·Basic Research·

Neuroprotective effect of edaravone in experimental glaucoma model in rats: a immunofluorescence and biochemical analysis

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Abstract

• **AIM: To evaluate the neuroprotective activity of systemically administered edaravone in early and late stage of experimental glaucoma in rats.**

• **METHODS: In this study, 60 Wistar albino rats were used. Experimental glaucoma model was created by injecting hyaluronic acid to the anterior chamber once a week for 6wk in 46 of 60 subjects. Fourteen subjects without any medication were included as control group. Edaravone administered intraperitoneally 3 mg/kg/d to the 15 of 30 subjects starting at the onset of glaucoma induction and also administered intraperitoneally 3 mg/kg/d to the other 15 subjects starting at three weeks after the onset of glaucoma induction. The other 16 subjects who underwent glaucoma induction was administered any therapy. Retinal ganglion cells (RGCs) have been marked with dextran tetramethylrhodamine (DTMR) retrograde at the end of the sixth week and after 48h, subjects were sacrificed by the method of cardiac perfusion. Alive RGC density was assessed in the whole-mount retina. Whole-mount retinal tissues homogenized and nitric oxide (NO), malondialdehyde (MDA) and total antioxidant capacity (TAC) values were measured biochemically.**

• **RESULTS: RGCs counted with Image-Pro Plus program, in the treatment group were found to be statistically significantly protected, compared to the glaucoma group (Bonferroni, P <0.05). The neuroprotective activity of edaravone was found to be more influential by administration at the start of the glaucoma process. Statistically significant lower NO levels were determined in the glaucoma group comparing treatment groups (Bonferroni, P<0.05). MDA levels were found to be highest in untreated glaucoma group, TAC levels were found to be lower in the glaucoma induction groups than the control group (Bonferroni, P<0.05).**

• **CONCLUSION: Systemic administration of edaravone in experimental glaucoma showed potent neuroprotective activity. The role of oxidative stress causing RGC damage in glaucoma was supported by this study results.**

• **KEYWORDS:** antioxidant; edaravone; glaucoma; neuroprotection
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INTRODUCTION

Glaucoma is a progressive, multifactorial optic neuropathy involving optic nerve head cupping and visual field defects caused by the damage of Retinal ganglion cell (RGC) bodies and axons and death due to apoptosis. Sixty million people have been affected by the glaucoma in worldwide, and this number is expected to reach 80 million in 2020 [1]. Even when lowering intraocular pressure (IOP), the most important risk factor in the development of glaucoma, the disease can be progress [2]. Therefore, it is necessary that reveal the underlying pathology of glaucoma, to determine an ideal treatment strategy.

The term of neuroprotection is a common pathway developed against excitotoxicity of free radicals, oxidative stress, mitochondrial dysfunction and apoptosis that the influential factors on pathophysiological mechanisms of Alzheimer's disease, stroke, glaucoma and many neurodegenerative diseases [3]. Astrocytes, glial cells are also as well as other targets of neuroprotection. However, the final common pathway of glaucomatous optic neuropathy is the loss of RGCs, thus priority of neuroprotection must preserve these

cells[4]. Neuronal loss occurs within minutes in the process of stroke whereas glaucomatous optic neuropathy progress in over the years. In both disease use of neuroprotective agents are known to be effective in preventing neuronal loss and delaying the process leading to cell death. Also it is obvious that early initiation of neuroprotective therapy prolongs the process of cell death[5].

Edaravone (3-methyl-1-phenyl-2-pyrazoline-5-one, MC-186, EDA) have been used to treat acute ischemic stroke as a free radical scavenger in Japan, since 2001. Neuroprotective, free radical scavenger, anti-apoptotic and anti-inflammatory effects of edaravone have been shown in recent studies. With the use of edaravone therapeutically effective results have been obtained in cerebral infarction, aneurysmal subarachnoid hemorrhage and acute stroke [6,7]. Edaravone protects the brain and heart from reperfusion injury by suppressing the production of reactive oxygen radicals[8].

The effect of edaravone has been demonstrated in many acute and chronic neurodegenerative diseases. Glaucoma is a neurodegenerative disease, involving free radical cell damage and oxidative stress in its pathogenesis. Therefore, we have tried to show the effectiveness of edaravone as a new neuroprotectant in the experimental glaucoma model for the first time.

MATERIALS AND METHODS

This work was conducted in Kocaeli University animal experiment research laboratory after obtaining approval from the local ethics committee and in accordance with the Declaration of Helsinki.

