

IMPACT OF ELEVATED INTRAOCULAR PRESSURE AND 0.15% BRIMONIDINE TARTRATE ON AQUEOUS HUMOR AND RETINA OF EXPERIMENTAL ANIMAL

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ABSTRACT

The effect of elevated intraocular pressure (IOP) and topical antiglaucoma medication Brimonidine tartrate "0.15%" on aqueous humor and retina of rabbit's eyes was investigated. Male New Zealand rabbits were subjected to subconjunctival injections of phenol in almond oil (5%), causing elevation in the IOP. One group kept without receiving any treatment, and the other group was subjected to topical drug treatment with Brimonidine tartrate "0.15%." Electroretinogram (ERG) and Fourier transform infrared spectroscopy (FTIR) were performed for the retina. Protein concentration, refractive indices (RI) and protein structure by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were performed for aqueous humor. In glaucomatous eyes group, there is an increase in protein concentration of aqueous humor and change in its molecular weight. The protein concentration matches the control after treatment with Brimonidine tartrate. The refractive index of aqueous humor also shows the same behavior. The FTIR measurements show distinct change in NH-OH bands of glaucomatous retina. The ERG record show no significant change of amplitude of b-wave, while there was obvious reduction of the photopic negative response (PhNR) amplitude of glaucomatous group. Glaucoma may lead to changes in membrane structure of retinal cells attributed to oxidative stress. Brimonidine tartrate induces protective effect resulted in improvement in protein concentration, RI and aggregation of high molecular weight proteins. The improvement in PhNR may be attributed to the ability of Brimonidine tartrate to attenuate retinal ganglion cell death.

Keywords: Intraocular pressure, Brimonidine tartrate, SDS-polyacrylamide gel electrophoresis, Electroretinogram, Fourier transform infrared spectroscopy.

INTRODUCTION

Glaucoma represents a group of neurodegenerative diseases characterized by structural damage to the optic nerve and slow progressive death of retinal ganglion cell (RGCs). It is the second most common cause of blindness all over the world¹. Elevated intraocular pressure (IOP) is traditionally considered the most important risk factor for glaucoma that might progressively hurt visibility²⁻³. Pharmacological neuroprotection refers to the situation in which drug is deployed to interact with neuronal or glial elements within the retina and optic nerve head, and thereby facilitate the survival of RGCs.

Brimonidine tartrate "0.15%" (Alphagan®P) is a highly selective α_2 -adrenoceptor agonist, which reduce the IOP primarily by decreasing aqueous humor production and uveoscleral outflow³. α_2 -adrenoceptors have been identified in the ganglion cell layer of the retina. The protective effects of Brimonidine tartrate against injury are likely to be mediated via activation of these receptors⁴. Brimonidine might protect the RGCs, and was associated with a significant reduction in RGCs loss in case of ocular hypertension and decrease apoptosis loss⁵⁻⁷.

Electroretinography is an objective technique used to measure retinal electrical responses, which directly reflects retinal function⁸. The photopic negative response (PhNR) of the photopic flash electroretinogram (ERG) appears as a negative-going wave after the b-wave. The PhNR was first identified in monkeys with the use of red flashes on a rod-saturating blue background⁹. These late negative waves were absent in animals with experimental glaucoma that had lost their retinal ganglion cells¹⁰⁻¹¹. Others have used white flashes on a white background. This observation suggested that, the PhNR was probably a reflection of the spiking activity of ganglion cells though; it might also include contributions from other spiking cells (amacrine cells) in the inner retina¹².

The aim of this prospective work is to use an experimental model of glaucoma to study the effects of elevated intraocular pressure (IOP) and topical antiglaucoma medications on aqueous humor protein. In addition, to evaluate the sensitivity of flash ERG and the retinal tissue characteristics studied by infrared spectroscopy (FTIR).

MATERIALS AND METHODS

All the experiments were done in compliance with the Public Health Guide for the Care and Use of Laboratory Animals and accordance with the protocols approved by the local experimental ethics committee in ophthalmic and vision research. Thirteen adults New Zealand rabbits weighted 2-2.5 Kg were used in this study. The animals were selected from the animal house and fed on the laboratory balanced diet and in a central temperature of 20-25°C.

