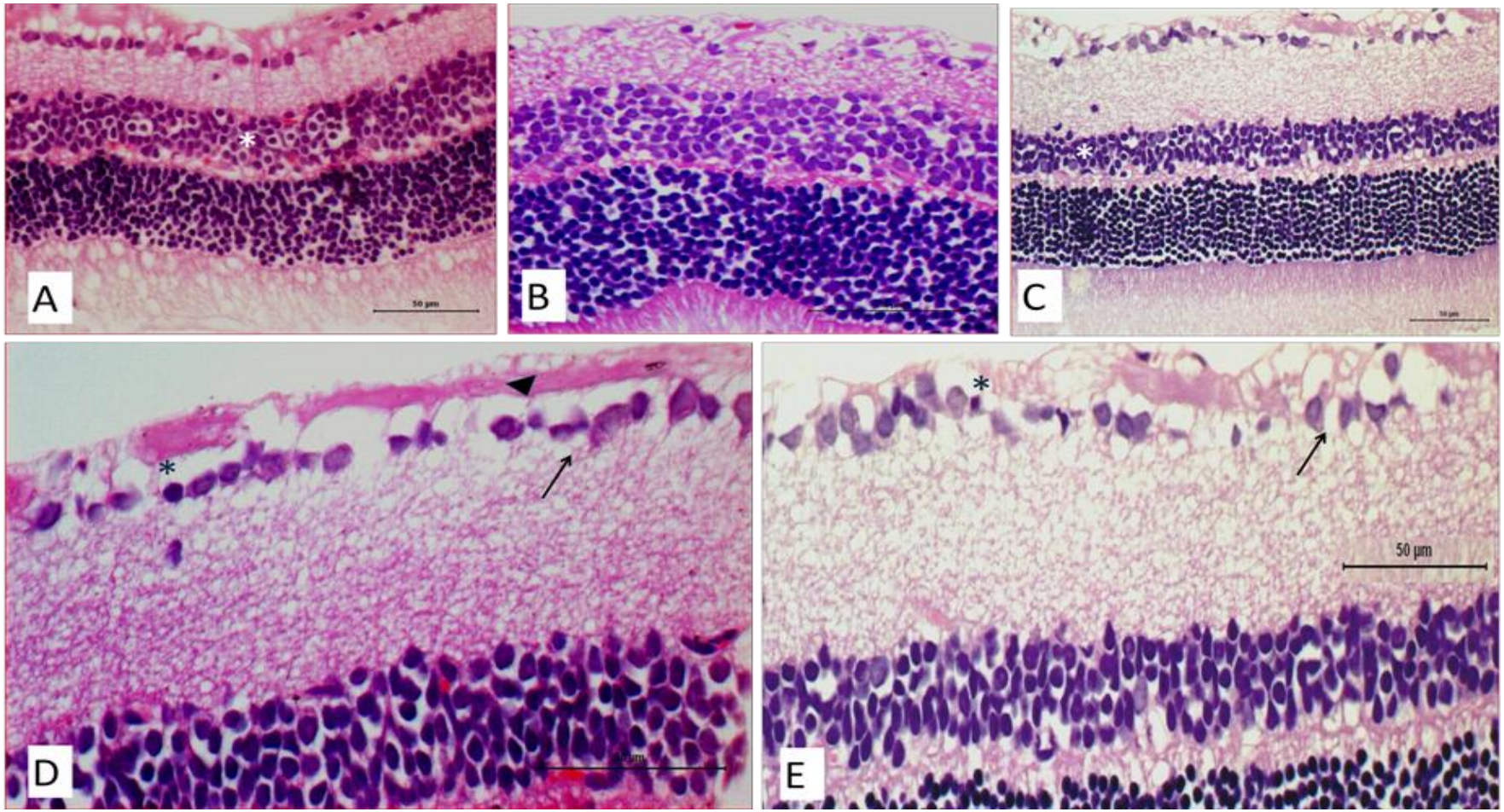


Figure 3. Retina from rats with induced acute glaucoma and treated with Sildenafil for 7 days. (A) Internal nuclear layer with mild to moderate edema– white asterisk (A) and, mild retinal atrofy. Loss RGC and moderate retinal atrophy (B). 7 days sildenafil showed mild edema (C). GCL nuclear picnosis (Black asterisk, cariolysis (head arrow) and cromatholisis (arrow) (D). GCL nuclear picnosis (Black asterisk and cromatholisis (arrow) (E). (A; B; C: HE, 40x; D; E: HE, 40x 1.6).

