INTRODUCTION

Glaucoma is the second leading cause of blindness worldwide[1] and is characterized by an optic neuropathy accompanied by typical structural and functional defects (optic disc damage and visual field loss).[2] It is widely accepted that the death of a substantial number of retinal ganglion cells (RGCs) in the inner retina and loss of their axons in the optic nerve are pathophysiologic characteristics of glaucoma.[2] Glaucoma is a multifactorial condition with intraocular pressure (IOP) elevation as the major risk factor.[3] However, the pathophysiological relationship of elevated IOP with RGC death remains unknown.

Abstract

Purpose: In this experimental study, the effects of systemic memantine administration on the retinal ultrastructure of experimentally induced glaucomatous rats were investigated.

Methods: Twenty-four Wistar albino rats were included in this study. Glaucoma was induced by injecting sodium hyaluronate into the anterior chamber of the rats for a period of three weeks. As a control, 8 rats were sham treated (Group C). Glaucoma induced animals were divided into two groups; Group M (n = 8) received a single daily dose of 10 mg/kg memantine, and Group G received the same volume of saline (n = 8), via intraperitoneal route for a period of six weeks, starting with the induction of glaucoma. Then, all rats were sacrificed and the retinas were prepared for electron microscopic examination. Electron microscopic damage findings were graded between 0 and 4 and mean damage scores for each cell or layer was calculated for each group. Statistical comparison was made between group G and group M.

Results: Including the photoreceptor cells, marked ultrastructural changes were observed in the retinas of the animals in group G. The ultrastructural changes in group M were modest and there was no significant cell death. Statistical findings indicated these results.

Conclusion: Results of the present study suggest that memantine treatment, when started in the early phase of glaucomatous process, may help to preserve the retinal ultrastructure and thus prevent neuronal injury in experimentally induced glaucoma.

Keywords: Electron Microscopy; Glaucoma; Memantine; Neuroprotection; Rat

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unclear. Understanding the underlying pathogenesis is necessary to determine the ideal treatment strategy.

Neuronal death occurs by several different mechanisms including growth factor deprivation, blockade of axonal transport in the optic nerve fibers due to IOP elevations, hypoxia-ischemia, oxidative stress, inflammatory cytokines, aberrant activity of the immune system, and glutamate excitotoxicity. Glutamate receptor activational excitotoxicity has been associated with mitochondrial dysfunction in acute and chronic neurodegenerative disorders, and has been suggested to cause RGC death in glaucoma. However, the relationship between IOP elevation and glutamate excitoctotoxicity and mitochondrial dysfunction remains unclear. IOP elevations lead to mechanical stress or ischemic conditions, resulting in RGC death and glaucoma.

Currently, the only management strategy for glaucoma is IOP reduction. However, despite successful IOP control, some patients continue to lose vision. Therefore, it is clear that reduction of IOP may be inadequate for treatment of glaucoma patients, and the focus of research is now shifting toward other strategies, such as neuroprotection of RGCs and other neurons in the central visual pathway.

Memantine is an uncompetitive N-methyl-D-aspartate (NMDA) glutamate receptor antagonist and has been shown to be neuroprotective in mouse, rat, and monkey models of glaucoma. Memantine has demonstrated some benefits in animal models, but there has been less convincing evidence in human studies to date. Two phase 3 trials on the safety and efficacy of memantine failed to show statistical significance for the primary endpoint. Kersey et al reported that perhaps the benefits of memantine were underestimated due to study design. According to Osborn, the result of a phase III clinical trial in the human open-angle glaucoma was disappointing, which may be due to inappropriate endpoint and insufficient duration of treatment. In spite of this study, the neuroprotective efficacy of memantine is still under investigation.

The protective effects of memantine on the ultrastructural construction of glaucomatous retinas have not been fully elucidated. In the current study, we aimed to investigate the effects of systemic memantine administration on the retinal ultrastructure in an experimental model of glaucoma in rats.