In this study, a total of 60 adult male Wistar albino rats were used. Wistar rats (male), in active condition, 12-16 weeks old and weight is about (350-400 g) are included in the study. Rats showing eating and behavior disorder, died before the completion of the experiment were excluded from the study.

The mean weight of the subjects was 383±21 g. Subjects were housed in standard cages with in groups of seven and were fed with standard food and water, in temperature (21±2 ℃) and humidity controlled rooms. Room lighting was provided by fluorescent lights, and every 12h opening and closing cycles were performed. The subjects were divided into two groups, glaucoma induction groups (46 subjects) and control group (14 subjects).

Experimental Glaucoma Model Hyaluronic acid (Sigma catalog no. H1751, Sigma Chemical Co., St. Louis, MO, USA) was injected to both anterior chambers of subjects with 30 gauge needle under general anesthesia after administration of intramuscular ketamine hydrochloride (25 mg/kg, Ketalar®, Pfizer) and xylazine hydrochloride (10 mg/kg, Rompun®, Bayer). Injections was performed at corneoscleral limbus by blocking the needle tip in contact with the iris and the lens, one week intervals for 5wk moving towards from 12 o'clock to 6 o'clock[9]. The same amount of hyaluronic acid, balanced salt solution was injected into the anterior chamber of rats in the control group with 30 gauge needle.

Edaravone Administration and Groups Single dose of 3 mg/kg/d edarovone was injected intraperitoneally, dissolving with 0.5% dimethylsulfoxide (DMSO) in saline with beginning simultaneously of onset glaucoma induction on the same day time (10:00 to 12:00) daily. This group was identified as early edaravone group (EE, 15 subjects). During the study, a total of 42 doses Edarovone were applied to this group of subjects.

Single dose of 3 mg/kg/d Edarovone was injected intraperitoneally dissolving with 0.5% DMSO in saline with beginning 3wk after onset glaucoma induction on the same day time (10:00 to 12:00) daily. This group was identified as late edaravone group (LE, 15 subjects). During the study a total of 21 doses edarovone were applied to this group of subjects.

DMSO (0.5%) in 0.9% physiological saline were injected intraperitoneally on the same day time (10:00 to 12:00) daily with beginning simultaneously of onset glaucoma induction to other 16 subjects (glaucoma group-G) as control solution. Any drugs were administered to the control group (control group-C).

Intraocular Pressure Measurement All subjects IOP were measured and recorded following anesthesia with 10 mg/kg ketamine hydrochloride and administration of topical 0.4% proparacaine hydrochloride 0.5% (Alcaine®, Alcon) with Tono-Pen (Medtronic Solan XL) before and after each hyaluronic acid injection weekly, lasted for five weeks. All injections and measurements were performed on the same day time (08:00 to 10:00). At the end of six weeks the subjects were sacrificed by cardiac paraformaldehyde perfusion method and fixed globes were enucleated.

Biochemical Analysis After extraction by the method of whole mount, retina tissues were homogenized, nitric oxide (NO), malondialdehyde (MDA) levels were measured with cadmium method. To evaluation of total antioxidant capacity (TAC), hydrogen peroxide solution was prepared in accordance with kit catalog (Antioxidant Assay Kit, Cayman) for the measurement of antioxidant buffer, antioxidant chromogen, antioxidant metmyoglobin, antioxidant Trolox and antioxidant. The measurement was carried out by the ELISA device. The data was converted to concentration after forming trolox standard curve as noted in kit prospectus.

Statistical analysis of data was performed with IBM SPSS package program for windows version 19 software. In the statistical analysis for comparison of variables, multivariate variance analyzes test (ANOVA) was used for all groups and in the comparison groups among themselves, the post-hoc evaluation test (Bonferroni test) was used. Limit of statistical significance was considered $P<0.05$. Results were expressed as mean±standart deviation (SD).

