Neuroprotection in Acute Ischemia and Ischemia/Reperfusion in Rat Retina

1Renato Guizzo, 1Marcelo Araújo Rodrigues Cairrão, 2Joaquim Coutinho-Netto,
3Antônio Renato Meirelles e Silva, 4Norberto Cysne Coimbra and 1Wagner Ferreira dos Santos
1Laboratory of Neurobiology and Venom, Department of Biology,
Faculty of Philosophy, Sciences and Literature of Ribeirão Preto
2Laboratory of Neurochemistry,
Department of Biochemistry and Immunology, Ribeirão Preto School of Medicine,
3Laboratory of Applied and Experimental Neurology, Department of Neurology,
Psychiatry and Medical Psychology, Ribeirão Preto School of Medicine,
4Laboratory of Neuroanatomy and Neuropsychobiology,
Department of Pharmacology, Ribeirão Preto School of Medicine, University of São Paulo, Brazil

Abstract: We compared qualitative and quantitatively the neuroprotective effects of ketamine, nimodipine and lamotrigine in rat retinas submitted to acute ischemia and ischemia followed by reperfusion. Ischemia was induced by increasing of intraocular pressure above systolic blood pressure for 60 min. In reperfusion, intraocular pressure was reduced to normal levels for 45 min. Histological evaluation was performed by computer-assisted measurements from histologic sections. Ischemic retinas revealed a decrease in cells densities, cytoplasm vacuolization, pyknotic nuclei, edema and cell disorganization, when compared to contralateral-control eyes. Further, ischemia/reperfusion showed more evident retinal damage when compared to ischemic retinas. Lamotrigine pretreatment (25 mg kg	extsuperscript{-1}) protected neurons mainly in ganglinar cell layer. Nimodipine pretreatment (20 μg kg	extsuperscript{-1}) protected neurons of ganglion cell layer in ischemia and in ischemia/reperfusion the inner nuclear layer. Ketamine pretreatment (20 mg kg	extsuperscript{-1}) protected neurons mainly in outer nuclear layer and inner nuclear layer in ischemia and ischemia/reperfusion. Present results suggest that lamotrigine, nimodipine and ketamine have potential neuroprotective properties in acute ischemia and ischemia/reperfusion models and all of them may have therapeutic implications for retina.

Key words: Lamotrigine, nimodipine, ketamine, neuroprotection, rat, retina

INTRODUCTION

Glaucoma is an optic neuropathy, recognized as the second most common cause of blindness worldwide[1]. The most important risk factor for this disease is the high intraocular pressure (IOP) that leads to ganglionar cell death associated with changes in the morphology of the optic nerve. The death of ganglionar cells is a crucial feature of glaucoma, optic neuropathies and retinopathies, and as it happens with glaucoma, some of these disorders have an ischemic cause[2].

Neuronal and ganglionar cell death may occur via a variety of mechanisms involving, for example, a decrease of ATP, release of excitatory amino acids[3], sodium and calcium overload[4], neuronal transporters failure[5], nitric oxide production[6], necrosis and apoptosis[3]. These types of damage can be prevented by various agents, such as glutamate receptor antagonists[7], calcium channel blockers[8], inhibitors of free radical production[9], sodium channel blockers[10] and drugs that prevent apoptotic cell death[8].

Since glaucoma may lead to blindness, experimental models are important because they mimic the disease, supplying reasonable requirement for studying of neuroprotection[11]. Several publications have described different experimental paradigms of retinal ischemia. The most utilized are high IOP[12,13], middle cerebral artery occlusion[14] and photothermogenesis[15]. Thus, in this study the neuroprotective properties of ketamine, nimodipine and lamotrigine were investigated in the acute ischemia and ischemia/reperfusion in rat retinas through qualitative and quantitative analyses.

Corresponding Author: Dr. Wagner Ferreira dos Santos, Universidade de São Paulo, Av. Bandeirantes, 3900, FFCLRP-USP, Cep. 140-9901, Ribeirão Preto, São Paulo, Brazil
Tel: +55 16 602-3657 Fax: +55 16 633-1758 E-mail: wagnerf@usp.br
MATERIALS AND METHODS

Animals: Adult male Wistar rats (230-250 g) were used. The animals were housed in individual cages, with free access to food and water. They were kept in the experimental room for 48 h prior to the experiment on a 12 h light/12 h dark cycle (lights on at 7:00 am) at 23-25°C. All experiments were performed in compliance with the recommendations of Brazilian Society of Neuroscience and Behavior (SBNBeC), which are based on the US National Institute of Health Guide for Care and Use of Laboratory Animals.

