

Investigation of the Impact of Nerve Growth Factor Intravitreal Administration on Ganglion Cell Degeneration in an Experimental Model of Elevated Intraocular Pressure in Rabbits

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Abstract: Nerve Growth Factor (NGF) has been reported to possess beneficial therapeutic potential in optic neuropathies. The aim of the present study was to assess the impact of NGF administration on the retinal alterations due to experimentally induced elevated intraocular pressure in rabbits. The increased intraocular pressure was induced in the right eyes of adult male New Zealand White rabbits by injection of 2% methylcellulose solution. NGF and VH groups (n = 9 animals per group) were treated with either right eye intraocular application of NGF or vehicle solution. The 2.5 S subunit NGF powder diluted in 10 µg normal saline was intravitreally injected on the 5 and 10th day after the induction of increased intraocular pressure. Control group (n = 4) consisted of the untreated left eyes of some animals belonging to the two first groups. Retinal Ganglion Cells density was assessed by immunohistochemical staining of the retina 20 days post-induction of the elevated intraocular pressure. Elevated intraocular pressure resulted in Retinal Ganglion Cells loss in the NGF and VH groups as compared with the controls. The number of Retinal Ganglion Cells was reduced in the NGF-treated group as compared with the VH group. These findings demonstrate that NGF administration in specific dosage and at different times following experimentally-induced increased intraocular pressure displays a protective role against Ganglion Cell layer degradation.

Key words: Nerve growth factor, intraocular hypertension, rabbits, neuropathies, injection

INTRODUCTION

Glaucoma is a progressive optic neuropathy characterized by peripheral visual field loss which is caused by Retinal Ganglion Cell (RGC) apoptosis and optic nerve damage (Lambiase *et al.*, 2009). Glaucoma is reported as the first common cause of blindness worldwide (Resnikoff *et al.*, 2004).

A wide group of risk factors such as the age, the gender and the genetic background have been implicated in glaucoma's pathophysiology (Coleman and Kodjebacheva, 2009). Elevated Intraocular Pressure (IOP)

contributes significantly to glaucoma pathogenesis (Garway-Heath *et al.*, 2012) and the main concern of ophthalmological scientists is to decrease IOP with the use of topical or systemic agents, laser or surgical treatment (Coleman and Brigatti, 2001). New therapeutic agents target to achieve the balance between neuroprotective and neurotoxic mechanisms involved in glaucoma generation (Bai *et al.*, 2010).

Neurotrophins are polypeptide growth factors that promote neuronal development, differentiation and survival (Ichim *et al.*, 2012). Nerve Growth Factor (NGF) is the earliest determined and characterized member of the

family of neurotrophins such as the BDNF, NT-3, NT-4/5, NT-6 and zebrafish NT-7 (Cuello, 2012). NGF is produced by a wide range of cells including those of the visual system (Levi-Montalcini, 1987; Carmignoto *et al.*, 1989; Mantyh *et al.*, 2011; Cuello, 2012). Impairment in the expression of two main receptors on the surface of NGF responsive cells, tyrosine kinase TrKA and p75 (Meakin and Shooter, 1992) can alter the functional activity of NGF cells, leading to degeneration of the nervous system. It has been reported that retinal injury is associated with upregulation in the expression of these NGF receptors (Bai *et al.*, 2010). Furthermore, recent studies (Sposato *et al.*, 2009) have shown that intraocular administration of NGF in experimentally-induced ocular hypertension reduces the grade of RGC damage. However, it remains to extensively examine the optimal dosage scheme of NGF administration.

The purpose of the present study was to investigate the impact of exogenous NGF administration in various dosages and at various times different from those suggested in literature, afterwards the induction of increased IOP on histopathology of RGC layer in rabbits. This animal model was chosen according to other relevant studies (Lambiase *et al.*, 1997). The results obtained provided significant evidence for the dose but also time-dependent protective action of NGF against RGCs degradation.

MATERIALS AND METHODS

Animal model: Eighteen, 2 months old, male New Zealand White rabbits (*Oryctolagus cuniculus*) (body weight 2.54 ± 0.13 kg, mean $\pm$ SD) were individually housed in stainless steel wire-bottom cages. The animals were provided by a farm breeding rabbits for experimental purposes in the Attica Region. The use and treatment of rabbits in all studies were in accordance with ethical recommendation of the European Communities Council Directive of November 24, 1986 (86/609/EEC) and the protocol was approved by the competent Veterinary Directorate of Athens Prefecture (Approval No.: K.7181/07). The animals were housed one per cage and were kept in a temperature-controlled environment ($19 \pm 1^\circ\text{C}$ with $55 \pm 5\%$ humidity) with a 12 h light/dark cycle (5:30 am to 5:30 pm) in an air-conditioned room and had free access to standard rabbit feed and tap water. The animals were clinically examined by a veterinarian during the entire experimental period. All possible steps were taken to avoid animal suffering at each stage of the experiment. All interventions that could cause suffer or pain to the animals were performed under ketamine-xylazine general anesthesia and all efforts were

made to minimize suffering. After 2 weeks of acclimatization, the animals were randomly assigned as follows:

