Research Article

Anti-Inflammatory and Antioxidative Stress Effects of Oryzanol in Glaucomatous Rabbits

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Purpose. y-Oryzanol works by anti-inflammatory and radical scavenging activity as a neuroprotective, anticancer, antiulcer, and immunosuppressive agent. The present study was conducted to investigate effect of oryzanol in acute and chronic experimental glaucoma in rabbits. Methods. Effect of oryzanol was evaluated in 5% dextrose induced acute model of ocular hypertension in rabbit eye. Chronic model of glaucoma was induced with subconjunctival injection of 5% of 0.3 ml phenol. Treatment with oryzanol was given for next two weeks after induction of glaucoma. From anterior chamber of rabbit eye aqueous humor was collected to assess various oxidative stress parameters like malondialdehyde, superoxide dismutase, glutathione peroxidase, catalase, nitric oxide, and inflammatory parameters like TNF-α and IL-6. Structural damage in eye was examined by histopathological studies. Results. In acute model of ocular hypertension oryzanol did not alter raised intraocular pressure. In chronic model of glaucoma oryzanol exhibited significant reduction in oxidative stress followed by reduction in intraocular pressure. Oryzanoltreatmentreducedlevel of TNF-α and IL-6. Histopathological studies revealed decreased structural damage of trabecular meshwork, lamina cribrosa, and retina with oryzanol treatment. Conclusions. Oryzanol showed protective effect against glaucoma by its antioxidative stress and anti-inflammatory property. Treatment with oryzanol can reduce optic nerve damage.

1. Introduction

Oxidative stress is the major contributing factor for cellular and metabolic deregulation. It is persuaded by reactive oxidizing agents and free radicals. In recent years, naturally occurring phytochemicals with antioxidant property have been an emerging interest because of their expedient effect on human health as they provide protection against oxidative degradation which is responsible for cell dysfunctions and neurodegeneration. Lessening of oxidative stress due to free radicals, exclusively at mitochondria level, appears to be defensive. Antioxidants, that is, gingko, ubiquinone, and melatonin, polyphenolic flavonoids in green tea, coffee, or red wine, and anthocyanosides existing in bilberries, have been proved to have therapeutically beneficial effect in such context [1].

Glaucoma is stated as extremely elevated intraocular pressure (IOP) and progressive degeneration of the optic nerve, which is characterized by selective apoptosis of retinal ganglion cells (RGCs) and optic nerve head structural modulation [2]. It is the major cause of blindness after cataract worldwide. According to WHO report 2012, around 285 million people are estimated to be visually impaired worldwide. About 90% of visually challenged population reside in developing countries and 82% of population existing with blindness are at and above 50 years [3]. Numerous mechanisms that have been concerned for RGCs death in glaucoma include deprivation of neurotrophic factor, ischemia or hypoperfusion, activation of glial cells, glutamate offered excitotoxicity, and unusual immune reactions [4]. In glaucoma RGC apoptosis follows oxidative stress, ROS generation, ATP deprivation, and cytochrome C release.
No matter how the axon degenerates, RGC’s loss follows apoptotic pathway and neurotrophin deficiency due to suspended axoplasmic transport. Further axonal collapse occurs because of native astroglial cells participation and resides in head of the optic nerve and lamina cribrosa. Hence axons suffer extensive damage and follow Wallerian degeneration with severe microfilament and microtubule breakdown as well as mitochondrial swelling [5]. Existing therapies are aimed at managing the illness but not cur- ing or reducing the advancement of glaucoma. The U.S. Food and Drug Administration has approved five catego ries of drugs that temporarily improve the symptoms, that is, alpha-agonist, beta-blockers, carbonic anhydrase inhibitors, parasympathomimetic agents, and prostaglandin analogs. Oryzanol (OZ) is a physiologically active com pound present in Oryza sativa (Rice) bran oil (RBO) at level of 1-2%. It is a mixture of 4-hydroxy-3-methoxy cinnamic acid (ferulic acid) esters like cycloartenyl fer- ulate, 24-methylene cycloartenol ferulate, β-sitosterol fer- ulate, and campesterol ferulate [6]. OZ has been stated to possess comprehensive assortment of biological actions; in specific it retains antioxidant/free radical scavenging activity [7]. In experimental rats OZ has been reported for anticarcinogenic and antihyperlipidemic activity [6]. Anti-inflammatory activity possessed by OZ is by virtue of its NF-kB inhibitory nature, so it can inhibit activation and nuclear translocation of nuclear factor kappa-B (NF-kB) in macrophages, which predominantly plays an important role in exacerbation of inflammatory reactions in neurons followed by damage of optic nerve and RGCs loss [8, 9]. Recently an investigation conducted on SH-SY5Y cells by oryzanol-rich fraction against H2O2 induced neurotoxicity has revealed its neuroprotective activity [10]. Ferulic acid downregulates ICAM-1 mRNA expression, level of microglia, and macrophages and simultaneously reduces the inflammation induced oxidative stress and consequential apoptosis due to cerebral ischemia and reperfusion injury in rats [11]. Ulcerative colitis murine model investigation has stated nitric oxide (NO), TNF-α, and IL-1β attenuation aspects of OZ [12]. Keeping this in mind the study has been targeted towards implementation of OZ treatment in glaucoma.