Drugs: The drugs used were lamotrigine (Lamictal®, Glaxo Welcome, 25 mg kg⁻¹), nimodipine (Oxygen®, Biosintética, 20 µg kg⁻¹) and ketamine (Kealar®, Parke-Davis, 20 mg kg⁻¹).

Induction of ischemia and ischemia/reperfusion: Rats were anesthetized intraperitoneally with thiopental (50 mg kg⁻¹) and the retinas were submitted to experimental ischemia according to Louzada-Jr. et al.[9]. Intracocular pressure was increased (155 mmHg) by cannulating in the anterior chamber of the eye, with a sterile 27-gauge needle attached to a manometer/pump and to an air reservoir. Ischemia was induced for 60 min. After this period, the intracocular pressure was reduced to normal levels for 45 min (reperfusion period). The left retina of each animal was subjected to the experimental condition, while the right retina served as control (contralateral eyes). The drugs were injected intravenously 15 min before elevating the pressure.

Histology: Animals were euthanized immediately after retinal ischemia or ischemia/reperfusion by an overdose of sodium pentobarbital, the left and right eyes were rapidly enucleated and immersed fixed in Bouin’s solution (75% picric acid, 25% formalin and 5% acetic acid) for 24 h. After fixation, corneas, aqueous humor, lens, vitreous humor were removed and the eyecups dehydrated in 70-100% ethanol and embedded in paraffin. Retinas were sectioned sagittally at 5 µm, in the superior nasal pars beginning approximately 1 mm from optic nerve emergency, stained with hematoxilin-eosin and examined using a Zeiss Axioshot microscope.

Data analysis: From each retina, five microscopic fields (1 microscope field= 160X or 636x474 pixels) of one transverse section at the superonasal retina (beginning 1 mm above the optic nerve) were analyzed by light microscopy (Axiohot, Zeiss) and digitalized by an analogic camera (JVC TK1270) connected to the microscope and a computer. Qualitative analysis was performed to characterize histological damages as decrease of cell number, cytoplasm vacuolization, edema, disorganization and pyknotic nuclei. The KS 400 computer program (Carl Zeiss Vision, Germany) was used to quantify the cell counts in a masked fashion[13] on the same digital images from identical areas in both the retinas. The results were expressed as densities (cells/mm²±SEM). Significance differences between the groups were determined by using One Way Analysis of Variance (ANOVA) and p<0.05 indicated a statistically significant difference.

RESULTS

Contralateral-control eyes (n=10) were evaluated. There was no histological evidence of edematous changes, pyknotic nuclei and cell disorganization (Fig. 1A). The cell densities in ONL, INL and GCL were 76007±3413, 34641±1182 and 5634±189, respectively (Fig. 3).

Five eyes were submitted to 60 min of ischemia. The cell densities in ONL, INL and GCL were 4702±1156, 1586±797 and 247±148, respectively (Fig. 3). Compared to the control retinas, there was significant difference between the cell densities in ONL, INL and GCL (Fig. 3). All five eyes had histological evidence of edema with cell disorganization in INL and ONL, as well as pyknotic nuclei in GCL and INL compared to the control retinas (Fig. 1A and 1B). GCL also presented cytoplasm vacuolization and it was observed edema and matrix disorganization in inner plexiform layer (IPL) (Fig. 1B).

Five eyes were submitted to 60 min of ischemia and 45 min of reperfusion. The cell densities in ONL, INL and GCL were 45746±2408, 12827±194 and 2376±76, respectively (Fig. 3). Compared to the control retinas, there was significant difference between cell densities in ONL, INL and GCL (Fig. 3). All five eyes had vacuolization, pyknotic nuclei and disorganization in INL and GCL compared to the control retinas (Fig. 1A and 1C). Edema was found in INL. Compared to 60 min of ischemia, there was significant difference between the means nuclei counts in INL. In GCL there was more pronounced edema of the cytoplasm.

Lamotrigine pretreatment: In lamotrigine pretreatment, the cell densities in ONL, INL and GCL in ischemia were 58632±4824, 22202±2719 and 4051±382, respectively (Fig. 3). Compared to control retinas there were fewer pyknotic nuclei in INL (Fig. 1A and 2A). Compared to ischemic eyes without pretreatment, there were less signs of damage and a significant difference between the cell densities in GCL, showing a protection by 48% (Fig. 3C).

