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Effect of pycnogenol on ethanol-related oxidative retinal injury: an experimental study

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Abstract. – OBJECTIVE: We aimed at determining the protective effects of Pycnogenol on ethanol-induced retinotoxicity in an experimental model.

MATERIALS AND METHODS: 30 male Wistar albino rats were randomly divided into three groups: an untreated healthy control (HC group), a group in which only ethanol was daily administered for six weeks (EtOH group), and a group in which ethanol + 40 mg/kg Pycnogenol was daily administered orally for six weeks (PEtOH group). The same volume (0.5 ml) of distilled water as solvent was applied in the same manner to the rats in the HC and EtOH groups. With the rats in the PEtOH and EtOH groups, 32% ethanol at a dose of 5 g/kg was administered by oral gavage one hour after the application of pycnogenol or distilled water. At the end of the experimental period, tissue samples were obtained for biochemical examination of malondialdehyde (MDA) and total glutathione (tGSH) levels, and afterwards, the eyes were removed for histopathological examination.

RESULTS: Histopathological evaluations in the EtOH group showed significant destruction of retinal tissue with marked edema, decomposition and degeneration in layers, polymorphonuclear cell infiltration, dilatation and congestion in blood vessels. However, it was observed that MDA values increased and tGSH values decreased in the EtOH group. In the PEtOH group, MDA values decreased and GSH values increased. Again, degenerative changes were considerably less in this group.

CONCLUSIONS: In the light of biochemical markers and histopathological evaluations, it was observed that ethanol exposure caused a significant degeneration in the retinal tissue. It was found that Pycnogenol administration significantly reduced the destructive effects seen histopathologically. Biochemical results also coincided with other findings. It was concluded that ethanol-induced rethytosis can be prevented to a large extent by Pycnogenol administration.

Key Words:
Ethanol, Pycnogenol, Retinotoxicity, Oxidative damage.

Abbreviations
tGSH: Total glutathione; MDA: Malondialdehyde; ONL: Outer nuclear layer; INL: Inner nuclear layer; IPL: Inner plexiform layer.

Introduction

The two-carbon alcohol ethanol (CH_3CH_2OH) is a central nervous depressant system that is widely available to adults; its use is legal and accepted in many societies, and its abuse is a societal problem. The relevant pharmacological properties of ethanol include effects on the gastrointestinal, cardiovascular, and central nervous systems, effects on disease processes, and effects on prenatal development. Ethanol disturbs the fine balance between excitatory and inhibitory influences in the brain, producing disinhibition, ataxia, and sedation. Understanding the cellular and molecular mechanisms of these myriad effects of ethanol *in vivo* requires an integration of knowledge from multiple biomedical sciences[1]. Long term consumption of ethanol may induce damage to many organs. Ethanol induces its noxious effects through reactive oxygen species production, and lipid peroxidation and apoptosis induction in different tissues and cell types[2]. Alcohol can exert a toxic action due to its direct effect on the generation of free radicals or through the metabolites thereof, mainly acetaldehyde[3]. It has been proven that chronic ethanol intake leads to an increase in lipid peroxidation products and

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a decrease in antioxidative factors such as glutathione (GSH)[4,5]. It has been reported that many eye diseases, including retinopathy and uveitis, are associated with oxidative stress[6].

It has been shown by Johnsen-Soriano et al[3] that chronic alcohol consumption increases the amount of malondialdehyde (MDA), which is the end product of lipid peroxidation (LPO) and decreases the amount of glutathione (GSH) in the retinal tissue of the eye. The retina is the neuro-sensory tissue of the eye, and its membranes are extremely rich in polyunsaturated lipids. This feature makes it sensitive to ROS and LPO[7]. This information obtained from the literature suggests that ethanol may cause oxidative stress in retinal tissue by increasing oxidant parameters and decreasing antioxidant parameters.