2. Methods

2.1. Chemicals and Reagents. All bioanalytical assay kits were acquired from Lab care diagnostics Pvt. Ltd. India. The oryzanol was purchased from Tokyo chemical industry Co. Ltd., Tokyo, Japan. Pilocarpine was procured from FDC limited, India. Xylocaine was obtained from Astra Zeneca Pharma India limited. Phenol and almond oil were procured from central drug house, India. TNF-α and IL-6 assay kits were purchased from B D Biosciences, USA.

2.2. Animals. The experimental protocol involving animals were approved by the Institutional Animal Ethics Com mittee (IAEC) of Institute of Pharmacy, Nirma University. (IP/PCOL/MPH13-1/006). Adult New Zealand White Rabbits of either sex weighing 2-3 kg were selected for the study. The animals were familiarized to the laboratory sur- roundings for 2-week period. They were sustained at 25 ± 4°C and 12/12 h of light-dark cycle and were given standard rabbit feed (Pavan Agro Industries Ltd., Pune, India) and water ad libitum.

2.3. Acute Glaucoma Model. Animals were randomly divided into normal control (n = 6), acute control (n = 6), OZ treated (n = 6), and pilocarpine treated (n = 6) groups. Acute model of glaucoma was developed by earlier described procedure [13]. Before induction of acute glaucoma, the basal IOP was measured by Schiotz Indentation tonometer in all animals after instillation of the local anesthetic Xylocaine 2% eye drops. This process was repeated each time before the measurement of IOP. After IOP recording, 1% CMC was administered in normal control and acute control animals. Two to three drops 1% OZ solution prepared in 1% CMC was instilled topically into the eye for treatment as a single dose. 1% pilocarpine was used as standard treatment in animals. It was applied topically as a single dose of two to three drops. After 15 minutes of vehicle and drug administration, intravenous 5% dextrose solution (15 ml/kg) was infused through marginal ear vein to induce ocular hypertension. The intraocular pressure (IOP) changes were noted by tonometer at 15, 30, 45, 60, 90, 120, 180, 240, 300, and 360 minutes (till normal pressure is achieved). The tonometer used for IOP measurement was calibrated by open manometric calibra- tion.