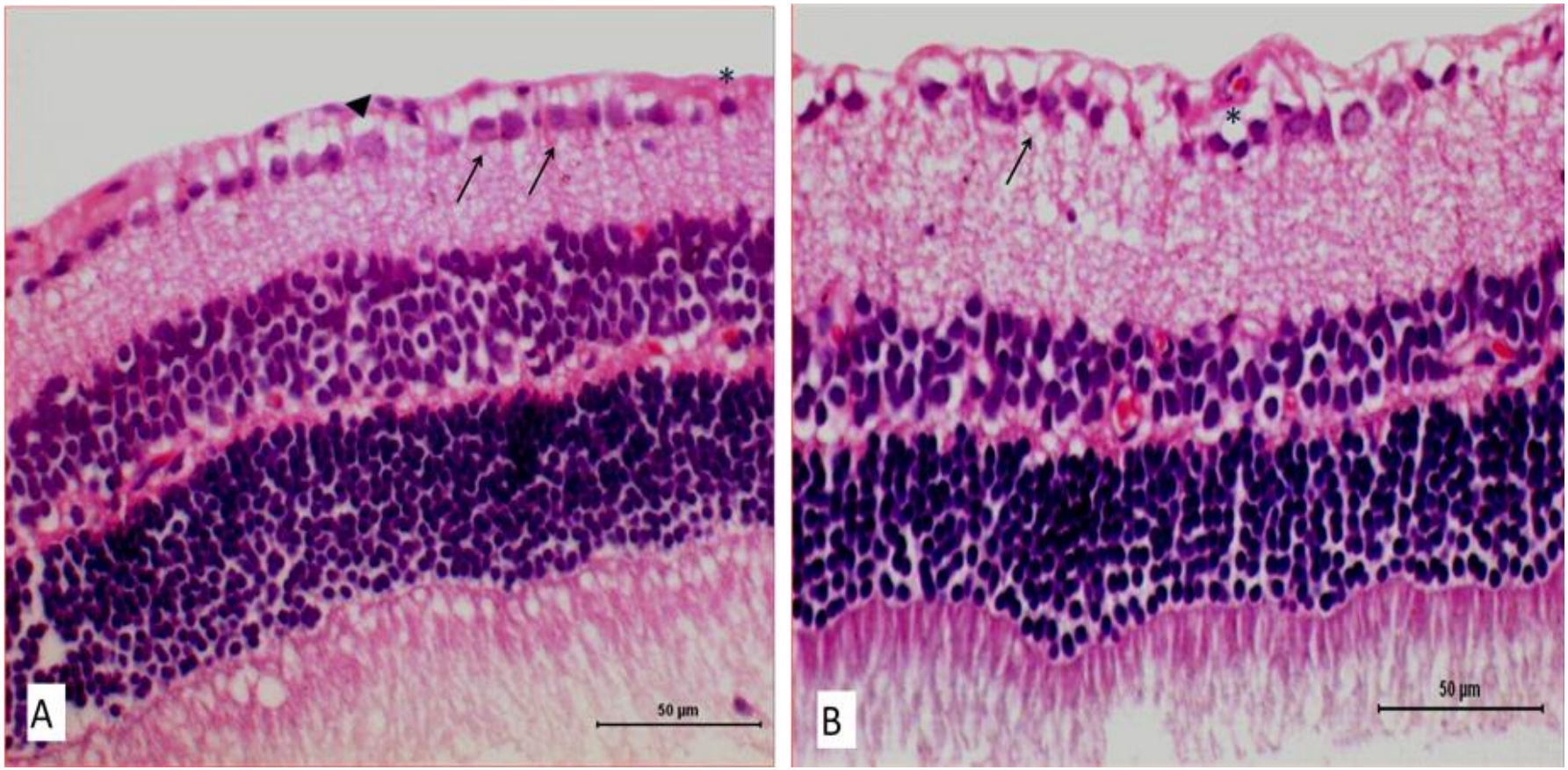


Figure 4. Retina from rats with induced acute glaucoma and treated with Sildenafil for 21 days. (A) represent 21 days placebo with nuclear picnosis (Black asterisk, cariolisis (head arrow) and chromatolisis (arrow), (B) represent 21 days sildenafil with nuclear picnosis (Black asterisk) and cromatolisis (arrow) (HE, 40x).