Induction of glaucoma

Both eyes of the animals were anesthetized using 0.4% Benoxinate eye drops (Bausch & Lomb, Australia, Pty Limited) for local anesthesia to prevent eye movement during measurements of intraocular pressure. Using a standardized Schiötz tonometer, the intraocular pressure of rabbits was recorded for 3 days before induction of glaucoma to determine the base line. Three rabbits were used as a control group, and the other ten rabbits were subjected to subconjunctival injections of 150µl phenol in almond oil (5%) in the four quadrants of the eye to produce scarring in the aqueous humor pathway-causing increase in IOP with no apparent macroscopic or microscopic damage to the eye¹³. The intraocular pressure was monitored daily and recorded. If the IOP did not elevate through one week, the injections were repeated at equal intervals (one week each).

When the IOP was raised, the rabbits were classified into two glaucomatous groups. One group kept without receiving any treatment, and the other group was installed with Brimonidine tartrate "0.15%" (Allergan, Waco, Texas, USA) three times daily for two weeks.

Electroretinogram (ERG) measurements

After two weeks of topical drug treatment with Brimonidine tartrate, ERG was performed for the control, glaucomatous and treated glaucomatous groups in both eyes. Pupil dilatation was performed by instillation of Mydracyl eye drop "0.5%." After the dilation, light adaptation was achieved using background illumination for ten minutes to measure the cone response. Extracellular ERG was recorded by using wick electrode as an active electrode placed on the cornea; Ag-AgCl electrode was placed on the

lid as reference electrode and Ag-AgCl electrode on the ear of the animal as an earthed electrode. The obtained ERG signals were then amplified and delivered to a computer system. A white flash of light with fixed intensity (4 Lux) and duration (0.2 sec) was used. The PhNR amplitude was defined as the difference between the baseline and the peak of the negative wave following the b-wave. The b-wave time to the peak (implicit time) was measured from the time of the flash to the peak of the wave. The b-wave, implicit time and the PhNR parameters in the three groups were measured and compared.

Protein analysis

The rabbits were sacrificed; the eyes were enucleated and aqueous humor samples were aspirated by inserting insulin syringe into the anterior chamber of the eye. Aqueous humor total protein concentration was determined using a colorimetric assay¹⁴. The developing color was measured with a spectrophotometer at 750 nm. The protein composition of the aqueous humor samples was analyzed according to its molecular weight by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 3% stacking gel and 10% separating gel¹⁵. The gel was scanned using scanner model SG-700 Imaging Densitometer (Bio Rad). The refractive indices of aqueous humor were measured using Abbe refractometer with its attached temperature control at $37 \pm 0.02^\circ\text{C}$.

Fourier transformation infrared spectroscopy (FTIR)

Retinas from each group were separated, weighed, lyophilized and mixed with KBr powder (98mg KBr: 2mg retina) to prepare the KBr disks for FTIR analysis. FTIR spectra were measured using Shimadzu

infrared spectrometer with an effective resolution of 2cm^{-1} . Hundred sample interferograms were recorded for each spectrum. The spectrometer is operated under a continuous dry nitrogen gas purge to eliminate interference from atmospheric carbon dioxide and water. The data was baseline corrected, and smoothed by Savitzky-Golay to remove the noise before Fourier transformation. The average of three spectra for each group was obtained using Origin Pro 7.5 software.

Statistical evaluation

Protein levels and refractive indices were compared between treated and untreated eyes by student t-test¹⁶. Where "t" is the measure of significance, differences were considered significant at $P < 0.05$.

RESULTS

IOP measurements

The mean intraocular pressure for the studied groups was shown in Fig.1. The obtained data revealed that, the IOP mean of normal rabbit eyes was 20.1 ± 0.49 mm Hg which was in the normal pressure range (11.1 - 20.9 mm Hg) confirmed by the previous study¹⁷. After one week of injection, the data indicated a high significant increase in the IOP (32.3 ± 1.35 mmHg) for all groups injected with phenol in almond oil ($P < 0.01$). After treatment with Brimonidine tartrate "0.15%" for two weeks, the reduction in the IOP was approximately equal to the baseline levels ($18.5.2 \pm 0.9$ mmHg).