**METHODS**

This study was conducted according to the principles regarding the care and use of animals adopted by the American Physiological Society and the Society for Neuroscience.

### Animal Selection

A total of 24 young adult male Wistar albino rats (mean weight 220 ± 40 grams) were used for the purpose of the study. The animals were housed in a standard animal room with food and water ad libitum under controlled conditions of humidity and temperature (21 ± 2°C). The room was lit by fluorescent lights that were turned on and off automatically every 12 hours (on from 6 AM to 6 PM).

The animals were divided into three groups of 8 rats as follows: Group G, Group M, and Group C. In groups G and M, glaucoma was induced by injecting sodium hyaluronate into the anterior chamber of the rats for a period of three weeks. The rats in Group C were sham treated. Rats in Group M received a single daily dose of 10 mg/kg memantine, while rats in group G received the same volume of saline via the intraperitoneal route for a period of six weeks, starting with induction of glaucoma.

### Glaucoma Induction

All rats were anesthetized using intraperitoneal ketamine hydrochloride (15 mg/kg) and xylazine hydrochloride (0.3 mg/kg). Glaucoma was induced in the right eyes of the rats as described previously. Briefly, 25 μL of sodium hyaluronate (23 mg/ml, Healon-5) was injected into the anterior chamber of the rats, through the corneoscleral limbus via a Hamilton syringe (Hamilton, Reno, NV, USA) using a 30-gauge needle, after drawing an equal volume of the aqueous humor. Injections were performed under a surgical microscope (Topcon OMS 75, Topcon Corp., Tokyo, Japan). Glaucoma was induced by weekly injections for 3 weeks. In the sham-treated animals (Group C), an equal volume of saline solution was injected.

### Intraocular Pressure Measurement and Animal Sacrifice

Intraocular pressure (IOP) measurements before and after the procedures were recorded during the 3 weeks of glaucoma induction, and weekly for another 3 weeks. IOP was measured with the Tono-Pen (Tono-Pen XL, Medtronic Solan, Jacksonville, FL, USA) once mild anesthesia was achieved by 3 mg/kg of asepromazine (0.3 mg/kg). Glaucoma was induced by weekly injections for 3 weeks. In the sham-treated animals (Group C), an equal volume of saline solution was injected.
At the end of 6 weeks, all rats were sacrificed by and intracardiac perfusion of glutaraldehyde, and the globes were enucleated.

**Electron Microscopic and Ultrastructural Evaluation**

The posterior wall of the globes were cut into 1 mm pieces and fixed in 2.5% glutaraldehyde for one hour at +4°C and then washed in cold cacodylate buffer (0.05 M; pH 7.4) twice, for 10 minutes each. Samples were then post-fixed in 1% osmium tetroxide in 0.05 M Na cacodylate for one hour and were dehydrated in a series of graded alcohols (50%, 70%, 90%, 96%, and 100%, respectively). Propylene oxide-Epon 812 treated specimens were embedded in Epon–812 embedding media. Semi thin sections (1 µm thick) were obtained using a microtome (Reichert Optische Werke AG, Vienna, Austria) and stained with toluidine blue. Using retinal sections, uranyl acetate-lead citrate-stained ultrathin sections were prepared using LKB ultramicrotome (LKB, Sweden). Transmission electron microscopic examination was performed using a Jeol JEM ‑ 1011B electron microscope (Peabody, MA, USA) with Megaview digital imaging system. Electron microscopic damage findings were graded between 0 and 4 (0 representing no damage and 4 representing the highest level of damage) and mean damage scores for each cell or layer was calculated for each group.

**Statistical Analysis**

Data for IOP measurements were expressed as mean ± standard error of mean (SEM). Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) software for Windows (version 12.0; SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) test and Post Hoc Tukey’s test were used for comparing the three groups in terms of IOP changes. Electron microscopic grades were compared between group G and group M employing the Mann-Whitney U test. P values less than 0.05 were considered as statistically significant.