Pycnogenol is a bark extract of the French maritime pine (*Pinus maritima*), produced by a validated water extraction procedure. Its main constituents are monomeric phenolic compounds (catechin, epicatechin and taxifolin) and condensed flavonoids (procyanidins/proanthocyanidins). Pycnogenol has antioxidant and free radical scavenging activities, which are higher than those of green tea extracts, *Ginkgo biloba* and other vegetable extracts[8]. This indicates that Pycnogenol may be useful in the treatment of ethanol-induced oxidative retinal damage. No studies investigating the protective effect of Pycnogenol against ethanol-induced oxidative retinal damage were found in the literature. The aim of our study was to investigate the biochemical and histopathological effects of Pycnogenol on ethanol-induced oxidative retinal damage in rats.

Materials and Methods

Animals

We used thirty 280-296 g Wistar albino male rats obtained from the Ataturk University Laboratory Animals Breeding and Experimental Research Center, Turkey. All animals were housed in groups in plastic cages at 21-22 °C, 55-60% humidity and a 12 h light: 12 h dark cycle (lights on at 07:00) and rats were allowed free access to food and water. All animal experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by the Local Animal Experiments Ethics Council of Ataturk University, Erzurum, Turkey (Ethics Committee Number: 77040475-000-E. 1800201965, Dated: 07 April 2018). All animal experiments were performed in accordance with the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research.

Experimental Design

The rats were randomly divided into three groups, with 10 rats in each group as follows: healthy controls (HC group), a group in which only ethanol was administered (EtOH group) and a group in which ethanol + Pycnogenol (40 mg/kg) was administered (PEtOH group). In this experiment, 40 mg/kg Pycnogenol was administered to the rats in the PEtOH group by oral gavage[9]. The same volume (0.5 ml) of distilled water as solvent was applied in the same manner to the rats in the HC and EtOH groups. One hour after application of thiamine pyrophosphate or distilled water, 32% ethanol at a dose of 5 g/kg was administered *via* oral gavage to the rats in the EtOH and PEtOH groups. Various doses of ethanol have been used to produce oxidative stress in the organs and tissues of animals. In our study, this dose was preferred because 32% ethanol had a greater effect at a dose of 5 g/kg[10].

This procedure was repeated once a day for six weeks with the same dose and volume in the same manner. At the end of this period, the animals were sacrificed with a high dose (50 mg/kg) of i.p. thiopental sodium (IE Ulagay, Istanbul, Turkey) anesthesia and their eyes were enucleated. The retinal tissue was examined histopathologically. In addition, malondialdehyde (MDA) and total glutathione (tGSH) measurements were made on retinal tissue samples taken from the animals' tail veins. The biochemical and histopathological results obtained were compared and evaluated between the groups.

Biochemical Analysis

Preparation of Samples

As oxidative stress indicators, we investigated in tissue malondialdehyde (MDA) and total glutathione (tGSH). After the animals were sacrificed, the eye tissues were homogenized in ice-cold 2 mL phosphate buffers (50 mM, pH:7,4) and centrifuged at 1,200 xg for 20 minutes at 4°C[11]. Following that, the levels of tGSH and MDA in the supernatants were determined. The protein concentration in the supernatant was also determined using the Bradford MM method, and all tissue values were represented by dividing by

G-protein[12]. A microplate reader was used for all spectrophotometric analyses (Bio-Tech, Minneapolis, MN, USA).

MDA Analysis in Tissue

The Ohkawa et al[13] technique and Kurt et al[14] modification were used to detect MDA, which involved spectrophotometrically measuring the absorbance of the pink-colored complex generated by thiobarbituric acid (TBA) and MDA. 25 µL of tissue homogenate were mixed with 25 µL of a solution containing 80 g/L sodium dodecyl sulfate and 1 mL of a combination solution (20 g/L acetic acid + 1.06 g 2-thiobarbiturate + 180 mL distilled water). For 60 minutes, the mixture was incubated at 95°C. After cooling, the mixture was centrifuged at 1,200 xg for 10 minutes. At 532 nm, the absorbance of the supernatant was measured. The standard curve was created with 1,1,3,3-tetra methoxy propane.

tGSH Analysis in Tissue

According to the approach outlined by Sedlak and Lindsay[15], 5,5'-dithiobis (2-nitrobenzoic acid) disulfite (DTNB) is chromogenic in the medium and is quickly reduced by sulfhydryl groups. The yellow color generated during the reduction is detected using spectrophotometry at 412 nm. A cocktail solution (5.85 mL 100 mM Na-phosphate buffer, 2.8 mL 1 mM DTNB, 3.75 mL, 1 mM NADPH, and 80 mL 625 U/L Glutathione reductase) was prepared for measurement. Prior measuring, 100 µL of meta-phosphoric acid were added to 100 µL of sample and centrifuged for 2 minutes at 1,000 xg for deproteinization. The 150 µL cocktail solution was mixed with 50 µL of supernatant. L-gluthathione oxidized (GSSG) was used to generate the calibration curves.