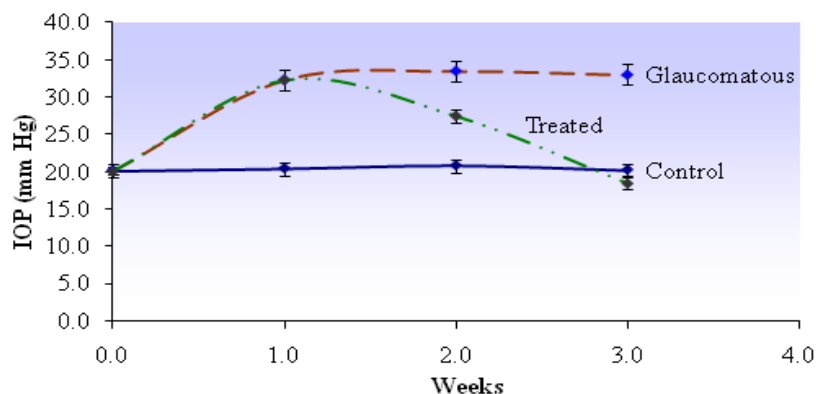


Fig. 1: Intraocular pressure (IOP) of the control, glaucomatous and treated rabbit's eyes with Brimonidine tartrate "0.15%".

Protein content and refractive index of aqueous humor

In Fig.2, the total protein concentration of the control sample of aqueous humor was 8.65 ± 0.08 mg/ml. This value was significantly increased to 11.67 ± 0.26 mg/ml in the glaucomatous group ($P < 0.001$). After treatment with Brimonidine tartrate for two weeks,

the protein content reduced nearly to the control value (8.8 ± 0.03 mg/ml).

The refractive index of the studied groups showed the same behavior (Fig.3). The values for the control, glaucomatous and Brimonidine treated groups were 1.32363, 1.32417 and 1.32367 respectively.

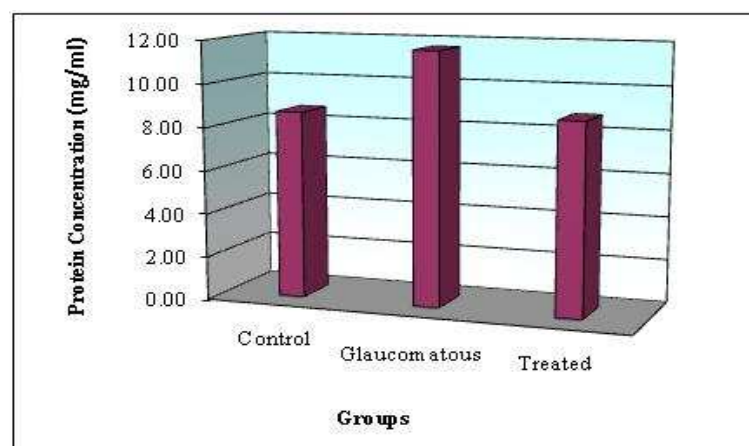


Fig. 2: Protein concentration of rabbit's aqueous humor for control, glaucomatous and treated group with Brimonidine tartrate

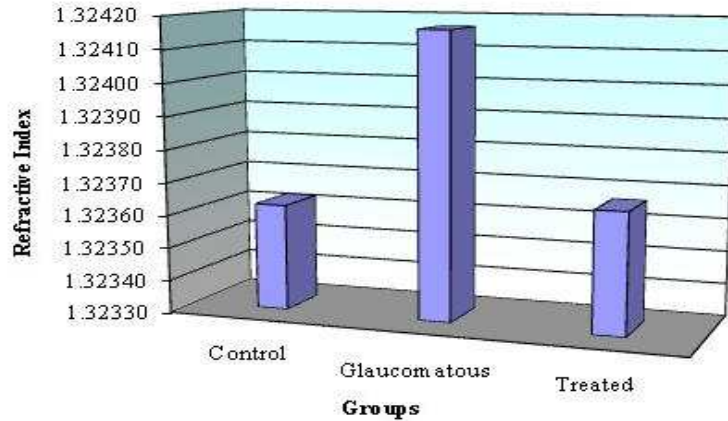


Fig. 3: Refractive index of rabbit's aqueous humor for control, glaucomatous and treated group with Brimonidine tartrate

SDS- PAGE of aqueous humor

Fig.4 showed the scanning pattern of SDS- PAGE for the rabbit's aqueous humor for control and glaucomatous samples. Significant differences in aqueous humor samples were found between the glaucomatous and the control groups. These differences characterized by decreases in the molecular weights of the low mobile group (high molecular weight region). Moreover, the high mobile group (low molecular weight region) indicated changes in peaks intensities especially for the molecular weight ranged from 16-77 KD.