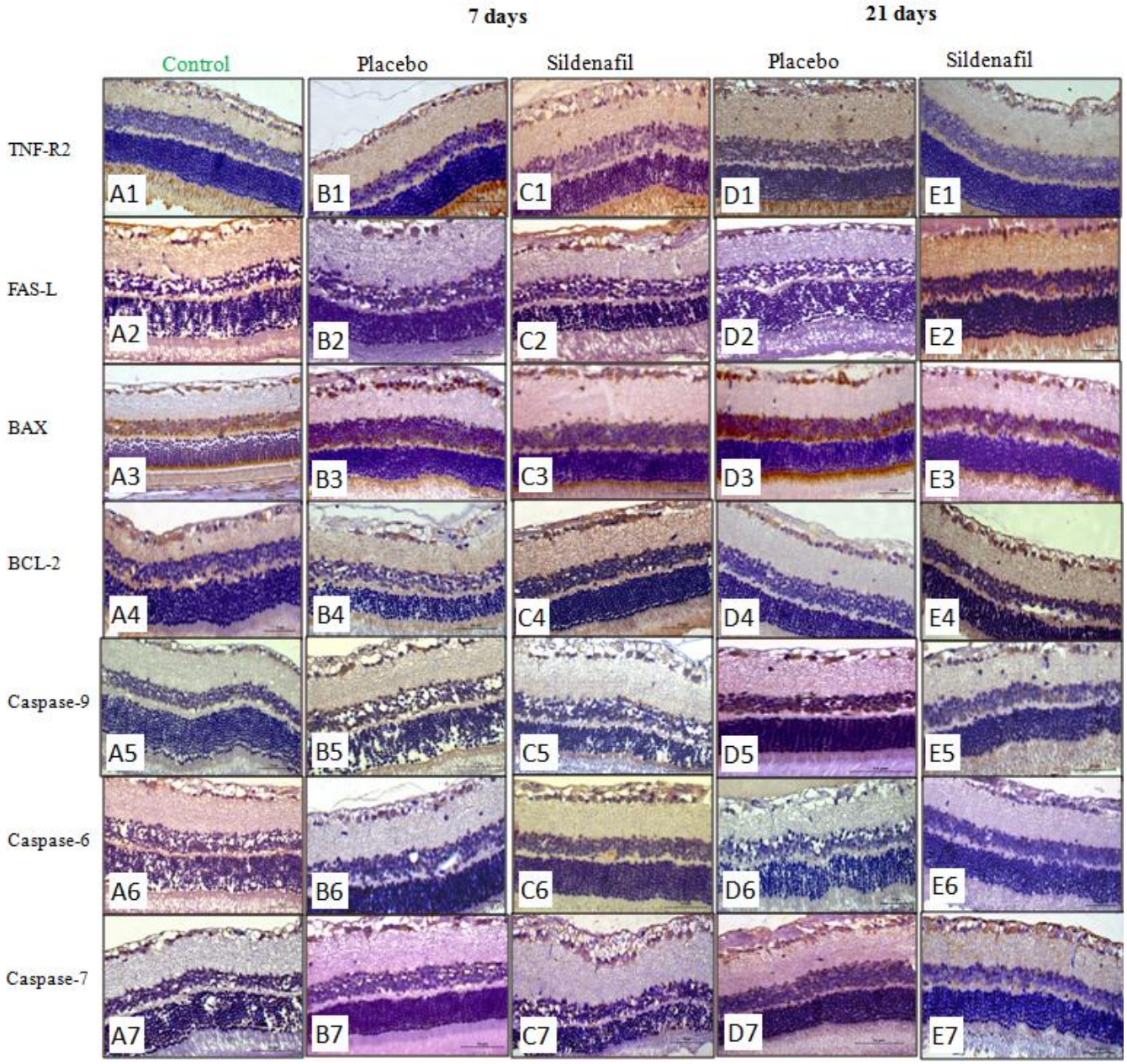
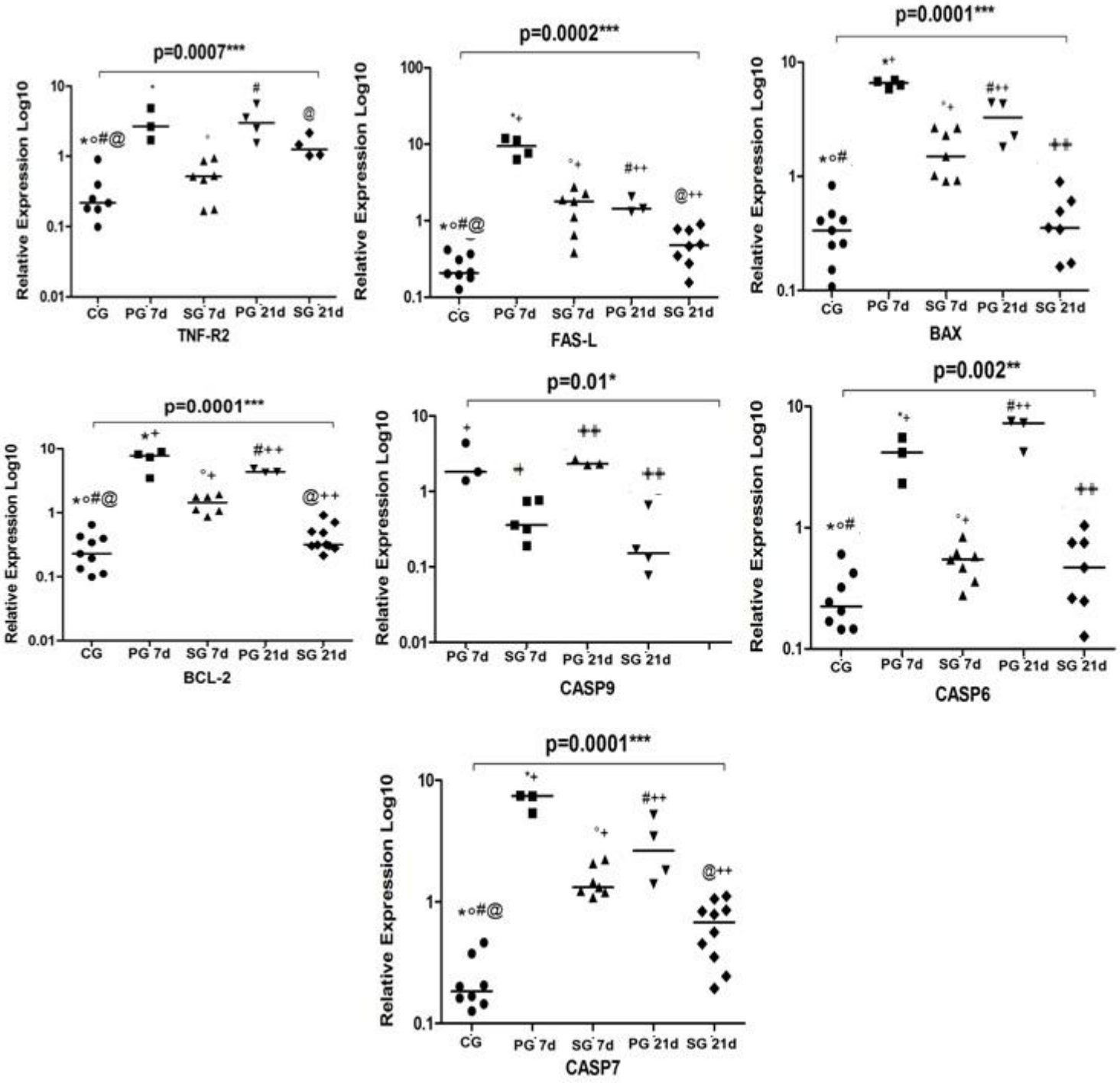