Histopathological Examination

All of the tissue samples were first identified in a 10% formaldehyde solution for light microscope assessment. Following the identification process, the tissue samples were washed under tap water in cassettes for 24 hours. The samples were then treated with a conventional grade of alcohol (70%, 80%, 90%, and 100%) to remove the water within the tissues. Tissues were then passed through xylol and embedded in paraffin. Four-to-five-micron sections were cut from the paraffin blocks and hematoxylin-eosin staining was performed. Photographs of the sections were taken following the Olympus DP2-SAL firmware program (Olympus® Inc. Tokyo, Japan) assessment. At this stage, 100 serial sections were taken from the paraffin blocks obtained from each tissue. Counting and scoring were performed in six areas, one central and five peripherals, photographed at 400X magnification in 10 sections randomly selected from the 100 serial sections taken. Using this program, we measured total retinal thickness and outer nuclear layer (ONL), inner nuclear layer (INL) and inner plexiform layer (IPL) thicknesses to reveal the effect of ethanol on retinal layer thickness changes. In addition, cell counting was performed in the retinal ganglion cell layer (RGC) at 400X magnification. Qualitative analysis of histopathological examinations was evaluated as retinal destruction, edema, vascular congestion and polymorphonuclear cell presence, and scored between 0-3. Histopathological assessments were carried out by two observers blinded to the experimental data and the study groups.

Statistical Analysis

IBM SPSS 22 (IBM Corp., Armonk, NY, USA) was used for statistical analysis. The results were presented as mean ± standard error of the mean (SEM). The assumption of normalcy was confirmed with the Kolmogorov-Smirnov test. For comparison of groups, one-way ANOVA was used. After ANOVA, Tukey's HSD or Games-Howell's tests were used as post hoc, according to the homogeneity of variances. When variables were not normally distributed, the Kruskal-Wallis' test was used with Dunn's test as a post-hoc test. The statistical level of significance for all tests was taken as $p = 0.05$.

Results

Biochemical Results

The MDA and tGSH values are given in Figure 1. It was found that the amount of MDA in the tissue samples of the EtOH group was significantly greater than in the HC and PEtOH groups ($p < 0.001$). However, the MDA difference between the HC and PEtOH groups was found to be statistically insignificant ($p > 0.05$) (Figure 1A). In addition, ethanol application caused a decrease in the amount of tissue tGSH. The amount of tGSH in the EtOH group was significantly greater than in the HC and PEtOH groups ($p < 0.001$). However, the quantity of tGSH in the HC and PEtOH groups showed no significant difference ($p > 0.05$) (Figure 1B).