Fig. 5 showed the scanning pattern of SDS- PAGE for the rabbit's aqueous humor for control and glaucomatous eye treated with Brimonidine tartrate. The treated sample showed significant protein changes compared to the control group. There was an increase in the molecular weight of the low mobile group gathering at 312 KD (high molecular weight region).

Moreover, the high mobile group (low molecular weight region) showed some sorts of recovery indicated by a reduction in peaks intensities especially for the molecular weight ranged from 62-31 KD.

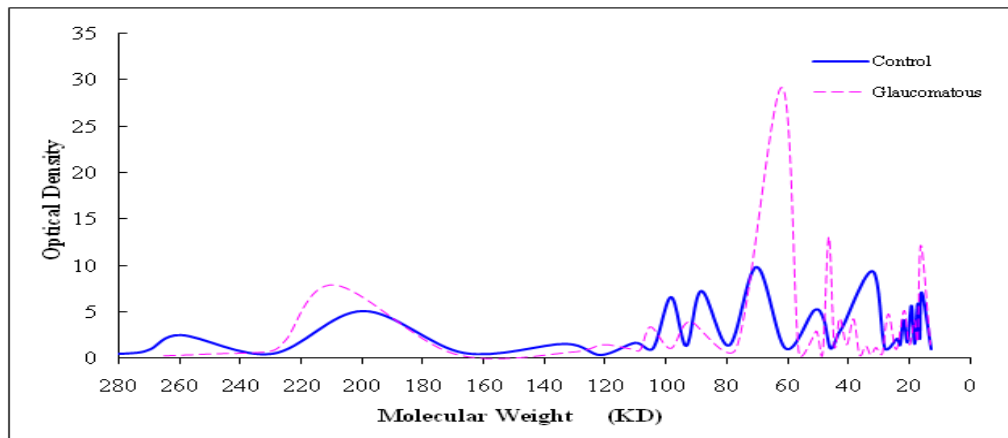


Fig. 4: Scanning pattern of SDS- PAGE for the aqueous humor of control and glaucomatous rabbits

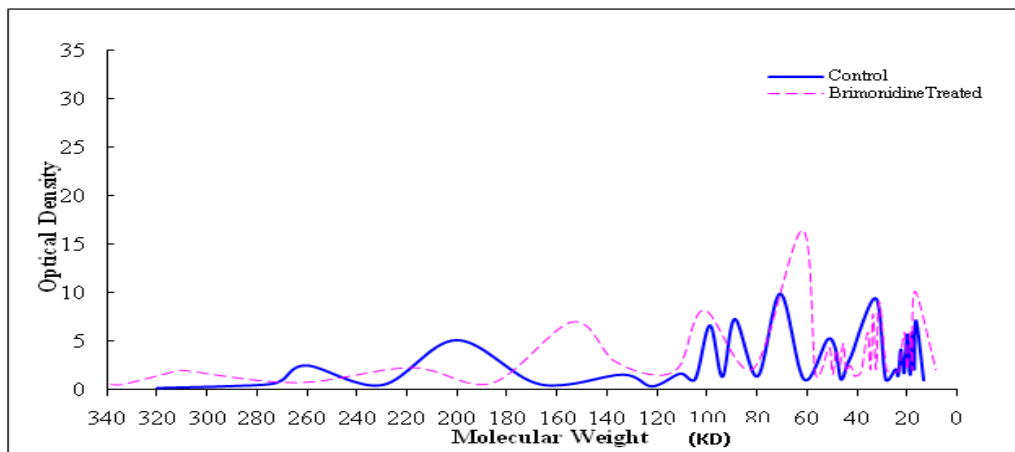


Fig. 5: Scanning pattern of SDS- PAGE for the aqueous humor of control and glaucomatous rabbits treated with Brimonidine tartrate