Figure 5. Retina from rats with induced acute glaucoma, illustrating the immunostaining. Note marking in the cytoplasm of retinal ganglion cells (obj. 40x). A1 to A7 represent control groups. B1 and D1 has more marked cells and, too increasing marking intensity of the PG 21 days. In C1 and E1 was similar to A1; FAS-L showed tendency of more marked cells in PG 7 and 21 days (B2 and D2), compared to SG (C2 and E2) and CG (A2); BAX had a tendency of discrete intensity to moderate marking in the SG (C3 and E3), compared to PG (B3 and D3) with a tendency of high intensity; The Bcl-2 had expression increased in SG (C4 and E4) compared to the PG (B4 and D4) and CG (A4); Caspase-9 has more marking in PG 7 and 21 days and, too increasing marking intensity of this groups (B5 and D5), but C5 and E5 was similar to CG (A5); Caspase-6 showed more number of marking cells in PG 21 days (D6), compared to SG (E6) and CG (A6) and, there wasn't difference marking intensity in all groups compared to the control group; Caspase-7 showed a marking more cells in PG (B7 and D7) compared to SG (C7 and E7) and CG (A7).



Legend: *; °; #; @: Statistic difference compared to the control; +; ++: Statistical difference in relation to the treatment groups.

Figure 6. PCR expression of group studed for Tnf-r2, Fas-l, Bax, Bcl-2, Caspase-9, Caspase-6 and Caspase-7. See over expression in untreatment groups comparative with sildenafil group.

GENERAL CONCLUSION

Based on these results, treatment with sildenafi proved to be neuroprotective in the retina in this glaucoma model (ischemia / reperfusion). Cell survival was evident in the retinal ganglion cell layer of animals treated with the drug and shown in histology and morphometry. For immunohistochemistry and qRT-PCR was observed protective effect as it the apoptosis pathways showed similar expression when compared to control, which may be related to down regulate apoptotic pathway.

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