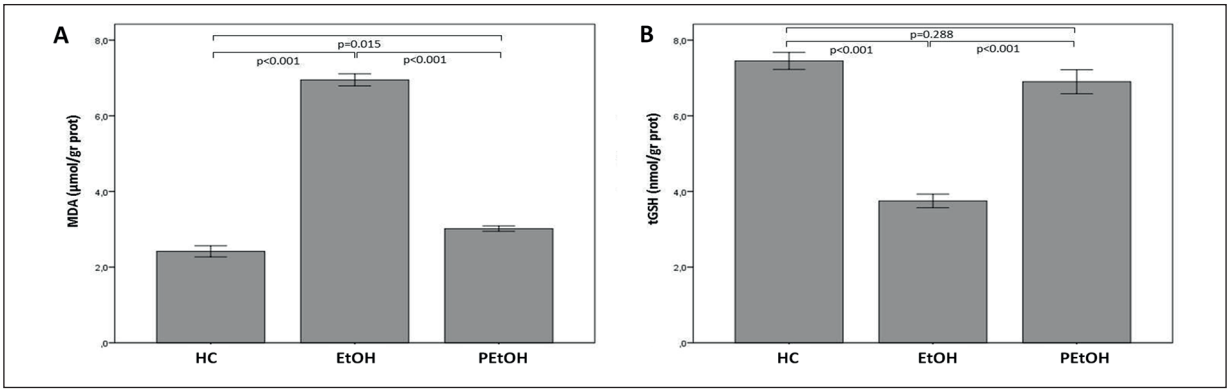

Figure 1. Differences in MDA **(A)** and tGSH **(B)** levels between the healthy control (HC), ethanol (EtOH), and ethanol + Pycnogenol (PEtOH) groups.

Histopathological Results

In the HC group, all the layers of the retina showed normal histological appearance. All of the retinal layers that were examined, the ganglion cell layer (GCL), the inner plexiform layer (IPL), the inner nuclear layer (INL), the outer plexiform layer (OPL) and the outer nuclear layer (ONL), were well defined. Inner and outer nuclear layer nuclei were stained basophilic and blood capillaries were rarely seen in the ganglion cell layer (GCL) and the inner plexiform layer (IPL) (Figure 2A). In the EtOH group, the ganglion cell layer was highly edem-

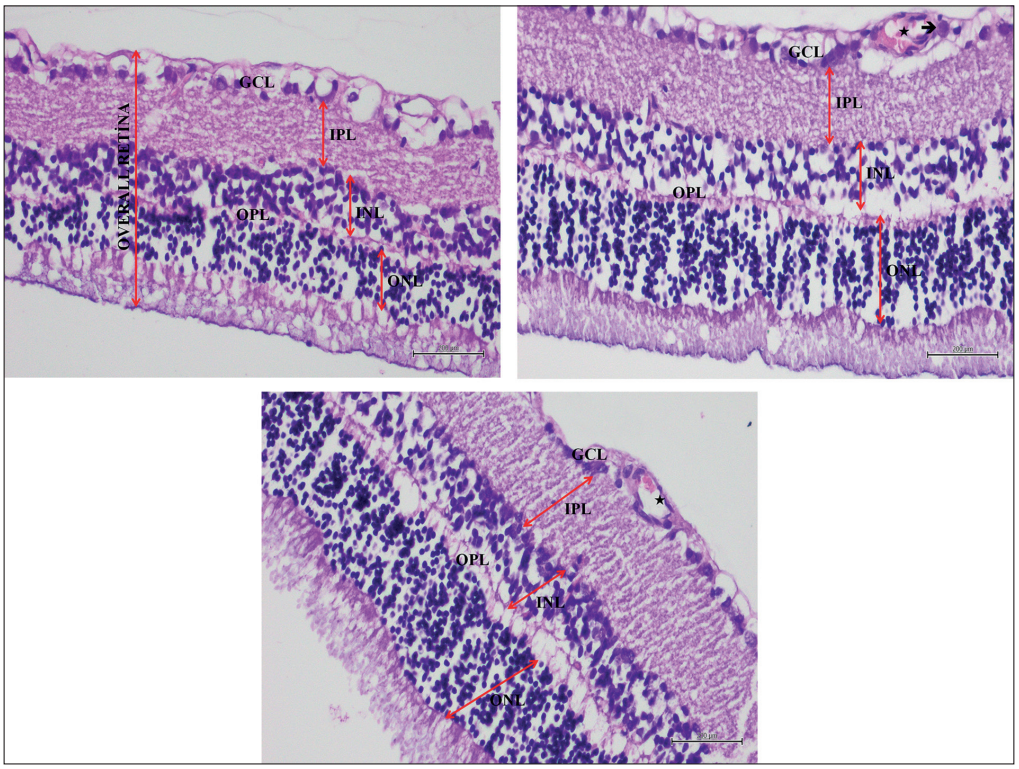

Figure 2. A, Hematoxylin-eosin staining in retina tissue in the control group; **GCL:** ganglion cell layer, **IPL:** inner plexiform layer, **INL:** inner nuclear layer, **OPL:** outer plexiform layer, **ONL:** outer nuclear layer, 200×. **B,** Hematoxylin-eosin staining in retina tissue in the ethanol group; **GCL:** ganglion cell layer, **IPL:** inner plexiform layer, **INL:** inner nuclear layer, **OPL:** outer plexiform layer, **ONL:** outer nuclear layer, →: polymorphonuclear cell, ★: dilated and congested blood capillary, 200×. **C**, Hematoxylin-eosin staining in retina tissue in the ethanol + pycnogenol group; **GCL:** ganglion cell layer, **IPL:** inner plexiform layer, **INL:** inner nuclear layer, **OPL:** outer plexiform layer, **ONL:** outer nuclear layer, ★: less dilated and mild congested blood capillary, 200×.