2.4. Chronic Glaucoma Model. Animals were randomly di- vided in to normal control (n = 6), chronic control (n = 6), OZ treated (n = 6), and pilocarpine treated (n = 6) groups. Chronic model of glaucoma was developed by formally described procedure [14]. 150 μL phenol, prepared in almond oil (5%), was injected in subconjunctival region in the four quadrants of rabbit eye to produce scarring in the aqueous humor (AH) pathway-causing increase in IOP without apparent macroscopic or microscopic damage to the eye. The injections were repeated (once a week) for next two weeks till IOP was elevated completely. IOP was recorded every day by Schiotz Indentation tonometer during induction period. After 3 weeks of induction period 1% CMC was administered in normal control and disease control group while 1% OZ solution (prepared in 1% CMC) and 1% pilocarpine were instilled topically as test and standard in their respective groups for 2 weeks once a day, two to three drops.

2.4.1. Aqueous Humor Collection. Aqueous humor (AH) samples were collected by method described by Gupta et al. [15]. In brief, AH was collected carefully from the anterior chamber of rabbit’s eye using 27-gauge needle after adminis- tration of diazepam 1 mg/kg i.v. and ketamine 25 mg/kg i.v. without causing any injury to the iris or lens throughout the procedure. After collection, samples were immediately stored at temperature (−20 °C) till the performance of various biochemical analysis from AH.
2.4.2. Estimation of Total Protein. Total protein was estimated in AH as per Lowry method [16]. Briefly, AH (10 μL) was allowed to be diluted using 1 N NaOH (990 μL) and kept for reaction with copper reagent (4 mL). 10 min later, Folin’s reagent (0.5 mL) was added and mixed using vortex mixture. Next, the samples were placed in dark at room temperature for 30 min. At 620 nm absorbance was estimated using spectrophotometer. Bovine serum albumin (BSA, sigma chemicals, USA) was used as a standard.

2.4.3. Estimation of Antioxidant Parameters. Malondialdehyde (MDA) levels were measured as per the method described by Ohkawa et al. In brief, 0.1 ml tissue homogenate was mixed with 0.2 ml 8% sodium dodecyl sulphate. Resultant solution was mixed with 1.5 ml 20% glacial acetic acid and 1.5 ml 0.8% thiobarbituric acid; thereafter pH 3.5 was adjusted with NaOH. Mixture was diluted up to 4 ml with distilled water. Solution was heated at 95°C for 60 min and then cooled by running tap water. Next to this 5 ml of 15:1 v/v n-butanol and pyridine was added. The mixture was mixed thoroughly and shaken rapidly. Mixture was centrifuged for 10 min at 4000 RPM and the supernatant was collected. Its absorbance was measured at 532 nm [17].

Nitrite (NO−2) levels were measured by method reported by Tracey et al. The method utilizes Griess reagent. In brief, 6 μL of plasma sample, 44 μL dH2O, 20 μL 0.31 M pH 7.5 phosphate buffer, 10 μL 0.86 mM NADPH, 10 μL 0.11 mM FAD, and 1.0 U/ml nitrate reductase were mixed together in well plates. Resultant mixture was incubated for 1 hr at room temperature. Next, 200 μL Griess reagent was added to each well and allowed to incubate at room temperature for 10 min. Then absorbance was measured at 549 nm [18].

Glutathione peroxidase (GPx) level was measured by method described by Mohandas et al. In brief to the reaction mixture composed of 0.05 M pH 7.0 phosphate buffer, 1 mM EDTA, 1 mM sodium azide, 1 U glutathione reductase, and 1 mM GSH, 0.2 mM NADPH 100 μL of the tissue homogenate equivalent to 6 mg protein/ml was added. The final mixture was treated with 0.25 mM hydrogen peroxide and the enzyme activity was initiated at 37°C. The absorbance was measured at 340 nm [19].

Superoxide dismutase (SOD) was estimated by the method suggested by Kovačeva et al. In brief, 750 μL tissue homogenate was assayed with 600 μL ice-cold ethanol-chloroform mixture mixed at 5:3 v/v. Resultant solution was thoroughly mixed and then centrifuged for 45 min at 4°C. Supernatant was diluted with 50 mM pH 7.8 phosphate buffer, 5 mM EDTA, 40 mg/L bovine serum albumin, 0.2 mg/ml nitroblue tetrazolium, and 0.1 mM xanthine oxidase and xanthine. The absorbance was taken at 546 nm for 10 min [20].