FTIR for the retina

The FTIR results were analyzed for the following spectral regions: 3700 – 3000 cm^{-1} (NH-OH stretching region), 3000 – 2800 cm^{-1} (CH stretching region) and 1800 – 900 cm^{-1} (fingerprint region). Fig.6 illustrated the spectra of the NH-OH region of control, glaucomatous and treated rabbit's retina. The main band of the normal pattern was found at 3323 \pm 3 cm^{-1} . The curve enhancement procedure resolved this band into four components centered at 3544 \pm 3 cm^{-1} , 3441 \pm 2 cm^{-1} , 3312 \pm 3 cm^{-1} and 3185 \pm 3 cm^{-1} .

These bands corresponded to stretching OH (labeled as 1), stretching OH symmetric (labeled as 2), stretching NH asymmetric (labeled as 3) and stretching NH symmetric (labeled as 5) respectively as described previously¹⁹. The NH-OH region of glaucomatous retinas resolved into three structural bands centered at 3505 \pm 3 cm^{-1} (stretching OH), 3337 \pm 2 cm^{-1} (stretching NH asymmetric) and 3277 \pm 3 cm^{-1} (stretching OH symmetric, labeled as 4). The treated retina was resolved into three structural bands centered at 3465 \pm 2 cm^{-1} , 3313 \pm 2 cm^{-1} and 3185 \pm 3 cm^{-1} which

corresponded to stretching OH, stretching NH asymmetric and stretching NH symmetric respectively (Table 1).

In Fig. 7 the CH stretching region (3000 – 2800) cm^{-1} in control retina indicated the presence of three bands centered at 2961 \pm cm^{-1} with band width of 16 \pm 1, 2924 \pm 3 cm^{-1} with band width of 30 \pm 2 and 2859 \pm 3 cm^{-1} with band width of 32 \pm 2. As mentioned by previous work, these bands were corresponded to CH₃ asymmetric, CH₂ asymmetric and CH symmetric respectively¹⁹. Induction of glaucoma and treatment with Brimonidine tartrate "0.15%" has no significant effect on either band position or bandwidth.

The bands in the fingerprint region (1800 – 1000 cm^{-1}) for all the studied groups were illustrated in Fig.8. The normal pattern was characterized by eight bands as shown in table 2. No change in the position of the amide I, amide II and $\nu_{\text{sym}}\text{PO}_2$ was found in all the studied groups. CH₂ bending has the same frequency for control and glaucomatous retina while disappeared in retina treated with Brimonidine tartrate "0.15%." The absorption band that centered on 1305 cm^{-1} and corresponded to CH₃ deform was disappeared in glaucomatous retina.

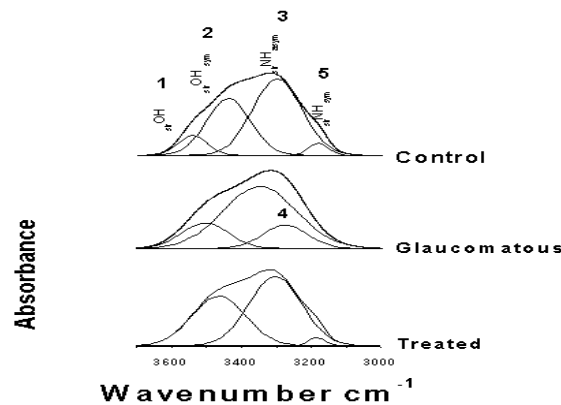


Fig. 6: FTIR of control, glaucomatous and treated rabbit's retina in the NH-OH region (3700- 3000 cm^{-1})

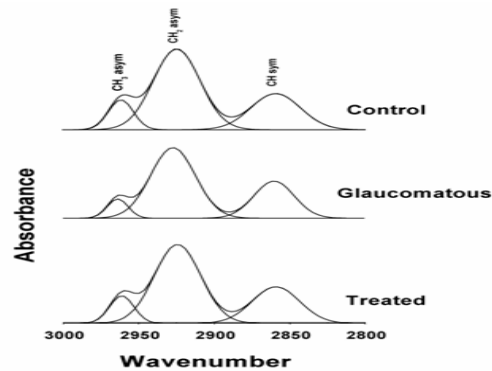


Fig. 7: FTIR of control, glaucomatous and treated rabbit's retina in the CH stretching region (3000-2800 cm^{-1})

