

Photopic negative response in diagnosis of glaucoma: an experimental study in glaucomatous rabbit model

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Abstract

• **AIM:** To determine whether the photopic negative response (PhNR) elicited by transient white flash on white background is characterizing for glaucoma model in rabbits.

• **METHODS:** Glaucoma was induced in twelve rabbits by subconjunctival injection of 0.05 mL of betamethasone in right eyes (each 1 mL contain betamethasone dipropionate 5 mg and betamethasone sodium phosphate 2 mg). The intraocular pressure (IOP), electroretinogram (ERG) and visual evoked potential (VEP) were measured successively prior and on the 3, 7d, two weeks and four weeks postglaucoma induction. After four weeks, the animals were sacrificed and the globes were histopathologically examined.

• **RESULTS:** The IOP increased significantly after one week ($P=0.0001$), then it gradually returned to the control level. In ERG examination, the means of a and b wave amplitude and latency were not affected significantly. PhNR amplitude decreased significantly within one week ($P=0.0001$), but its latency was not affected significantly ($P=0.132$). The means of VEP latency and amplitude were significantly affected after two weeks and four weeks of glaucoma induction ($P=0.0001$ and 0.02 , respectively). The histopathologic examination of the globes showed reduced number of cells in the retinal ganglion cell layer with multiple vacuoles in the retinal nerve fibre layer. There was significant positive correlation between ganglion cell layer cells and PhNR amplitude ($r=0.8$, $P=0.002$).

• **CONCLUSION:** The rise in IOP resulted in irreversible changes or incomplete recovery of VEP and PhNR amplitude. Both PhNR and VEP represented good additional tools in early diagnosis of glaucoma.

• **KEYWORDS:** glaucoma; electrophysiology; photopic negative response; rabbits

INTRODUCTION

Glaucoma is a chronic neurodegenerative disease characterized by loss of retinal ganglion cells with distinctive changes in the optic nerve head (ONH) and retinal nerve fiber layer (RNFL). Early diagnosis is critical to prevent permanent structural damage and irreversible vision loss. Techniques such as confocal scanning laser ophthalmoscopy, scanning laser polarimetry, and optical coherence tomography have been extensively studied as adjuncts to subjective ONH evaluation. Similarly, selective perimetry techniques, including short-wavelength automated perimetry (SWAP) and frequency-doubling technology (FDT) perimetry, are being explored as replacements to standard automated perimetry (SAP) to provide earlier detection of visual field deficits^[1].

Many reports suggested the use of electrophysiologic tests in early diagnosis of glaucoma^[2,3]. The pattern electroretinogram (PERG) assesses the function of retinal ganglion cells (RGCs) by isolating the magnocellular ganglion cell response using a reversing checkerboard or grating pattern with no change in average luminance over time. PERG was used successively in early diagnosis in primary open angle glaucoma (POAG) and ocular hypertension^[2,4]. PERG amplitude reduction can appear before any significant visual field changes^[5]. PERG was not used widely because it requires refractive correction, clear ocular media, good patient co-operation for proper placement of electrodes otherwise erroneous results can be produced.

In conventional flash ERG, the photopic negative response (PhNR) is the slow negative potential following the b-wave, originating from the inner retina^[6]. The PhNR amplitude is associated with cone-related RGCs function and it may be compared with the N95 of the PERG. Full-field PhNR amplitude correlated with the mean deviation of the visual field and with RNFL thickness in OCT. Therefore, its use as a relative measure of the retinal ganglion cells function in glaucoma patients was suggested^[7]. For generating PhNR a brief long-duration red Ganzfeld flashes on a rod-suppressing

blue-adapting background were used [5]. Also, a white Ganzfeld flashes on white [8] or blue [9] background was described. Rapid-on and rapid-off sawtooth flicker light pulses were even tried [10].

This study aimed to determine whether the PhNR elicited by transient white flash on white background is characterizing for glaucoma model in rabbits.