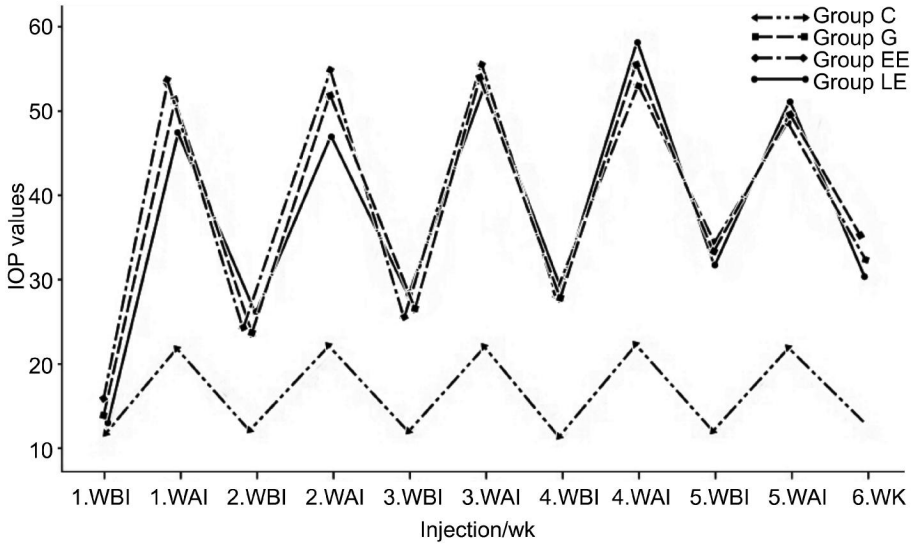

Figure 1 IOP values of the subjects WBI: Week before injection; WAI: Week after injection.

RESULTS

Demographic Characteristics of Rats At the beginning of the study, mean weights were 367±14 g in group C, 360±9 g in group G, 364±16 g in group EE and 368±7 g in group LE. At the end of the study, the average weights in the groups were 398±14, 379±7, 382±14 and 386±6 g, respectively. Any behavioral disorders and loss of appetite were not detected with the rats.

Intraocular Pressure Values IOP elevation was observed in all subjects, after the glaucoma induction. Adequate IOP values were achieved in all groups for the duration of six weeks. There was a statistically significant difference between the IOP values in G, EE, LE groups compared with group C (P<0.05). Besides there was no statistically significant difference in terms of mean IOP between each other G, EE and LE groups (P>0.05, Figure 1).

Retinal Ganglion Cell Count RGC cell count was performed with Image Pro Plus (IPP 5.1 for Windows®; Media Cybernetics, Silver Spring, MD, USA) program. DTMR marked average RGC density (average number in mm^2 ±SD), and the differences between the groups are presented in the Table 1. A significant reduction was determined in number of RGC in the subjects with glaucoma induction. The average number of RGC of the groups G, EE and LE were found to be significantly lower compared to group C (Bonferroni, P<0.000, P<0.033, P<0.000). RGC counts were significantly higher in groups LE and EE compared to group G (Bonferroni, P<0.000, P<0.000). Furthermore, average RGC numbers in group EE were significantly higher compared to the group LE (Bonferroni, P<0.000).

Six weeks after glaucoma induction, average RGC reduction was 45%±5% in group G, 10%±3% in group EE and 25% ±4% in group LE compared with the group C. Considering parameters of total retinal cell number, cell number in mm^2 and percentage reduction in cell number,

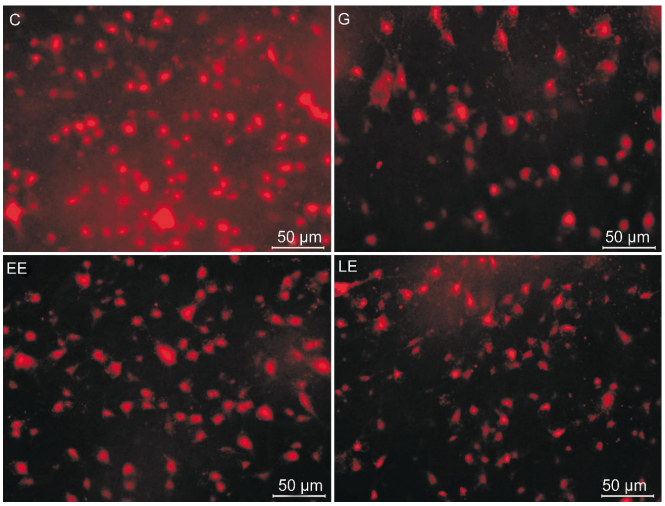

Figure 2 DTMR marked immunofluorescence images of RGCs Group abbreviations was placed in picture.

RGCs were found to be significantly protected in group EE and LE compared to group G. DTMR marked RGC immunofluorescence images of the groups are presented in Figure 2.

Biochemical Analysis of Retinal Tissue After excising whole mount average wet weight of retina tissues were 18.08±5.25 mg in group C, 15.80±4.12 mg in group G, 14.56±3.52 mg in group EE and 15.28±5.71 mg in group LE, respectively. There was no statistically significant difference between the groups in terms of wet tissue weight. TAC, MDA and NO levels measured in all groups are presented in Table 2.