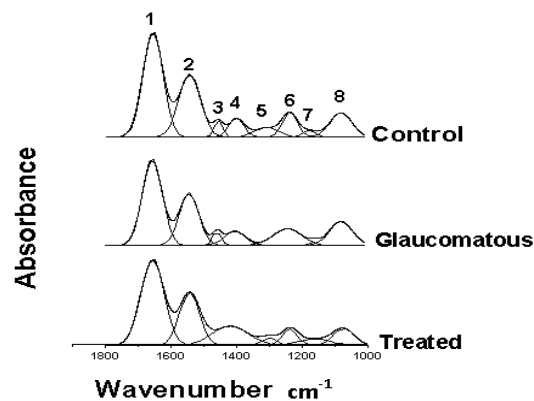


Fig. 8: Fingerprint region (1800-1000 cm^{-1}) of control, glaucomatous and treated rabbits' retina.

Table 1: Wavenumber and bandwidth of NH-OH region for all the studied groups

	str-O-H	str-O-H _{sym}	str-N-H _{asym}	str-O-H _{sym}	str-O-H _{sym}
Control	3544 ± 3	3441 ± 2	3312 ± 3	-----	3185 ± 3
	82 ± 4	125 ± 6	141 ± 4		55 ± 3
Glaucomatous	3505 ± 3*	-----	3337 ± 2*	3277 ± 3	
	92 ± 7		128 ± 6	125 ± 5	
Treated	3465 ± 2*	-----	3313 ± 3	-----	3185 ± 3
	154 ± 5		151 ± 6		46 ± 4

First line indicates the frequency of the band in cm⁻¹, while the second line indicates the bandwidth in cm⁻¹. * Statistically significant.

Table 2: Wavenumber and bandwidth of fingerprint region for all the studied groups

	Amide I	Amide II	CH ₂ Bending	COO _{sym}	CH ₃ Deform	asym PO ₂	COOC _{asym}	symPO ₂
Control	1665 ± 2	1541 ± 3	1453 ± 2	1397 ± 4	1305 ± 2	1233 ± 3	1171 ± 3	1076 ± 1
	61 ± 2	65 ± 5	26 ± 1	47 ± 3	23 ± 4	48 ± 4	42 ± 4	56 ± 5
Glaucomatous	1665 ± 3	1542 ± 2	1457 ± 3	1401 ± 1	-----	1240 ± 2	1159 ± 2	1076 ± 2
	59 ± 5	59 ± 3	22 ± 5	43 ± 4		50 ± 3	20 ± 2	48 ± 3
Treated	1655 ± 2	1541 ± 4	-----	1416 ± 3	1302 ± 2	1236 ± 2	1166 ± 3	1076 ± 2
	62 ± 4	64 ± 5		51 ± 4	19 ± 2	38 ± 3	36 ± 5	43 ± 4

First line indicates the frequency of the band in cm⁻¹, while the second line indicates the bandwidth in cm⁻¹.

ERG Measurements

ERG was recorded after light adaptation in response to white bright flash of white background. A representing ERGs for control, glaucomatous, and treated groups showing the peak for b-wave, the implicit time of b-wave, and PhNR (Fig. 9). The amplitude and implicit time of b-wave have mean values of 2.14 mV and 36 msec respectively, while the amplitude of PhNR was 1.8 mV.

From Fig.9, it was noticed that the PhNR was not well defined as the b-wave, but it had relatively broad trough, making it difficult to determine its exact implicit time. It was clear from Fig.10 that,

there was non significant change in the amplitude of b-wave for control, glaucomatous, and treated groups. Moreover, there was a significant reduction of PhNR amplitude ($p < 0.05$) of glaucomatous group and some sort of improvement in PhNR amplitude after treatment with Brimonidine tartrate. The percentage difference of PhNR for glaucomatous and treated groups was 27.7 % and 10% respectively. The implicit time of b-wave (Fig.11) was significantly longer in the glaucomatous group than in the normal group ($p < 0.01$) the percentage change of implicit time for glaucomatous and treated groups was -13.8% and -8.3 % respectively compared with control.