atous, and the blood capillaries of the ganglion cell layer were dilated and congested. Some polymorphonuclear cells were identified around the capillaries, and a trace of them had spread into the ganglion cell layer. In the inner nuclear layer, the density of cell nuclei was relatively low compared to the control group sections. The outer plexiform layer was separated, and the connection processes of cells seemed to be disrupted. Retinal tissue was edematous in general histological appearance. In addition, outer nuclear layer nuclei were aberrational (Figure 2B). In the PEtOH group, sections of retina tissue cells were neat and regular, and the accompanying blood capillaries were less dilated and mildly congested. No polymorphonuclear cells were seen around the blood capillaries or GCL. Cell nuclei were more organized and regular in the inner nuclear layer. Layer decomposition was lower in the outer plexiform layer when compared with the cisplatin group samples. The outer nuclear layer showed a similar appearance to the control group (Figure 2C).

We measured total retinal thickness and outer nuclear layer (ONL), inner nuclear layer (INL) and inner plexiform layer (IPL) thicknesses in all groups. Retina thickness data results are given in Figure 3. Total retinal thickness and ONL, INL and IPL thicknesses in the EtOH group were statistically significantly greater than in the control group ($p < 0.001$). When the PEtOH group was evaluated, it was observed that the greater retinal layer thicknesses, which were thought to be caused by ethanol-related edema, were significantly lower ($p < 0.001$). The GCL number in the HC group was found to be 8.4 ± 0.2, while in the EtOH group it was measured as 4.9 ± 0.1. This lower value was statistically significant ($p < 0.001$). The GCL level in the PEtOH group was 7.2 ± 0.1, and this level was higher than that of the EtOH group ($p < 0.001$), and it was seen to approach the level of the control group (Figure 4). The qualitative analysis of histopathological examinations is presented as retinal destruction, edema, vascular congestion and polymorphonuclear cell presence in Table I.