**RESULTS**

**Intraocular Pressure Measurements**

Mean IOP values of the study groups at different time points are shown in Figure 1. Mean IOP in Group M and Group G were significantly higher than that of the control group after glaucoma induction (P < 0.001).

**Electron Microscopic and Ultrastructural Evaluation**

Fine structural changes in retinal layers, indicating reversible and irreversible cellular degeneration, were evaluated. Mitochondria and other membranous organelles of the retinal pigmented epithelium (RPE) were intact in Group C [Figure 2a], whereas partial degeneration of mitochondrial cristae was noted in Group M [Figure 2b]. The inclusion bodies in the mitochondria and other organelles were more prominent in Group G as compared to Groups C and M [Figure 2c]. The structural organization of photoreceptor lamellae as well as mitochondrial cristae was preserved both in groups C and M [Figure 3a and b, respectively], whereas, the lamellar structure and mitochondria of the inner segment of the photoreceptors were damaged in Group G [Figure 3c].

Microvilli of Müller cells, and the mitochondria of cone and rod cells in the inner segment of the retina were preserved in Group C [Figure 4]. However, in Group M lamellar inclusions and focal loss of cristae were observed in some mitochondria, and obliteration was observed in the microvilli of Müller cells in the inner retina [Figure 5]. Loss of mitochondrial cristae and swelling of inner segments were more prominent in Group G [Figure 6].

### Table 1. Intraocular pressure change at different time points

| Week | Control group | Glaucoma group | Memantine group |
|------|--------------|----------------|-----------------|
| 0    | 14.02±0.69   | 16.0±0.74      | 14.07±0.86      |
| 1    | 14.01±0.56   | 31.0±0.75      | 32.0±0.82       |
| 2    | 14.02±0.70   | 32.0±0.83      | 32.0±0.96       |
| 3    | 14.02±0.54   | 37.0±0.74      | 37.0±0.76       |
| 4    | 14.01±0.58   | 35.0±0.56      | 38.0±0.56       |
| 5    | 14.03±0.82   | 31.9±0.92      | 37.0±0.89       |
| 6    | 14.04±0.83   | 33.0±0.96      | 35.0±0.52       |

IOP, intraocular pressure; SD, standard deviation

![Figure 1. The mean intraocular pressure of the groups at different time points. Note that the mean intraocular pressure of Group M and Group G remained remarkably higher than that of Group C.](http://www.jovr.org)
Attachments amongst outer nuclear layer cells were preserved in Group C [Figure 7a]. Cellular attachments forming the external limiting membrane were intact and there were minimal detachments between the cells in Group M [Figure 7b]. Severe cytoplasmic and nuclear vacuolization was detected in the cells of the outer nuclear layer in Group G [Figure 7c].

Cell attachments, membranous compartments, synaptic contacts, and synaptic vesicles in the outer plexiform layer were preserved in Group C [Figure 8a and b]. Synaptic contacts were preserved, but synaptic vesicles were diminished and mitochondrial cristae were partially destructed in Group M [Figure 8c]. In contrast, severe mitochondrial degeneration and separation of the intercellular junction were evident in the cells of the outer plexiform layer in Group G [Figure 8d].

In Group M, vacuolization and edema was observed in the cells of the inner plexiform layer [Figure 9a] but cellular organelles, such as the endoplasmic reticulum, were intact in both interneurons and ganglion cells [Figure 9b]. Nuclear membrane invaginations, dilatation in endoplasmic reticulum cisternae, and autophagosomes were common in bipolar and ganglion cells of Group G [Figure 10a-c, respectively]; however such findings were detected only in two animals in Group M.

In Group G, apoptotic cells characterized by fragmented nuclei and dark cytoplasm were mostly observed in the ganglion cell layer and in inner plexiform layer [Figure 11]. The number of apoptotic cells was smaller in the outer plexiform layer and they were rare in the photoreceptor cell layer.

One of the major findings noted in Group M was the presence of widely scattered Müller cells, exhibiting increased number of phagosomes, among the degenerated neurons. In addition, structural integrity of the mitochondria was preserved in Group M.