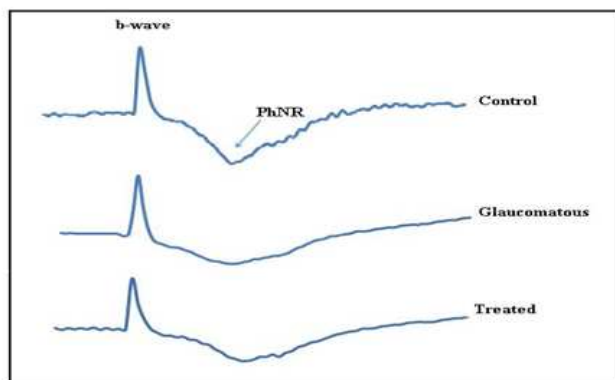


Fig. 9: Representative photopic flash ERG for control, glaucomatous, and treated groups

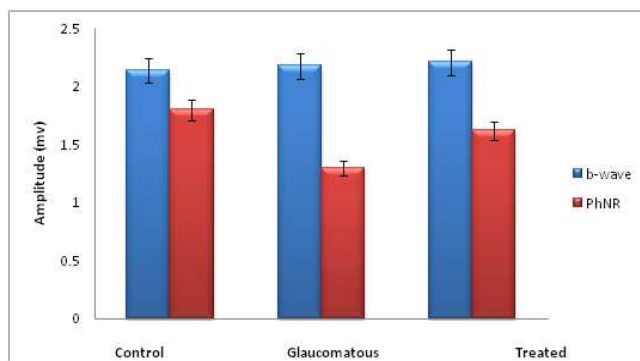


Fig. 10: Amplitude of b-wave and PhNR for control, glaucomatous, and treated groups

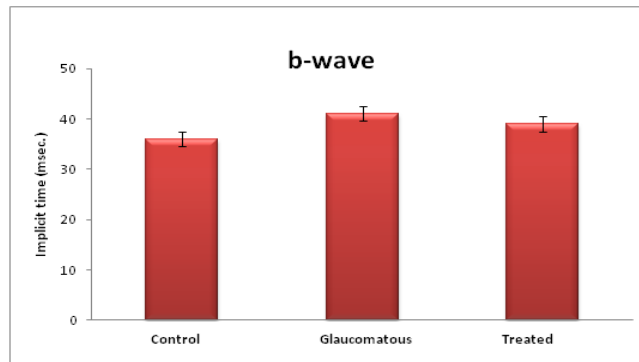


Fig. 11: The implicit time of b-wave for control, glaucomatous and treated groups

DISCUSSION

To analyze the obtained data for the aqueous humor and the retina, one may layout some suggestions and theories about the possible mechanisms triggering retinal ganglion cell injury and death in glaucoma, and the interaction of Brimonidine tartrate with the ocular tissue. The mechanisms that have been proposed for glaucoma includes compromise to blood flow at the optic nerve, mechanical compression, loss of neurotrophic factors, autoimmune mechanism, nitric oxide induce injury to the optic nerve and glutamate excitotoxicity. In addition to these primary mechanisms, number of studies has provided evidence that oxidative stress and damage contributed to degeneration of retinal ganglion cells in glaucoma²⁰⁻²¹.

Brimonidine tartrate is a highly selective α_2 -adrenoceptor agonist, which reduce IOP primarily by decreasing aqueous humor production and stimulating aqueous humor outflow. α_2 -adrenoceptor has been identified in ganglion cell layer of the retina, and the protective effect of brimonidine is likely to be mediated via activation of these receptors. Brimonidine attenuates the release of glutamate in the eyes with elevated IOP and increased survival of retinal neurons in culture⁴.

In the present work, different techniques were applied in order that, the output data can be piled together to create a good understanding of what is going on the molecular level for the aqueous humor proteins and retina in case of glaucoma. The total protein concentration in glaucomatous aqueous humor is approximately 26% higher than that in non-glaucomatous rabbit's eye. This result agrees with previous investigation²². The anterior segment of the eye is isolated from the blood stream by anatomic barrier known as the blood aqueous barrier (BAB) which employs both molecular sieve effects and active transport mechanisms to regulate the qualitative and quantitative protein structure²³. The blood - aqueous barrier is located in the anterior part of the eye and is formed by the endothelial cells of the blood vessels in the iris and the non-pigmented cell layer of the ciliary epithelium²⁴. These barriers break down after induction of glaucoma causing an influx of proteins from serum into the aqueous humor²⁵.