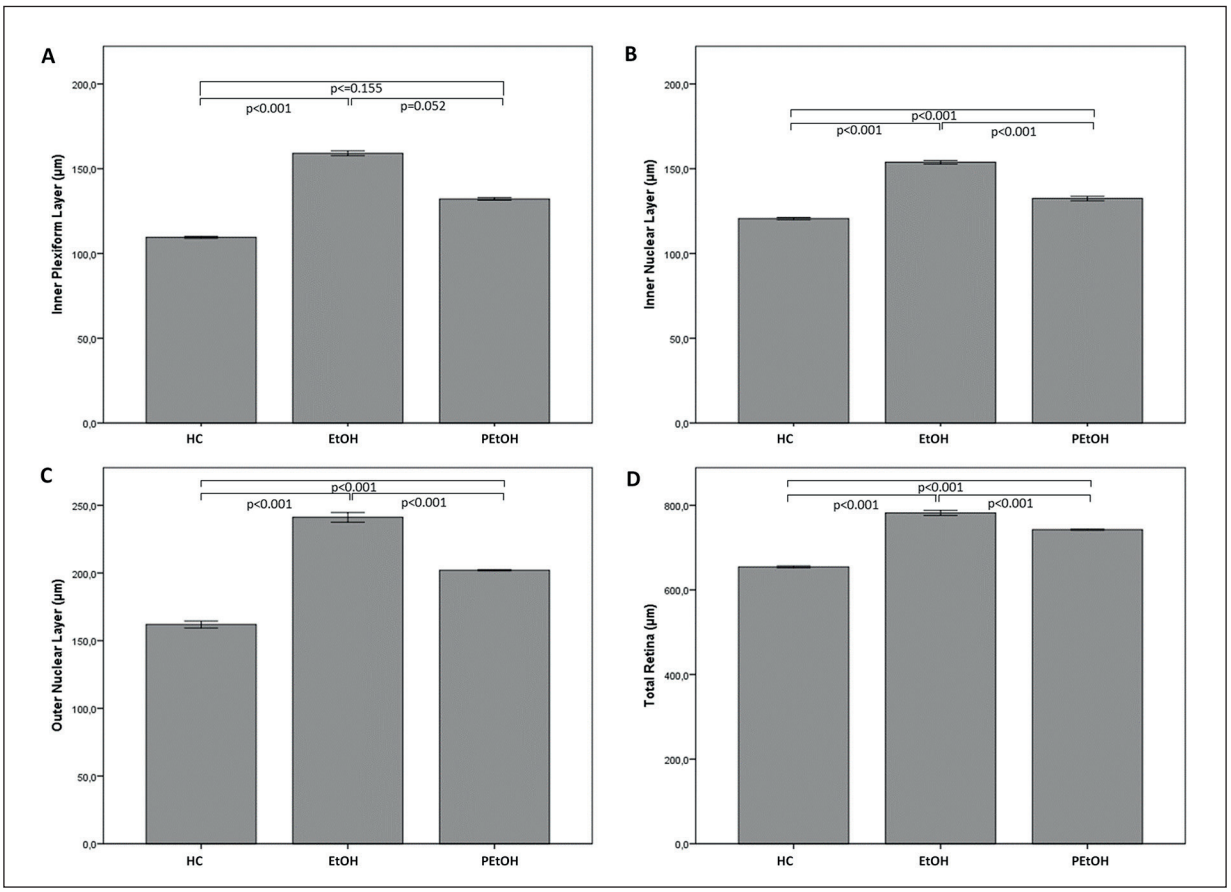

Figure 3. The graphics show the comparison of inner plexiform layer **(A)**, inner nuclear layer **(B)**, outer nuclear layer **(C)** and total retinal thickness **(D)** between groups.

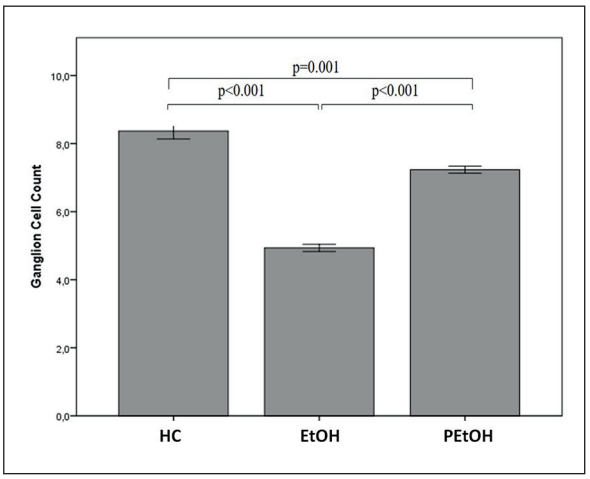

Figure 4. The graphic shows the ganglion cell numbers counting (GCL) differences between groups.

Discussion

In this study, we investigated the effects of Pycnogenol on protection from the destructive effects of chronic ethanol administration on retina tissue in an experimental model. Our biochemical experiment results showed that the MDA value in the retinal tissue of the animals treated with ethanol was higher and the tGSH value was lower than the healthy control and Pycnogenol groups. Johnsen-Soriano et al[3] reported that alcohol administration increases the amount of MDA in retinal tissue and decreases the amount of glutathione. It is known that cell damage begins with ROS peroxidation of cell membrane fatty acids[16]. The best known of the various aldehydes resulting from LPO is MDA. By causing cross-linking and polymerization of membrane components, MDA can also cause serious damage to membrane proteins by inactivating receptors and membrane-bound enzymes in membranes[17]. However, excessively produced reactive oxygen radicals are neutralized by GSH and other enzymatic and non-enzymatic antioxidant defense systems in order to maintain tissue integrity and functions at normal levels[18]. GSH is one of the most widely known antioxidants in living tissues. It is a tripeptide found in many cells, and consists of L-glutamate, L-cysteine and glycine. GSH is catalyzed by GPO, an enzyme that contains selenium in its active site. It reacts with H_2O_2 and organic peroxides, and acts as an antioxidant and removes H_2O_2 from cells. GSH chemically detoxifies hydrogen peroxide or organic oxides and protects cells from SOR damage[19]. In a study by Icel et al[9], it was reported that Pycnogenol protected optic nerve tissue from oxidative damage by cisplatin by stopping the increase in the amount of MDA and decreasing tGSH. Our study results and information gathered from the literature show that Pycnogenol effectively prevents the alteration of the oxidant-antioxidant balance, which is disturbed by ethanol exposure, in favor of oxidants. It is known that the oxidant/antioxidant balance is maintained by the superiority of antioxidants under normal physiological conditions. The disturbance of this balance in favor of oxidants is evaluated as oxidative stress[20].