In ischemia/reperfusion, in ONL, INL and GCL, the cell densities were 49502±6363, 18738±2314 and 4081±510, respectively (Fig. 3). Compared to control retinas there
Fig. 1: Photomicrograph of transverse sections of rat retinas stained with hematoxyline-eosin. Arrows, pyknotic nuclei; Arrowhead vacuolization. (A) Control; (B) Ischemia; (C) Ischemia/reperfusion; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglionar cell layer. Bar= 50 μm.

were few pyknotic nuclei in INL (Fig. 1A and 2B). Compared to ischemia/reperfusion without pretreatment, GCL showed less signs of damage and a significant difference between the cell densities showing a protection by 72% (Fig. 3C).

**Nimodipine pretreatment:** In nimodipine pretreatment, the cell densities in ONL, INL and GCL in ischemia were 60426±4928, 23567±436 and 3441±215, respectively (Fig. 3). Compared to control retinas the means of the cell densities in the ONL, INL and GCL was decreased, as well as pyknotic nuclei in INL (Fig. 1A and 2C). Compared to ischemia without pretreatment, there was a significant difference between the cell densities in GCL showing a protection by 20% (Fig. 3C). In ischemia/reperfusion, the cell densities in ONL, INL and GCL were 57596±4035, 22283±2555 and 2315±298, respectively (Fig. 3). Compared to control retinas there was decreased in the cell densities and pyknotic nuclei in INL (Fig. 1A and 2D). Compared to ischemia/reperfusion without pretreatment, there was a significant difference between the cell densities in ONL showing a protection by 48% and in INL a protection by 95% (Fig. 3A and B).

**Ketamine pretreatment:** In ketamine pretreatment, the cell densities in ONL, INL and GCL in ischemia were 69644±2065, 25305±1606 and 3685±295, respectively (Fig. 3). Compared to control retinas a decreased in the cell densities and vacuolization in the GCL were observed (Fig. 1A and 2E). Compared to ischemia without pretreatment, we observed a significant difference between in the cell densities in ONL, INL and GCL showing a protection by 48%, 60% and 34%, respectively (Fig. 3A-C). In ischemia/reperfusion, the cell densities in ONL, INL and GCL were 67818±3572, 25038±2595 and 2771±457, respectively (Fig. 3). Compared to control retinas there was decreased in the cell densities (Fig. 1A and 2F). Compared to ischemia/reperfusion without pretreatment, there was a significant difference between the cell densities in ONL showing a protection by 48% and in INL a protection by 95% (Fig. 3A and B).
Fig. 2: Photomicrography of transverse sections of rat retinas stained with hematoxyline-eosin. (A) Lamotrigine pretreatment in ischemia; (B) Lamotrigine pretreatment in ischemia/reperfusion; (C) Nimodipine pretreatment in ischemia; (D) Nimodipine pretreatment in ischemia/reperfusion; (E) Ketamine pretreatment in ischemia; (F) Ketamine pretreatment in ischemia/reperfusion. Bar—50 μm
**DISCUSSION**

The acute experimental glaucoma, a model of ischemic insult, simulates the enhancing of IOP for a desired period and has been employed in many studies\(^1\). The advantages of this model are: ischemic injury can be produced locally by a relatively simple procedure, it is easy to control and it also allows studying details of the ischemia/reperfusion. Yet, this model combines a relatively simple methodology, cost-effective execution and a fast, computerized method of quantitation.

An ischemic insult results in extracellular increased levels of glutamate and excessive stimulation of its ionotropic receptors. The increased extracellular glutamate levels lead to intracellular calcium overload and cellular membrane depolarization with opening voltage-sensitive sodium channels. This causes a secondary passive influx of sodium, cellular swelling and enzymes activation leading to neuronal death. Therefore, substances that improve these events are thought to be an effective therapeutic approach against ischemic insult.

Present results show that retinas submitted to ischemia with reperfusion were more damaged than those with ischemia without reperfusion. The most injured layer was INL, where there was a significant difference between the cell densities. Edema was verified in the inner layers, mainly in the INL, inside and outside the cells (data not show). Still in INL, we verified an increase in the layers thickness and an increase in the diameter of the cells, which was more pronounced after reperfusion. Despite the neuroprotection of the lamotrigine, nimodipine and ketamine, we verified a severe cellular edema, also located in the tissular matrix. At first, we can hypothesize that post or presynaptic blockade was one of the reasons for the edema. This includes the glutamate remaining in the synaptic cleft and a failure of the neuronal transporters, as well as glial reuptake\(^2\).