MATERIALS AND METHODS

Animals Eight-week-old NeoZealand albino rabbits (obtained from the laboratory animal research house, Research Institute of Ophthalmology) were used in this study. Twelve rabbits were housed in large spacious cages, and were given food and water ad libitum. The animal room was well ventilated, and a regular 12h:12h light/dark cycle was maintained throughout the experimental period. The experiment was performed in accordance to the ARVO rules for use of animals in ophthalmic and vision research. The intraocular pressure (IOP) and electrophysiological tests were measured successively before and on the 3, 7d, two weeks and four weeks postglaucoma induction.

Glaucoma was induced, as previously mentioned [11], by subconjunctival 0.05 mL betamethasone injection in right eyes [each 1 mL contains betamethasone dipropionate 5 mg and betamethasone sodium phosphate 2 mg, (Betafos, Amoun pharmaceutical Co. Egypt)]. Left eyes were used as controls.

Ophthalmological Examination Rabbit eyes were examined daily by hand held slit lamp (Zeiss, Germany) and by direct ophthalmoscope (Welch Allen, Germany). All animals were examined before and on the 1, 3, 7, 14 and 28d of the experiment. The IOP was measured using Schiotz indentation tonometer (Eichtabelle, Germany) under topical anaesthesia (benoxinate hydrochloride 0.4% eye drops, EIPICO, Egypt), with two weights (5.5 and 10 g) and the average IOP was taken.

Electrophysiological Examination Photopic ERG and flash visual evoked potential (VEP) recordings were performed before and after induction of glaucoma (after 1, 2 and 4wk), using Reti-com system (Roland-Consult). Full-field stimulation was produced with a miniganzfeld stimulator, positioned close to one eye. Pupils were dilated using tropicamide 1% eye drops (Alcon, Egypt) and phenylephrine hydrochloride 2.5% eye drops (Misr, Egypt).

Photopic Electroretinogram Single flash cone response was recorded with HK-loop conjunctival electrode moistened with carboxymethylcellulose sodium 1% lying in the lower eye lid. The reference electrode was placed on the upper rim of the orbit. Electrode placed on the right ear served as a ground electrode. The white Standard Flash was used, with a flash intensity of 3 cd.m² at frequency of 0.3 Hz and cycle time of 3.3s. A white background with a luminance of 25 cd.m⁻² was used to suppress the rods. The PhNR signal is

the slow negative potential following a- and b-waves in photopic ERG. Amplitudes of PhNR and the a-wave were measured from the baseline to the lowest point of the negative peak amplitude of b-wave was measured from the a-wave trough to the b-wave positive peak. The a-wave, b-wave and PhNR peak latencies were calculated from the flash onset to peaks of waves.

Flash VEP was recorded with skin electrodes. The active electrode was fit on the skin above the area of the visual cortex, midway between the two ears. The reference and ground electrodes were inserted in ears. The standard flash was used with +5 db intensity at frequency of 1.4 Hz and cycle time of 0.7s. The signal was amplified and filtered (1-30 Hz). The VEP amplitude was measured from the trough of the first negative wave to the peak of the following positive wave. Peak latencies were calculated from stimulus onset to the trough of the first negative wave. ERG and VEP values were expressed as mean±SD.

Histopathological Examination Animals were euthanized after four weeks with overdose of intracardiac ketamine and xylazine. Globes were enucleated, and a puncture incision was done on the central cornea, and fixed immediately in 10% buffered formalin. Eyes were sectioned horizontally to obtain a pupil-optic nerve section and examined macroscopically. Tissues were then processed and embedded in paraffin, sectioned at a thickness of 5 µm, and stained with hematoxylin and eosin (H&E). Light microscopy was used for histological examination of the globe; the cornea, anterior chamber angle, lens, retina and optic nerve was done.

Statistical Analysis Analysis of variance (ANOVA), posthoc test with multiple comparisons and Bonferroni correction were performed to compare the IOP and electrophysiological responses before and after induction of glaucoma. The Student's *t*-test was used to compare the retinal ganglion layer cells count before and after glaucoma. Pearson correlation test was done to evaluate the association of the continuous variables. A *P*-value less than 0.05 was considered statistically significant. For these estimations, SPSS software package version 10.0 (SPSS Inc., Chicago, IL, USA) was used.