Catalase (CAT) levels were measured using method described by Hugo. To 2 ml tissue homogenate 1 ml 30 mM H2O2, prepared in 50 mM pH 7 phosphate buffer, was added. Reduction in absorbance was observed at 240 nM [21].

2.4.4. Estimation of Inflammatory Mediators. TNF-α and IL-6 were estimated by commercially available kits (BD Biosciences, USA).

2.4.5. Histopathological Examination. The animals were sacrificed by overdose of xylazine 4.3 mg/kg (Indian immunological Ltd., India) and ketamine 25 mg/kg i.v. (Troikaa Pharmaceuticals Ltd., India). Eye ball was excised and fixed in 10% formalin solution. After 24 h, the tissues were washed thoroughly with repeated use of absolute alcohol. These cycles were repeated till the final sections cut with tissue. Later the eye ball was cut down in middle of optic nerve to the cornea. Then the sections of eye were kept in melted paraffin twice before drying. After they were dried, the block of paraffinized tissues was cut according to the size of tissue. Sections were deparaffinized in xylene and rehydrated through descending grades of alcohol. The sections were then stained with 10% hematoxylin for 3–5 minute and staining was intensified by placing in running water. Again sections were stained with 10% eosin for 2 minutes and quickly passed through ascending grade of alcohol. Finally they were treated with xylene followed by mounting and analyzing under OLYMPUS (trinocular-CX21FSI) microscope with 100X magnification.

2.4.6. Analysis of H&E Stained Slide. The hematoxylin and eosin (H&E) stained slides were analyzed under photo microscope with 100x magnification setting. Eye ball section was observed for presence of retinal cell, blocking of trabecular meshwork (TM), epithelial cell matrix in mesh of TM, optic nerve head, and apoptosis.

2.5. Statistical Analysis. All the values were expressed as mean ± SEM. Statistics was applied using graph pad prism 6.0 (San Diego, CA, USA) for windows and SPSS statistical software (version 22). ANOVA was used to determine statistical significance between various groups. Difference was considered statistically significant when p < 0.05.

3. Results

3.1. Effects on IOP. Rise in IOP is the characteristic feature of glaucomatous state. Following changes were observed in IOP of acute and chronic model animals.

3.1.1. Effect on IOP in Acute Model. After treatment with 5% dextrose through marginal ear vein, in acute control group, significant (p < 0.05) elevation in IOP was observed compared to normal control group. IOP level was at peak at 45 minute and normalized by 4–6 hrs. In this model treatment with OZ did not reduce IOP in its respective treatment group, while pilocarpine treatment led to reducing the IOP significantly (p < 0.05) as compared to acute control group (Figure 1(a)).

3.1.2. Effect on IOP in Chronic Model. After topical administration of 5% phenol for 3 weeks of induction period, IOP was found to be significantly (p < 0.05) increased to 38–42 mmHg as compared to normal control group (Figure 1(b)). In this model both pilocarpine and OZ treatment resulted in significant (p < 0.05) fall in IOP as compared to chronic control group.
3.2. Effects on Total Protein Level. Protein present in the AH has diagnostic significance as it is associated with pathogenesis of several ocular abnormalities. In current study significant \((p < 0.05)\) elevation in total protein level was observed in chronic control group as compared to normal control. Significant \((p < 0.05)\) reduction in total protein was observed in OZ treated group as compared to chronic control group (Figure 2).

3.3. Effect on Oxidative Stress Markers. Significant \((p < 0.05)\) elevation of MDA and NO levels as well as significant \((p < 0.05)\) reduction in SOD, CAT, and GPx levels was observed in chronic control group as compared to normal control group. Significant \((p < 0.05)\) reduction in MDA and NO levels as well as significant \((p < 0.05)\) elevation in SOD, CAT, and GPx levels was observed with OZ treatment when compared to chronic control animals (Figure 3).