The three drugs were injected in the animals 15 min before the ischemic insult. Although many neuroscientists have focused their attention on drugs that can have neuroprotective effects after an ischemic insult, a pretreatment therapy could be beneficial, because there is a very large population of patients who are at significant risk to develop neuropathies\(^3\).

Ketamine pretreatment in ischemic assays was more effective in comparison to lamotrigine and nimodipine, reducing partially the edema, mainly in the ONL and INL, as well as in ischemia/reperfusion. However, the literature show reports that antagonists of L-Glu receptors do not inhibit edema of glial cells\(^4\).

Although ketamine protected significant the ONL and INL, no significant differences between ischemia and ischemia/reperfusion were observed. Besides the

---

Fig. 3: Cell densities in the retinal layers. (A) outer nuclear layer; (B) inner nuclear layer; (C) ganglionar cell layer.
1- Pretreatment with lamotrigine. 2- Pretreatment with nimodipine. 3- Pretreatment with ketamine. *Significantly different from the control retinas (p<0.05, One way ANOVA). **Significantly different from ischemia and ischemia/reperfusion (p<0.05, One way ANOVA)
demonstration of the beneficial effect of this drug in the ischemia, it is well known that it might also act during reperfusion as a scavenger of free radicals. During an ischemic insult, the lack of energy depolarizes neurons, producing a large increase of excitatory neurotransmitters. The stimulation of excitatory receptors induces calcium influx, which indirectly activates voltage-dependent calcium channels. The accumulation of intracellular calcium causes activation of proteases, phospholipases and endonucleases that leads to destructive processes and cell death. In this study the administration of nimodipine, before the ischemia reduced significantly the neuronal death in the GCL. A great protection was also observed in ischemia/reperfusion in INL. In fact, the inhibition of calcium influx through the membrane, limiting and decelerating the excessive flux of calcium into the cell, could be a strategy for neuroprotection. One of the reasons is that these channels possess long duration action potentials and a great sensitivity for inhibitory dihydropyridine-like drugs. However, the neuroprotective effects of nimodipine was moderate in ischemia/reperfusion, see that in GCL nimodipine demonstrated less cell densities than ischemia/reperfusion without pretreatment and there was significant statistical difference between the cell densities in comparison to lamotrigine pretreatment.

The use of sodium channel blockers may alleviate, at least to some extent, ganglionar cells death during ischemia. Lamotrigine inhibits sodium influx by blocking voltage-sensitive sodium channels. The neuronal sodium channels blockade decreases the frequency of action potentials, reduces presynaptic glutamate release, diminishes the accumulation of intracellular sodium, preserves a sufficient sodium gradient used for the sodium-dependent glutamate transporters, terminating the glutamate action. Present results indicate that lamotrigine protected significantly neurons of GCL in ischemia and ischemia/reperfusion. In ischemia/reperfusion there were no statistical differences to the control retinas, indicating the best neuroprotection compared with the effects of nimodipine and ketamine. These findings suggest that lamotrigine might be useful against retinal damage, since neurons of GCL are particularly very vulnerable to the ischemic insult.

Present results indicate that ketamine, nimodipine and mainly lamotrigine blocked the function of L-Glu, attenuating neuronal death in the model of acute ischemia with reperfusion. Approaches aiming to an association of these drugs could be promising in experimental models and perhaps in the clinical practice, decreasing the penumbra area that consequently, would be helping the improvement of visual sharpness of patients that lose progressively vision, due to the glaucomatous frame.

ACKNOWLEDGMENTS

R. Guizzo was a Master fellowship from FAPESP (Proc. 99/01346-9). We thank Mr. Antônio J. Coluasso and Mr. Amauri R. Pinhal (Departamento de Biologia, FFCLRP-USP). We wish to thank Dr. Norberto Garcia-Cairasco (Departamento de Fisiologia FMRF-USP), Mrs. Andrea B. Pizzo and Mrs. Alexandra O. S. Cunha for a critical reading of the manuscript and Glaxo Welcome, Biosintéticas and Parke-Davis for the supply of drugs.

REFERENCES