RESULTS

Ophthalmological Examination Baseline slit lamp examination and on the 1, 2 and 4wk after induction of glaucoma did not reveal any abnormality in the anterior segment with clear lens. The steroid white precipitates were seen subconjunctivally. Fundus examination showed no significant retinal changes or optic disc cupping. In this animal model of experimental glaucoma, after one week of injection, the IOP was markedly increased as compared to control preinduction values (*P*=0.0001, ANOVA). On the following days of examination, after two weeks and four weeks, the IOP returned to the control level with no

Table 1 IOP, ERG and VEP results before and after 1, 2, 4wk of glaucoma induction in experimental rabbits

Variables	IOP	a-wave amplitude (μV)	a-wave latency (ms)	b-wave amplitude (μV)	b-wave latency (ms)	PhNR amplitude (μV)	PhNR latency (ms)	VEP amplitude (μV)	VEP latency (ms)
Control (n=12)	17±1.79	7.35±2.32	13.05±1.03	33.43±2.80	32.2±1.63	14.75±0.72	62.25±3.16	9.02±1.54	34.52±2.17
After 1wk (n=12)	35.67±2.16 ^a	7.4±2.16	13.17±1.02	33.4±3.75	32.35±0.97	6.15±0.91	61.97±4.78	7.05±1.36	34.82±2.93
After 2wk	19.5±2.07	7.65±2.25	13.4±1.27	33.08±4.07	31.47±1.08	4.12±3.15 ^a	62.07±4.65	6.87±1.28	43.27±6.82 ^a
After 4wk	16.83±1.72	8.2±2.75	13.15±1.33	33.02±6.81	32.02±1.45	4.25±2.68 ^a	66.6±1.91	6.85±0.83	49.33±5.20 ^a
<i>P</i> (ANOVA)	0.0001 ^a	0.92	0.98	0.99	0.67	0.0001 ^a	0.132	0.02 ^a	0.0001 ^a
<i>P</i> ¹	0.0001 ^b	0.99	0.96	0.97	0.86	0.0001 ^b	0.99	0.09	0.88
<i>P</i> ²	0.2	0.98	0.99	0.99	0.83	0.0001 ^b	0.87	0.041 ^b	0.024 ^b
<i>P</i> ³	0.98	1.0	0.86	0.95	0.78	0.0001 ^b	0.37	0.049 ^b	0.0001 ^b
<i>P</i> ⁴	0.16	1.0	0.89	0.96	0.79	0.85	0.31	0.99	0.21

IOP: Intraocular pressure; ERG: Electroretinogram; PhNR: Photopic negative response; VEP: Visual evoked potential; ^aSignificant *P* if less than 0.05, ANOVA test; ^bANOVA, Posthoc test with multiple comparisons, Bonferroni; *P*¹: Compare between 1wk and control; *P*²: Compare between 2wk and control; *P*³: Compare between 4wk and control; *P*⁴: Compare between 2 and 4wk.

significant difference from the baseline, (*P*=0.23, and 0.99, ANOVA, respectively), as shown in Table 1.

Electrophysiological Tests

Normal photopic electroretinogram and flash visual evoked potential Figure 1 shows a selected ERG recording from a control rabbit eye. The ERG is composed of a- and b-wave followed by a slow negative potential; PhNR waveform.

Photopic electroretinogram changes in experimental glaucoma model In all animals, the PhNR mean amplitude was significantly reduced after one week reaching 6.15±0.91 μV (*P* =0.0001, Posthoc test), as compared to the control pre-induction level. Meanwhile, its latency was not affected significantly (*P*=0.132, Posthoc test). Thereafter, the mean PhNR amplitude was reduced in the two successive measures at 2 and 4wk (Figure 2), in spite of return of IOP to normal limits. The PhNR amplitude reduction and delay in latency were not significantly correlated with the IOP changes (*P* = 0.42 and 0.47, respectively, Pearson correlation).