In contrast to the normal vascular structure [Figure 12c] in Group M, vacuolar degeneration in the nerve fiber layer [Figure 12a] and endothelial swelling...
and vacuolization in some retinal vessels [Figure 12b] were observed in Group G. Ultrastructural changes related with glaucomatous damage are summarized in Table 2.

DISCUSSION

In this study, a rat model of experimental glaucoma was used to investigate the effects of systemic memantine treatment on retinal ultrastructure. Although ultrastructural changes in experimentally induced glaucoma have been previously studied, there is no published data on ultrastructural changes in the retina during pharmacological treatment to prevent excitotoxic neuronal death in glaucoma.[32] The present study constitutes the first demonstration of ultrastructural changes in glaucomatous retinas treated with systemic memantine, an uncompetitive NMDA glutamate receptor antagonist for neuroprotection.

An increased number of phagosomes in the RPE, and nuclear membrane invaginations in ganglion cells without evident neuronal loss were observed in memantine treated animals, and these alterations were not as severe as those of non-treated animals.

A noteworthy finding in the present study was the presence of evident degeneration and apoptosis in photoreceptor cells. There are contradictions about photoreceptor damage in glaucoma in the literature.[33] In most studies, electroretinogram findings were reported to be normal in patients with glaucoma suggesting that photoreceptors remain intact in glaucoma.[33‑35] Quigley and Green[36] demonstrated damage only in the RGC and their axons in a light and electron microscopic study on postmortem glaucoma patients. In contrast to those studies, there are studies proposing that photoreceptors are affected as glaucoma progresses.[37] Data of the present study seem to support the idea that outer retinal layers including photoreceptors and RPE were all affected following sub-acute glaucoma induction. These data support the opinion that glaucoma may cause damage not only in RGCs and in their axonal extensions but also in all retinal layers, including the photoreceptors.

Müller cells are some of the most important glial cells of the vertebrate retina, with important functions including homeostasis, regulation of retinal angiogenesis and retinal blood flow, and development of reactive

Figure 5. Electron micrographs show lamellar inclusions and focal loss of cristae in some mitochondria, and obliteration of the microvilli of Müller cells in the inner retina in Group M. (a) Electron micrograph shows sparse lamellar inclusions and focal loss of mitochondrial cristae (arrows) and obliteration of microvilli of Müller cells (asterisk) in the inner retina in Group M. Note a cilium (black arrow). (b) Electron micrograph reveals loss of mitochondrial cristae (asterisk) and a part of lamellar body in the apical portion of inner segment (arrow) in Group M.

Figure 6. Electron micrograph showing loss of mitochondrial cristae (arrows) and swelling of inner segments (asterisk) in Group G.

Figure 7. Electron micrographs showing the structure of the outer nuclear layer in the study groups. (a) Electron micrograph of the outer nuclear layer shows attachments (arrows) between cells in Group C. (b) Electron micrograph from the outer nuclear layer shows minimal detachment between cells (asterisk) and intact cellular attachments forming the external limiting membrane (arrows) in Group M. (c) Electron micrograph shows severe cytoplasmic and nuclear vacuolization in the cells of the outer nuclear layer in Group G.
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It has also been reported that Müller cells are effective in preventing excitotoxicity, uptake of glutamate from the extracellular environment and the release of glutathione. We observed that in non-treated glaucomatous rats, Müller cells were scattered around the degenerated neurons, with their dense and long microfilamentous processes. However, Müller cells in memantine-treated animals did not show wide dispersion around the neurons and they contained dense phagosomes. Regarding the configuration of Müller cells, the difference between memantine treated and non-treated groups may be explained as a “workload relieving” effect by memantine on Müller cells.

Another finding, which was not observed in memantine-treated eyes but frequently noted in the non-treated glaucoma group, was villous transformation and vacuolization of endothelial cells in inner retinal layers. This finding indicates possible degeneration in retinal vessels that is ultrastructurally visible.