Total Antioxidant Capacity Measurement After the glaucoma induction, a significant decrease was observed in TAC values of retinal tissue. Mean TAC values of the groups G, EE and LE were found to be significantly lower compared to the group C (Bonferroni, P<0.000, P<0.013, P<0.009). Mean TAC values of the groups EE and LE treated with EDA, were found to be higher compared to the

Table 1 DTMR marked retinal ganglion cells count in the subjects

Groups	No. of cells in mm^2	Total number
Group C	1520±95	10856±684
Group G	822±110	5869±789
Group EE	1366±79[b]	9753±567[b]
Group LE	1125±74[b]	8033±534[b]

[b]P<0.01. Comparison of treatment groups with glaucoma group (ANOVA, P<0.05).

Table 2 Biochemical analysis of TAC, MDA and NO levels in the retinal tissue of subjects

Groups	TAC (μmol/g)	MDA (nmol/g)	NO (nmol/g)
Group C	0.31±0.12	4.15±0.6	25.73±5.3
Groupg G	0.14±0.04	7.95±1.2	18.39±6.0
Group EE	0.19±0.05	5.82±0.5	28.03±7.2
Group LE	0.18±0.05	7.93±0.2	27.61±7.5

untreated group G (Bonferroni, P>0.1, P>0.1), otherwise group EE values were found to be higher than group LE but all these differences were not statistically significant (Bonferroni, P>0.1).

Malondialdehyde Measurement As a result of glaucoma induction a significant increase in MDA levels were detected in retinal tissue of subjects. Mean MDA values were found to be significantly higher in group G, EE and LE compared to group C (Bonferroni, P<0.000, P<0.02, P<0.000). Mean MDA values of the groups G and LE, were found to be significantly higher compared to the group EE (Bonferroni, P<0.000, P<0.000), otherwise group EE values were found to be significantly lower than group LE(Bonferroni, P<0.000).

Nitric Oxide Measurement The lowest NO levels were measured in the group G. Mean NO values of the groups EE and LE, were found to be significantly higher compared to the group G (Bonferroni, P<0.025, P<0.047), otherwise group EE values were found to be higher than group LE but this difference was not statistically significant (Bonferroni, P>0.1) .

DISCUSSION

In this study neuroprotective efficacy of systemically administered edaravone have been attempted to show with RGC count on an experimental rat glaucoma model. In addition, NO, MDA and TAC values were measured in retinal tissue to show antioxidative activity of edaravone in glaucoma. Already the neuroprotective efficacy of edaravone has been shown in many studies, although there is no published study relevant to its effects in glaucoma[10-12].

The target effect of glaucomatous damage on RGCs and their axons is well known. RGCs and optic nerve are the integral parts of central nervous system. Previous studies have been suggested that glaucoma as a neurodegenerative disease like Alzheimer disease due to similar cell death mechanisms[13,14]. Therefore, we wonder edaravone to be effective in glaucoma. Neuroprotective efficacy of edaravone treatment has been evaluated in retinal ischemia and phototoxicity models, but DTMR marked RGC evaluation has not been performed previously[15,16].

In our study number of RGCs of the groups treated with edaravone were significantly preserved, comparing glaucoma group, on the other hand, average RGCs number were higher in group EE than group LE. This can be explained by the edaravone and similar drugs to be less effective on apoptotic death of ganglion cells in the chronic glaucoma model.

Edaravone provides neuroprotective activity by different mechanisms. Munakata *et al* [7] demonstrated a marked remission of late ischemic neurological deficits and cerebral infarction due to vasospasm in patients with aneurysmal subarachnoid hemorrhage treated with edaravone. Yasuoka *et al* [11] showed that edaravone inhibits cytochrome c release and caspase-3 activation in the hippocampus and cortex after ischemia. Edaravone prevents neural cell degeneration and death induced by free radicals after cerebral infarction[17].

Detoxification of increased oxygen metabolites due to glaucoma is dependent ocular antioxidants. Oxidative stress is involved in the pathogenesis of many neurodegenerative diseases [18-20]. Suppression of the formation of free oxygen radicals with edarovone therapy in Parkinson model has been shown in previous studies [21,22]. With edarovone therapy, a marked retardation was determined in progressive motor neuron injury in the experimental amyotrophic lateral sclerosis model in mice [23] 19 amyotrophic lateral sclerosis patients reported by Yoshino and Kimura [24] treated with edaravone also showed retardation in progressive deterioration of motor neuron functions. In an another experimental spinal cord injury rat model, inhibition of lipid peroxidation, reduction in MDA levels and better functional recovery was observed in the group treated with edaravone, thus secondary neuronal damage was prevented by edaravone [25]. Free radical scavenger, antioxidant and neuroprotective effects of edaravone are demonstrated by these studies.