The photopic a- and b-wave did not show significant changes before and after glaucoma induction. The means of a-wave amplitude and peak latency were 7.35 ±2.32 μV and 13.05 ±1.03ms, respectively. After four weeks of glaucoma induction, they were 8.2 ±2.75 μV and 13.15 ±1.33ms, respectively with no significant difference (*P*=0.92 and 0.98, ANOVA, respectively). The means of b-wave amplitude and peak latency were 33.43 ±2.80 μV and 32.2 ±1.63ms, respectively. After four weeks of glaucoma induction, they remained nearly unchanged (*P*=0.99, and 0.67, ANOVA, respectively), as shown in Table 1.

Flash visual evoked potential results Regarding VEP recordings, after one week of glaucoma induction, the mean of VEP amplitude did not decrease significantly (*P*=0.09, Posthoc test). After two weeks of glaucoma induction, there was significant reduction in the VEP mean amplitude with increased peak latency (*P*=0.041 and 0.024, Posthoc test, respectively). After four weeks, the animals exhibited

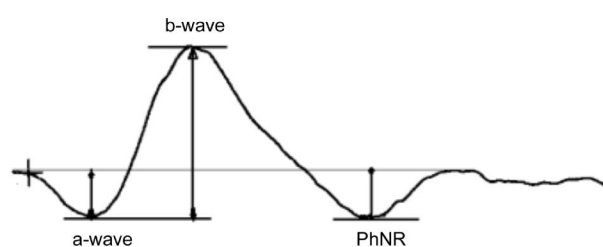


Figure 1 Single-flash cone ERGs recorded from a control animal The amplitude of a wave and PhNR was measured from the baseline to the wave trough. B-wave amplitude was measured from the a trough to b wave positive peak.

significant reduction of VEP amplitude and significant delay in peak latency (*P*=0.049, 0.0001, Posthoc test, respectively). VEP peak latency showed a significant positive correlation with PhNR peak latency (*P*=0.019, Pearson correlation). Meanwhile the VEP amplitude showed a significant positive correlation with PhNR amplitude (*P* =0.001, Pearson correlation; Table 1, Figure 2). Figure 3 showed the correlation as a sequence graphs between PhNR amplitude, VEP amplitude and IOP.

Histopathological Examination Sections revealed normal light microscopic appearance of the cornea, anterior chamber angle, iris and ciliary body in all studied animals.

Figure 4 shows photomicrographs of retina of a selected eye of the control group and the glaucoma model. The normal retinal layers of a control eye is shown in Figure 4A. The mean of glial and ganglion cells count in ganglion cell layer (GCL) within the central field surrounding the optic disc, examined at ×100, was 60.17±21.11 cells. After induction of glaucoma, photoreceptors, the outer nuclear layer (ONL), the inner nuclear layer (INL) appeared intact. GCL showed significant decrease of ganglion and glial cells count reaching a mean of 14.33±3.5 cells with multiple vacuoles (*P*=0.03, *t*-test; Figure 4B). The GCL cells count showed significant positive correlation with the PhNR amplitude (*r*=0.8, *P*=0.002, Pearson correlation). There was non significant positive correlation with VEP amplitude (*r*=0.31, *P*=0.33, Pearson