From our experiment results, it is seen that the biochemical findings coincide with the histopathological findings. In the EtOH group, in which biochemically high amounts of MDA and low amounts of tGSH were found histopathologically in our study, it was determined that ethanol administration caused edema in the retinal ganglion cell layer, dilatation in blood vessels, congestion, infiltration of polymorphonuclear cells, decrease in cell nuclei in the inner cell layer, separation in the outer plexiform layer, and rupture and irregularity in cell connections. However, histopathological findings were lower and milder in the Pycnogenol group, in which the oxidant-an-

Table I. Results of qualitative analysis in histopathological findings in the retina.

	Groups			
	HC	EtOH	PEtOH	*p*-value
Retinal Destruction	0.0 ± 0.0	2.89 ± 0.04[a]	0.47 ± 0.07	< 0.001**
Edema	0.0 ± 0.0	2.92 ± 0.04[a]	0.81 ± 0.11	< 0.001**
Vascular Congestion	0.0 ± 0.0	2.75 ± 0.06[a]	0.92 ± 0.21	< 0.001**
PMN Cell	0.0 ± 0.0	2.22 ± 0.07[a]	0.0 ± 0.0[b]	0.001**

Results are presented as Mean±SEM. *ANOVA or **Kruskal-Wallis' test was performed. aStatistically significant (p<0.01) when compared with HC, bwhen compared with EtOH.

tioxidant balance was changed against oxidants. Ucak et al[21] also stated that the ethanol group, in which the oxidant parameter was high, and the antioxidant parameter was low, showed marked destruction in the optic nerve tissue, edema, hemorrhage, and histopathological findings, such as dilated blood vessels. Han et al[16] reported that exposure to ethanol increased neuroapoptosis in the retinal ganglion cell layer in a dose-dependent manner. In a study by Ferriero et al[22], it was stated that 50% of the patients diagnosed with fetal alcohol syndrome had optic nerve hypoplasia, which was caused by an abnormality in the development of retinal ganglion cells.

Conclusions

In conclusion, the application of ethanol caused the oxidant-antioxidant balance to change in favor of oxidants. It was determined that the disruption of the oxidant-antioxidant balance by ethanol causes histopathological damage in the retinal tissue. Pycnogenol significantly prevented the change of the oxidant-antioxidant balance in favor of oxidants caused by ethanol, according to the parameters evaluated in our study. Pycnogenol administration minimized histopathological changes while reversing oxidative retinal damage. These findings show us that Pycnogenol effectively reduces ethanol-related oxidative retinal damage. Although further studies are needed, we believe that the therapeutic use of Pycnogenol would be beneficial.

Conflict of Interest
The Authors declare that they have no conflict of interests.

Acknowledgements
Not applicable.

Authors' Contribution
SM, YGN and SH designed the study. AYK performed the statistical analysis. YGN, SB and KN interpreted the results and wrote the manuscript. SH and YGN read and approved the manuscript. All authors read and approved the final version of the manuscript.

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Availability of Data and Materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics Approval
This experiment was performed in accordance with the National Guidelines for the Use and Care of Laboratory Animals and the study was approved by the Animal Care and Use Committee of Ataturk University, Erzurum, Turkey (Ethics Committee Number: 77040475-000-E. 1800201965, Dated: 07.04.18).

Consent for Publication
Not applicable.

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