In late 2008, some of the results of the second phase III trial on the clinical use of memantine in primary open-angle glaucoma were reported, and it was stated that while higher doses of memantine reduced progression of glaucoma, lower doses did not cause a significant effect. This author emphasized the fact that the results of this phase III trial on memantine should not be seen as a disappointment, but should be used as a basis for further studies and stated that memantine, might be effective in slowing down apoptosis during the glaucomatous process only in cells containing a defined density of NMDA receptors and also stressed the necessity of using agents with different mechanisms of actions for neuronal protection.

In the current study, by demonstrating the efficacy of memantine at the organelle level, we introduce data that may provide a basis for the combined use of memantine with different agents in order to enhance its efficacy. The employed dose of memantine showed no toxic effects at the level of the organelle. We assert that demonstration responses in pathologic conditions. It has also been reported that Müller cells are effective in preventing excitotoxicity, uptake of glutamate from the extracellular environment and the release of glutathione. We observed that in non-treated glaucomatous rats, Müller cells were scattered around the degenerated neurons, with their dense and long microfilamentous processes. However, Müller cells in memantine-treated animals did not show wide dispersion around the neurons and they contained dense phagosomes. Regarding the configuration of Müller cells, the difference between memantine treated and non-treated groups may be explained as a “workload relieving” effect by memantine on Müller cells.

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Table 2. Electron microscopic damage scores among the study groups

| Cell/layer | Ultrastructural findings         | Group C | Group G | Group M | P*     |
|------------|---------------------------------|---------|---------|---------|--------|
| RCE        | Vacuolization and loss of villi | 0       | 3.75±0.46 | 0.25±0.46 | <0.001 |
| RNFL       | Cellular edema                  | 0       | 3.00±0.75 | 1.13±0.64 | 0.001  |
| RNFL       | Inclusion bodies                | 0       | 3.62±0.51 | 0.50±0.53 | 0.001  |
| RNFL       | Lipid vacuolar degeneration     | 0       | 3.63±0.51 | 0.50±0.53 | 0.001  |
| GCL        | Mitochondrial damage            | 0       | 2.88±0.83 | 0.50±0.53 | 0.001  |
| GCL        | Mitochondrial inclusion bodies  | 0       | 3.38±0.74 | 0.50±0.53 | 0.001  |
| GCL        | Apoptotic cells                 | 0       | 3.63±0.51 | 0.88±0.35 | <0.001 |
| INL        | Condensation in cytoplasm and chromatin | 0       | 3.38±0.74 | 0.63±0.74 | 0.001  |
| INL        | Vacuole formation and edema     | 0       | 3.00±0.53 | 0.75±0.46 | <0.001 |
| INL        | Apoptotic cells                 | 0       | 3.00±0.75 | 0.50±0.53 | 0.001  |
| BCL        | Mitochondrial damage            | 0       | 2.75±0.71 | 0.75±0.71 | 0.001  |
| BCL        | Mitochondrial inclusion bodies  | 0       | 3.38±0.74 | 0.75±0.71 | 0.001  |
| BCL        | Dilatation in ER cisternae and axons | 0       | 3.50±0.53 | 0.50±0.53 | 0.001  |
| MC         | Phagosome content               | 0       | 0.38±0.51 | 3.00±0.75 | 0.001  |
| MC         | Cellular degeneration           | 0       | 3.00±0.75 | 0.50±0.53 | 0.001  |
| MC         | Mitochondrial damage            | 0       | 3.25±0.88 | 0.38±0.52 | 0.001  |
| MC         | Mitochondrial inclusion bodies  | 0       | 3.25±0.71 | 0.63±0.74 | <0.001 |
| OPL        | Detachment in layers            | 0       | 2.87±0.64 | 0.50±0.53 | 0.001  |
| ONL        | Apoptotic cells                 | 0       | 2.00±0.75 | 0.38±0.51 | 0.002  |
| PRC        | Degeneration in lamellar bodies | 0       | 3.75±0.46 | 0.50±0.53 | <0.001 |
| PRC        | Mitochondrial damage            | 0       | 3.38±0.74 | 0.75±0.71 | 0.001  |
| PRC        | Mitochondrial inclusion bodies  | 0       | 3.38±0.91 | 0.38±0.51 | 0.001  |
| PRC        | Apoptotic cells                 | 0       | 2.00±0.75 | 0.38±0.51 | 0.002  |
| RPE        | Loss of pigment granules        | 0       | 3.63±0.74 | 0.88±0.35 | <0.001 |
| RPE        | Formation of inclusion bodies   | 0       | 3.50±0.75 | 2.00±0.53 | 0.003  |
| RPE        | Vacuolization                   | 0       | 3.00±0.75 | 0.38±0.51 | 0.001  |
| RPE        | Detachment at intercellular junctions | 0       | 3.25±0.88 | 1.00±0.53 | 0.001  |
| RPE        | Loss of villous processes       | 0       | 3.75±0.46 | 0.50±0.53 | <0.001 |
| RPE        | Mitochondrial damage            | 0       | 3.75±0.46 | 0.38±0.52 | <0.001 |
| RPE        | Mitochondrial inclusion bodies  | 0       | 3.38±0.52 | 0.50±0.53 | 0.001  |