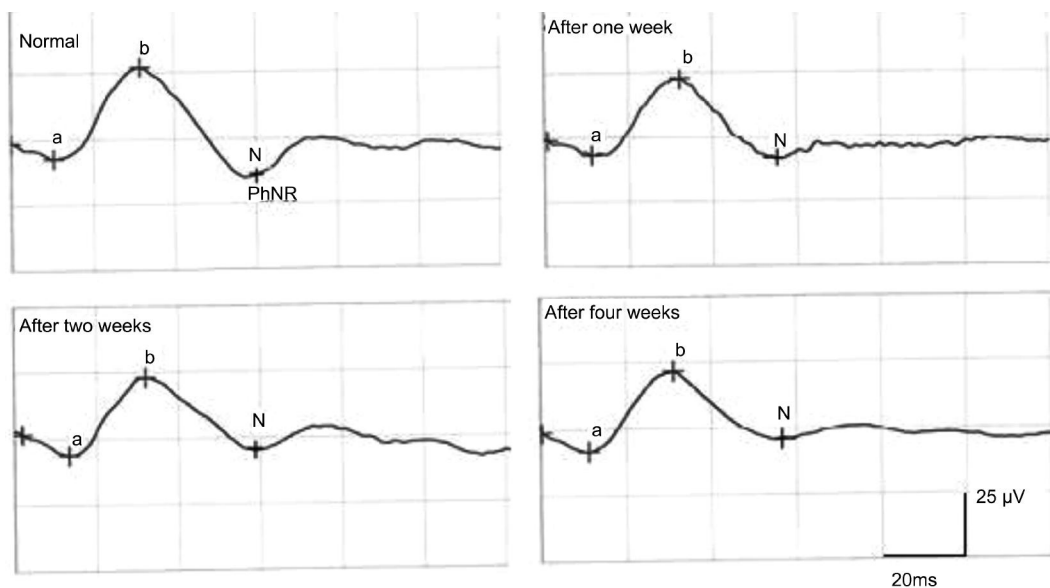


Figure 2 Photopic ERG responses to a white flash on a white background from a selected rabbit eye before injection (normal) and after; one, two and four weeks of glaucoma induction. It showed marked reduction of PhNR amplitude with intact a- and b-wave amplitude and latency.

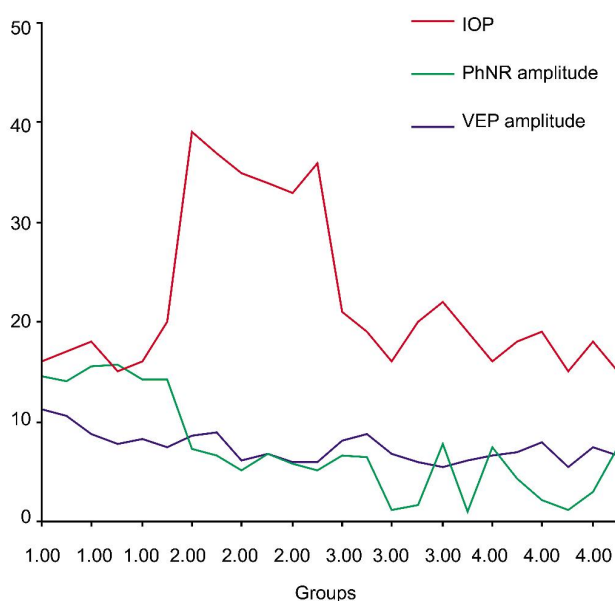


Figure 3 Sequence graphs showing the change in IOP, PhNR and VEP along the examination days (horizontal axis 1: Control group; 2: One week after induction of glaucoma; 3: Two weeks after; 4: Four weeks after).

correlation). VEP latency and PhNR latency showed significant negative correlation with RGC count ($r=-0.77$, $P=0.004$, $r=-0.8$, $P=0.002$, respectively, Pearson correlation). The optic nerve appeared normal with no cupping (Figure 4C).

DISCUSSION

The conventional ERG is the total retinal response to a brief flash of light from a Ganzfeld sphere. Under dark-adapted conditions, the negative a-wave is generated by rod photocurrents, and the positive b-wave is generated by a combination of depolarizing bipolar cell currents and bipolar cell-dependent K^+ currents in Müller cells. Under photopic