3.4. Effects on Inflammatory Mediators. TNF-\(\alpha\) and IL-6 are inflammatory markers. In our findings we observed significant \((p < 0.05)\) elevation in TNF-\(\alpha\) as well as IL-6 in chronic control animals as compared to normal control animals. OZ and pilocarpine treated groups showed significant \((p < 0.05)\) reduction in inflammatory markers in their respective treatment groups as compared to chronic control group (Figure 4).

3.5. Histopathology. Histopathological examinations of eyes were performed to study trabecular meshwork, optic nerve head, and retinal cells by 100x power lens under inverted microscope using eosin and hematoxylin stain. Under histopathology examination, TM section of normal control animals revealed open passage for AH drainage. In contrast, eye sections of chronic control and OZ treated groups revealed irregular TM meshwork cells and aggregation of extracellular tissue fibers in pore of TM and canal of Schlemm (Figures 5(a), 5(b), and 5(c)). Extracellular matrix aggregation was seen more on surface of TM in chronic control group as compared to normal control and OZ treated eyes. Canal of Schlemm and TM cellularity were found to be completely disturbed in both chronic control and OZ treated animals (Figures 5(b) and 5(c)). Further, the histopathological analysis of optic nerve head in normal control group showed no sign of cupping in lamina cribrosa (Figure 5(d)). Eye of chronic control animals exhibited complete change in the normal architecture of optic nerve head along with cupping and degradation of optic nerve head (Figure 5(e)). However, treatment with OZ exhibits less tissue degeneration and cupping on lamina cribrosa as compared to chronic control animal’s eye (Figure 5(f)). H&E staining of the normal control animal’s retina exhibited normal architecture (Figure 5(g)). Retina of both chronic control and OZ treated eyes revealed severe loss of RGCs in the temporal region, especially in mecula. The remaining structure and cellular components of the chronic control and OZ treated eyes appeared to stain within normal limits compared with staining pattern of normal control’s retina. Chronic control group showed clear decrease in cellularity of the retina, mainly rod, cones, and polar cell degeneration; moreover outer layer of the retina...
Figure 3: Effect of OZ on various oxidative stress markers. (a) Malondialdehyde (MDA) level, (b) nitrite (NO) level, (c) superoxide dismutase (SOD) level, (d) catalase (CAT) level, and (e) glutathione peroxidase GPx level. Data are expressed as mean ± SEM. (n = 6). Values are statistically significant at *P < 0.05 versus normal control; #P < 0.05 versus chronic control.

Figure 4: Effect of OZ on inflammatory mediators. (a) TNF-α level and (b) IL-6 level. Data are expressed as mean ± SEM. (n = 6). Values are statistically significant at *P < 0.05 versus normal control; #P < 0.05 versus chronic control.
was also disturbed completely as compared to normal control animal’s eye (Figures 5(h) and 5(i)).

4. Discussion

In acute model of glaucoma transient elevation in IOP (38–42 mmHg) was observed, when 5% dextrose (15 ml/kg) was administered through marginal ear vein. Pretreatment with 1% OZ in acute model (OA) failed to produce any alteration in the raised IOP. In contrast 1% pilocarpine significantly ($p < 0.05$) prevented the acute rise in IOP as compared to control. The ineffectiveness of OZ on acute elevation of IOP indicates that OZ may not act through direct action on AH outflow pathways.

Experimentally induced chronic glaucoma mimics the moderate IOP elevation, a characteristic of human open angle glaucoma. It had already been stated that exposure of phenol to the murine skin can stimulate glutathione oxidation, vitamin- E level, and total antioxidant assets in skin epidermis. Phenol had also been stated to elevate the free radical formation in RBCs in vitro [22]. After three weeks of induction period, significant elevation in the IOP was observed in all animals injected with 0.3 ml of 5% phenol in almond oil ($p < 0.05$) as compared to normal control animals. In our study after 2 weeks of 1% OZ treatment, significantly ($p < 0.05$) reduced IOP level was observed in chronic model of glaucoma. Pilocarpine treatment significantly ($p < 0.05$) reduced IOP as compared to disease control group. IOP, after treatment with pilocarpine, was comparable to that of normal control group. This activity is attributed to its cholinergic receptor agonistic activity. Pilocarpine decreases IOP by activating muscarinic receptors present on postsynaptic ciliary muscle and facilitating contraction of it and improves patency of fluid channels in the TM, therefore promoting trabecular outflow facility [23].