*Statistical comparisons were made between groups G and M. Electron microscopic findings of damage were graded between 0 and 4 (with 0 representing no damage and 4 representing the highest level of damage); mean damage scores for each cell or layer was calculated for each group. SD, standard deviation; RCE, retinal capillary endothelium; RNFL, retinal nerve fiber layer; INL, inner nuclear layer; BCL, bipolar cell layer; MC, Müller cells; OPL, outer plexiform layer; ONL, outer nuclear layer; PRC, photoreceptor cells; RPE, retinal pigment epithelium; GCL, ganglion cell layer; ER, endoplasmic reticulum

Figure 11. Electron micrograph shows an amacrine cell (A), diffuse vacuolization (asterisk) and an apoptotic cell (AP) in Group G.

of glaucomatous damage and the efficacy of agents, such as the NMDA receptor antagonist memantine, at the organelle level may be considered as an approach to elucidate the etiopathogenesis of glaucoma, although the reflection of its efficacy to clinical practice is controversial. It might be a rational method to reduce RGC loss due to glaucoma damage by the combined use of agents with different mechanisms of action and to effectively protect remaining neuronal populations by the combined use of multi-dimensional neuroprotective agents. Gabelt reported evidence for a neuroprotective effect by memantine on ganglion cell function in their experimental study on monkeys. Therefore, memantine is still considered to be valuable, and its neuroprotective efficacy is still under investigation.
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Figure 12. Electron micrographs show vacuolar degeneration in the nerve fiber layer and endothelial swelling in Group G and preserved vascular structure in Group M. (a) Electron micrograph of the nerve fiber layer shows destruction in mitochondria (arrows) and swelling (asterisk) in Group G. (b) Electron micrograph of a capillary in Group G shows endothelial swelling and platelets (P) in its lumen. (c) Electron micrograph of a capillary in Group M shows normal endothelium.

Data of the present study demonstrate that in an experimentally induced glaucoma model in rats, memantine demonstrated a neuroprotective effect without any significant impact on IOP. Our results seem to support the idea that glutamate-induced excitotoxicity may play a significant role in glaucoma induced neuronal injury. Currently, in addition to conventional glaucoma treatment, which directly aims to decrease IOP, anti-excitotoxic medications are being explored as new treatment strategies to prevent neuronal injury. Memantine treatment may preserve retinal ultrastructure and thus prevent neuronal injury in experimental glaucoma. Based on our knowledge, this is the first published report on qualitative ultrastructural changes in glaucomatous retinas treated with systemic memantine for neuroprotection. We hope that by demonstrating organelle protection in the neuronal cells, these observations contribute to further neuroprotective investigations and lead to a better understanding of the pathogenesis of glaucoma at the ultrastructural level.

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Nil.

Conflicts of Interest
There are no conflicts of interest.

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