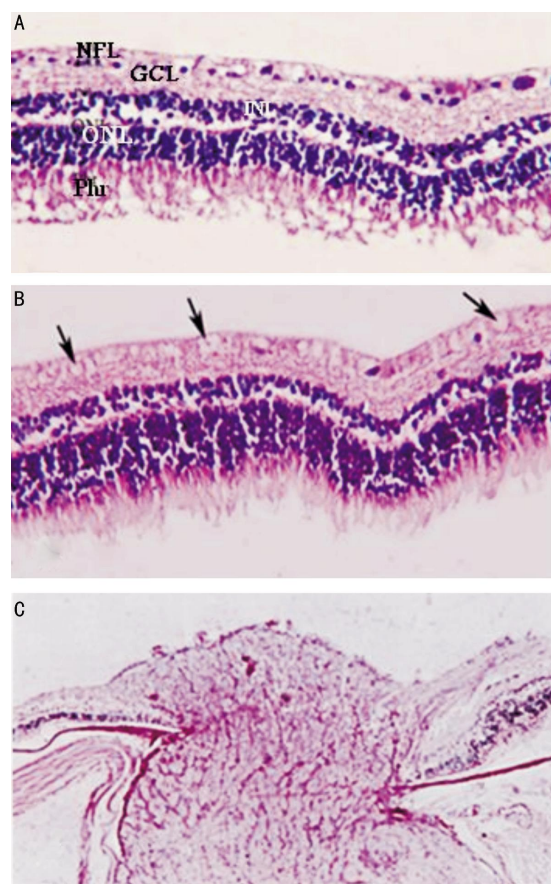


Figure 4 Normal retinal layers in a control eye showed in Figure A; photoreceptors (Phr), the outer nuclear layer (ONL), the inner nuclear layer (INL), the ganglion cell layer (GCL) and nerve fibre layer (NFL) (haematoxylin and eosin $\times 200$). After induction of glaucoma in Figure B, the examined retina showed marked loss of ganglion cells with multiple vacuoles (arrows) (haematoxylin and eosin $\times 200$). In glaucomatous group, the optic nerve in Figure C appeared normal with no cupping (haematoxylin and eosin $\times 50$).

conditions, the a-wave is generated by cone photoreceptor activity with contribution from hyperpolarizing cone bipolar cells. The photopic b-wave results from the combined activity of depolarizing and hyperpolarizing cone bipolar cells and perhaps Müller cells^[12]. Although RGCs do not contribute to the conventional flash ERG, but recording signals such as the PhNR consistent with RGC activity is possible. PhNR is a slow (time-to-peak longer than 100ms) negative potential following the positive b-wave of the ERG^[13].

In the present study, we found that the PhNR mean amplitude was reduced in the ganzfeld ERG of glaucomatous rabbits' eyes. The earlier involvement of PhNR than flash VEP, suggested the role of PhNR in earlier detection of ganglion cell dysfunction. These findings were consistent with previous studies of glaucoma both in experimental animals and in humans^[14,15].

The specific reduction of PhNR in glaucoma was explained by the reduced activity of retinal ganglion cells and glial cells. In macaques, PhNR was reduced by Intravitreal injections of tetrodotoxin (TTX), a voltage-gated Na⁺-channel blocker that eliminates spiking activity in amacrine and retinal ganglion cells^[13,16]. Further, intravitreal injection of Ba²⁺, an ion that blocks K⁺ channels in glia, and blocks glial-mediated responses in the ERG selectively eliminated the PhNR from the photopic ERG^[17].

As for the PhNR recording conditions, red flashes on a rod-saturating blue background was considered to be an optimal condition for producing PhNR^[5]. Herein, we used the standard white flash and rods were suppressed by a white background. PhNR amplitudes for normal control rabbits were $-14.75 \pm 0.72 \mu\text{V}$, which were adequate. The normal PhNR amplitudes in human were $-30.67 \pm 10.02 \mu\text{V}$, elicited by white flashes on a white background^[13]. Similar to our testing parameters, Horn *et al*^[8] found the PhNR amplitude in control subjects approximated studies using red flashes on a blue background.

In glaucoma, both flash and pattern VEP showed delayed subnormal parameters attributed to reduced conduction velocity of damaged optic nerve fibers and loss of retinal ganglion cells^[2,18]. Tsaousis *et al*^[2], observed a transient VEP amplitude drop in pattern VEP in glaucomatous eyes. The delayed affection of the flash VEP in our study agreed with previous work that suggested flash VEP was an indicator of retinal ganglion cell death rather than damage, which was confirmed by our histopathological examination. Hence it was suggested to be of little use in detecting early nerve dysfunction^[2].