Normally anterior portion of eye and blood stream both remain separated by anatomic barrier formed by endothelial layer of blood vessels in the iris and nonpigmented cellular layer of ciliary epithelium so-called blood aqueous barrier (BAB). It regulates transference of protein by active transport. Glaucomatous condition leads to collapse of the barrier and promotes protein influx from serum into AH [14]. These elevated protein aggregates can block the regular AH drainage and elevate IOP. Subconjunctival injection of 5% phenol produces irritation and inflammatory reaction in eye, which damages the anterior epithelial and blood vessels and subsequently increases the protein concentration in anterior chamber. In current findings total protein in AH
of glaucomatous animals was found to be approximately 18% higher than that in NC. 1% OZ treated animals showed significant \( p < 0.05 \) reduction in total protein level in AH as compared to chronic control animals. Moreover, the change in the protein concentration matches the change in IOP for all studied groups. 1% pilocarpine treated group exhibited only mild reduction in protein level, which did not significantly differ from eyes of chronic control group.

Previous findings have reported role of oxidative stress markers in open angle glaucoma pathogenesis. ROS are substantially associated with oxidative damage of DNA in TM of glaucomatous victims. This uneven oxidant and antioxidant divergence promotes damage of TM, RGCs, and neuronal death. These changes follow chronic alterations in AH as well as vitreous humor (VH) [24]. In previous studies there was significantly high MDA and reduced SOD, CAT, and GPx levels were reported in rats with elevated IOP having ischemic retinal tissues [25]. In our findings chronic control group exhibited significant \( p < 0.05 \) increase in the MDA level and relatively decreased SOD, CAT, and GSH as compared to normal control group. 1% OZ treated group exhibited significant \( p < 0.05 \) reduction in MDA level as well as significantly \( p < 0.05 \) improved SOD, CAT, and GPx levels. However 1% pilocarpine treatment did not show significant reduction in MDA level and increment in SOD, CAT, and GPx levels when compared to chronic control animal.

Former studies anticipated manifestation of inducible nitric oxide synthase (iNOS) in optic nerve of human as well as experimental glaucomatous rat eyes. Reactive astrocytes produce excessive NO which is followed by degeneration of RGCs. The fetal role of NOS-2 and NO was recognized by RGCs. The fetal role of NOS-2 and NO was recognized by produce excessive NO which is followed by degeneration of retina [26]. In our findings chronic control group exhibited significant \( p < 0.05 \) increase in the MDA level and relatively decreased SOD, CAT, and GSH as compared to normal control group. 1% OZ treated group exhibited significant \( p < 0.05 \) reduction in MDA level as well as significantly \( p < 0.05 \) improved SOD, CAT, and GPx levels. However 1% pilocarpine treatment did not show significant reduction in MDA level and increment in SOD, CAT, and GPx levels when compared to chronic control animal.

In support of given evidences it is clear that OZ treatment fail to provide structural support to optic nerve cells and produce matrix metalloproteinase that influences the matrix remodeling pattern [35, 36]. It is further strongly reported that interruption in ECM remodeling leads to augmented ECM material buildup in TM which resists the normal outflow of AH and raises IOP [37]. Our findings follow the reported cellular and structural modulation in glaucomatous state (Figure 5).

5. Conclusion

In support of given evidences it is clear that OZ treatment can prevent glaucoma by reducing elevated IOP and decrease optic nerve and retinal damage by controlling inflammatory mediators and scavenging free radicle.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.
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