Neurodegeneration and oxidative stress in the pathogenesis of glaucoma are well known. So in the light of previous studies we could imagine aderavone as a neuroprotective and antioxidant agent in the treatment of glaucoma. In our study, oxidative stress parameters were also evaluated. MDA is a product of lipid peroxidation occurring in the oxidative process. Elevated MDA levels were determined in vitreous and retina in experimental glaucoma model of rats [26,27]. Yildirim *et al* [28], reported higher plasma MDA levels in patients with primary open angle glaucoma (POAG) compared healthy control group. In an experimental rat retinal ischemia model performed by Song *et al* [29] lower MDA levels were determined in the retina of the group treated with edaravone compared to the untreated control group. In our work, MDA levels were significantly increased with the glaucoma induction. Furthermore, significant lower MDA levels were detected in group EE comparing groups G and LE.

TAC has evaluated as an indicator of retinal antioxidant activity. In our study protective antioxidant activity reduced due to oxidative stress after the glaucoma induction. Consequently highest TAC values were measured in the control group and lowest values in the glaucoma group. As well as higher TAC values were obtained in the groups treated with edarovone comparing untreared glaucoma group, but this difference was not statistically significant. Özgiray *et al* [30] compared methylprednisolone and edarovone treatment on experimental spinal cord injury model in rats. NO, MDA, TAC levels, superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activity were evaluated in blood and tissue. In comparison with the control group lower tissue NO and MDA levels, higher tissue TAC levels and increased SOD, GSH-Px activities were detected in the group treated with edaravone. Additionally lower NO levels, similar SOD and GSH-Px activities, higher MDA levels were detected in the blood.

Increased NO synthase (NOS), glutamin synthase levels and decreased glutathione transferase (GST), SOD levels were found in aqueous humor of patients with POAG in a study conducted by Bagnis *et al* [31]. In another study performed by Cho *et al* [32] inducible NOS (iNOS) levels were found to be significantly higher in the ischemia- reperfusion model of rats created by increasing IOP than the control group. Along with increased IOP, Park *et al* [33] demonstrated elevatedneuronal NOS (nNOS) and NO levels in the chronic glaucoma model in rats.

In our study, NO levels were found statistically significantly higher in the groups treated with edaravone than the glaucoma group. Unlike the previous studies, we found decreased NO levels in glaucoma. A reduction in the levels of NO may be due to NOS insufficiency as a result of apoptotic loss of cells, including NOS, and inhibition of NOS by the dimethyl arginine derivates during the glaucoma process [34,35]. Decreased NO levels in the aqueous humor of patients with POAG was emphasized by Doganay *et al* [36] in a study conducted in 2002 for the first time. Following this study, Galassi *et al* [37] found a significant decrease in NO_2, cGMP levelswhich the markers of NO, in the plasma and aqueous humor of patients with POAG, comparing normal eyes. NO makes vasodilatation and enhances vascular contractility on trabecular meshwork thus act an IOP lowering and antiapoptotic effect. After trabeculectomy NOS levels was found to be lower in trabecular meshwork fragments of the patients with POAG[38-40]. In this case, we can say that edaravone shows an antioxidant activity in glaucoma.

There were limited studies on topical use of edaravone in the literature. Hironaka *et al* [41] have achieved significant results with the intravitreal administration of submicron-sized liposomes (ssLips) containing edaravone against oxidative stress-induced retinal damage. Also Shimazaki *et al* [15] demonstrated neuroprotective effect of eye drops containing liposomes loaded with edaravone in light-induced retinal damage model in mice. In addition to our work, further studies are needed on the use of topical edarovone in glaucoma.

For today, IOP is the single factor can be controlled that slows progression of glaucoma. But despite the effective IOP control the disease can be progress. Priority in current researches focus on neuroprotection. Neuroprotective agents for treating glaucoma should slow down the process of retinal ganglion cell death. At this point, primarily it is necessary to know how the RGCs die. The purpose of neuroprotection studies conducted so far, both to find an appropriate agent for treatment and clarify the pathogenesis of glaucoma. The main objective of this study to present edarovone as a new neuroprotectan and antioxidan in the treatment of glaucoma. Considering our results edarovone showed a significant antioxidant and neuroprotective activity on the experimental glaucoma model. Therefore results of our work support the presence of oxidative stress in the pathogenesis of glaucoma. Researches in the literature are intended for using edaravone on acut and subacute deficits of neurodegenerative diseases. But glaucoma is a slowly progressing, chronic optic neuropathy compared to these diseases. Consequently, although short term experimental effectiveness of edaravone in our study, large-scale, randomized, long term clinical and experimental studies are needed to the clinical use of edaravone in glaucoma.

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