As regard our model of glaucoma, corticosteroid induced glaucoma is observed in ophthalmological practice after topical, periocular or systemic administration through

reduction of outflow facility. It increases the expression of the extracellular matrix protein fibronectin, glycosaminoglycans, and elastin in trabecular meshwork cells^[19]. Steroids also suppress phagocytic activity with increased deposition of material in the juxtacanalicular meshwork. As well as, actual angle obstruction by the steroid crystals has been postulated^[19]. We used betamethasone, which is a potent glucocorticoid with anti-inflammatory and immunosuppressant abilities. It has long half life (36-54h) and very potent than triamcinolone acetonoid (0.5 mg triamcinolone is equivalent to 0.07 mg betamethasone)^[19]. In our study, single subconjunctival injection of 0.35 mg betamethasone in 0.05 mL was sufficient to elevate the IOP within one week with gradual decline thereafter.

The loss of retinal ganglion cells, seen histopathologically, can be explained by various mechanisms. Elevated IOP may initiate death of RGC by obstructing the retrograde transport, inhibiting the delivery of neurotrophic substances to RGCs, thereby triggering apoptosis. An alternative hypothesis is that IOP elevation alters glial cells, resulting in damage to RGC axons. In addition, RGC death induced by elevated IOP involves caspase activation, a proteolytic enzyme facilitating apoptosis^[17].

Some limitations in the present work are considered. Concerning our model of glaucoma induction in rabbits, the IOP measurement is difficult to standardize. Also, the rabbit retina is partially myelinated by oligodendrocytes which may respond to increased IOP in a different way than humans^[20]. We observed that this glaucoma model represented a model for acute open angle glaucoma with retinal ganglion cells loss. The absence of optic disc cupping may be due to presence of excessive glial cells in rabbits' optic nerve which provide neuroprotection against injurious effect of increased IOP^[21] or it may simply not have adequate time to occur due to short duration of high IOP. The short IOP spike induced-only lasting one week seemed sufficient to mimic glaucomatous damage as evidenced by the pathological evaluation, but the same findings may not be present in a more chronic or low-grade type of IOP elevation. Contrary to our model, Melena *et al*^[22] used a rabbit model with subconjunctival injection of betamethasone weekly to maintain the high IOP for about four weeks. However, they did not mention if there was any lenticular opacity or optic disc cupping.

Secondly, in our model of open angle glaucoma, the PhNR amplitude and VEP amplitude were kept reduced although the IOP had returned to normal limits. This suggested that our model of glaucoma induced permanent loss of ganglion cells. Studies done by Ventura *et al*^[23] indicated that the abnormal PERG recorded in patients with early stages of glaucoma may improve after IOP reduction, however little

PERG improvement occurred in eyes with severe visual field defects. Also, Niyadurupola *et al* [15] reported improvement in PhNR after lowering of IOP. Reversible PhNR abnormality implies that in the early stages of glaucoma RGCs dysfunction may precede its death .

In conclusion, the PhNR amplitude reduction was not a transient change related to a sudden increase in IOP and it could be used for early diagnosis in glaucoma. The use of white flash on a white background could be adequate mechanism for eliciting PhNR. Also, we found that flash VEP was affected later than PhNR which ascertained the previous results of being not a good test for early glaucoma detection. Future reaserch studies on the use of newer S cone VEP and multifocal VEP to isolate the M (magnocellular) or P (parvocellular) cell channels may help in early glaucoma diagnosis. Additionally, our model of glaucoma caused a permanent retinal ganglion cell death, which elaborates the need for an optimal animal model to simulate human glaucoma with early RGC dysfunction that can be reversed after treatment requires more research.

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Conflicts of Interest: ElGohary AA, None; Elshazly LHM, None.

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