Topical application of Brimonidine tartrate leads to decreases in protein content in aqueous humor of the treated glaucomatous rabbit' eyes compared with glaucomatous. Moreover, the changes in protein concentration match the change in IOP for all studied groups. SDS-PAGE pattern of treated rabbits approximately resembles the control except for the presence of high molecular weight proteins (309 KD and 154 KD). This aggregation may be resulted from the combination between the native proteins and /or the breakdown products of the damaged proteins. Previous study revealed these differences in protein concentration and molecular weight to the topical anti-glaucoma medications used to treat glaucoma²⁶.

The FTIR results indicate that, induction of glaucoma and treatment with Brimonidine tartrate has no effect on the C-H stretching region (3000-2800 cm^{-1}). The most prominent changes in the rabbit retina have been registered in NH-OH bands of glaucomatous retina (3700-

3000 cm^{-1}). The NH bond exists in several membrane constituents that contain protein and lipid. Therefore, induction of glaucoma may lead to changes in membrane structure of retinal cells. In addition, there is marked change in bandwidth in the fingerprint region (1800 - 1000 cm^{-1}) of glaucomatous retina attributed to oxidative stress induced by glaucoma (Table 2). Oxidative changes have been reported in the retina of animals with experimentally induced glaucoma, including a decrease in endogenous antioxidant, enzyme activity, an increase in lipid peroxidation and protein oxidation²⁰.

In the present study, flash ERG is measured under light adapted (photopic) condition, when the rods are saturated, the ERG considers the electrical activity of cell in the cone circuits. Several electrophysiological studies have been performed to determine whether retinal structures other than the ganglion cell layer are affected by glaucomatous damage. The photopic b-wave may be resulted from combined activity of depolarizing and hyperpolarizing cone bipolar cell or horizontal cells and perhaps müller cell¹². Therefore, it can be regarded as a measure of the function of the middle retina, especially inner nuclear layer. Furthermore, PhNR was primarily a reflection of spiking activity of ganglion cell though; it might also contributions from other spiking cells in the inner retina as amacrine cells²⁷⁻²⁸.

The most impact of IOP elevation based on decrease blood flow, or pressure-induced compression of the retinal blood vessels could cause mild ischemia in certain retinal tissues²⁹. For example, the inner retina which has a high metabolic demand and blood flow of which is supplied by the central retinal artery, may be more vulnerable to metabolic stress induced by the insult when compared to the outer retina²⁹.

The current data indicates that, in flash ERG, the b-wave appears normal. This finding is similar to previous observations in macaques with experimental glaucoma. Moreover, significant reduction in b-wave amplitude generally occurred when the IOP reached a critical level (64-65 mmHg for rabbit)³⁰⁻³¹.

In glaucomatous group, the implicit time of b-wave is longer than control that is a good measure of inner nuclear layer function. Moreover, the reduction in PhNR is effective in early detection of glaucoma. Previous studies founded that this reduction was associated with the reduced activity of RGCs and glial cells³². When Brimonidine was applied at the time of IOP elevation, before pressure induced ganglion cell injury, the PhNR was improved. This improvement may assign to the ability of Brimonidine to attenuate ganglion cell loss.

CONCLUSION

This work concluded that, when IOP elevated due to induction of glaucoma, the BAB is intact, causing changes in aqueous humor protein concentration and composition these changes represented by shifting towards lower or higher molecular weights indicating degradation and aggregation of protein molecules. Glaucoma may lead to changes in membrane structure of retinal cells have been registered in NH-OH bands and the electrical properties of the retinal cells. This may be attributed to oxidative stress induced by glaucoma. In addition, the implicit time of b-wave is longer than

control, which is a good measure of inner nuclear layer function. Moreover, the reduction in PhNR is a reflection of spiking activity of ganglion cell and other spiking cells in the inner retina. Application of Brimonidine tartrate "0.15 %" for treatment of elevated IOP induces improvement in aqueous humor protein and retinal cells.

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