- NGF group (n = 9): IOP was induced in the right eye of each animal followed by two intravitreal injections of NGF
- VH group (n = 9): IOP was induced in the right eye of each animal followed by two intravitreal injections of vehicle solution consisting of 0.9% NaCl
- (Control group) (n = 4): No interventions were performed in the left eye of all the animals. The left eyes of four animals belonging to the above groups were used as non-glaucomatous controls

Induction of intraocular hypertension: The increased IOP in the animals was induced by one single injection of 2% Methylcellulose solution (MC) (Chemilab, Athens). MC is a viscous synthetic compound which can be dissolved slowly in cold water with maximum viscosity of 4,000 cps as previously described (Zhu and Cai, 1992). A single injection of MC was performed in the anterior chamber of the right eye of each rabbit using a 27-gauge needle on a micro-syringe. The left eyes remained untreated. All the injections were administered under general anesthesia with ketamine and xylazine (35 and 5 mg/kg/B.W., respectively).

NGF administration: The 2.5 S subunit of NGF powder from mouse submaxillary glands (Chemilab, Athens) diluted in 10 μg normal saline (0.9% NaCl) was twice injected intravitreally using a 27-gauge needle on a micro syringe, under aseptic conditions on the 5th and on the 10th day after the MC injection. Both injections were performed under general anesthesia (induced by Ketamine-Xylazine intramuscular injection).

IOP measurement: The IOP was recorded thrice at t0: 12 days, t1: 8 days, t2: 0 days before the injection of MC. After the injection (t2), IOP was measured at 4 different times; t3: 2 days, t4: 5 days, t5: 10 days post-operatively and t6: 20 days after the induction of hypertension before the euthanasia of the animals and the eyes enucleation. The IOP was measured using a Tonopen XL tonometer (Tono-pen XL, Reichert, Inc., Depew, NY). During the experimental procedure all the IOP measurements were performed 3-5 min after the induction of general anesthesia by intramuscular injection of Ketamine/Xylazine (35 and 5 mg/kg/B.W. for Ketamine and Xylazine, respectively) (Lipman *et al.*, 1990). All the surgical procedures were performed under antibacterial treatment pre and post-operatively (Ofloxacin 3 mg mL⁻¹ eye drops, Exocin-Galinos).

Animals euthanasia and tissue collection: The rabbits were euthanized by sodium pentobarbital overdose 20 days after the induction of hypertension and the eyes were enucleated and fixed in formalin solution 10% for 24 h.

Histopathological evaluation of the retinal damage

Immunohistochemistry: Retinal ganglion cells were indicated by the use of immunohistochemistry. Immunohistochemical staining for PGP 9.5 was performed on 4 μ m thick formalin-fixed paraffin sections of anatomically comparative areas of the retina using an avidin-biotin immunoperoxidase technique after overnight heating at 37°C and subsequent deparaffinization in xylene and rehydration through graded alcohols. After quenching the endogenous peroxidase activity using a methanol hydrogen peroxide solution (0.3% in TBS for 30 min), researchers proceeded to microwave-mediated antigen retrieval in 10 mM, pH 6, citrate buffer at 750 W for 5 min. A standard two-step technique (Envision, Dako, Glostrup, Denmark) was performed while diaminobenzidine was used as a chromogen. The slides were incubated overnight with a polyclonal antibody against PGP 9.5 (Ultraclone, RA 95101) at a dilution of 1:800. Finally, sections were counterstained with hematoxylin and mounted. For negative controls, the primary antibody was omitted and replaced with normal serum saline solution.

Evaluation of PGP 9.5 staining: Images of the immunohistochemically stained sections were captured with a Nikon DS-2MW digital camera attached to a Nikon Eclipse 80i microscope (Nikon Co., Tokyo, Japan) using a x400 objective and stored as high quality JPEG files. The 7-10 representative images per section were captured. The ganglion cell density (number of cells per mm²) was estimated with Image-Pro Plus 5.1 Software (Media Cybernetics, Silver Spring, MD). Color threshold settings of Diaminobenzidine (DAB)-stained pixels were set manually prior to analysis and left unchanged throughout. To determine the hue threshold values for DAB immunostaining, images of the positive and negative control slides were examined for optimal separation between blue and brown-stained areas. Averaging the quantitative computerized image analysis data from the 7-10 images of each tissue section yielded an average number of ganglion cells per mm².

Statistical analysis: Data is expressed as mean $\pm$ 1 Standard Deviation (SD) for continuous variables. The normality of the distributions was assessed with

Kolmogorov-Smirnov test and graphical methods. Comparisons between more than two groups were performed with Analysis of Variance (ANOVA) using Benjamini and Hochberg's False Discovery Rate (FDR) in order to assess between-group differences as well as to control family-wise error to <0.05 . Kruskal-Wallis's test was utilized as a non-parametric test for multiple group comparisons using Mann-Whitney's U-test and FDR for post hoc multiple testing. Comparisons between more than two measurements were performed using Friedman's test and Wilcoxon's signed rank test with FDR as Post Hoc tests. Pearson's correlation coefficient and Spearman's rho were calculated in order to examine linear relationships between variables. In all cases of multiple hypothesis testing, FDR was utilized in order to assess between-group differences as well as to control family-wise error to <0.05 . All tests were two-sided. Differences were considered as statistically significant if the null hypothesis could be rejected with $>95\%$ confidence ($p<0.05$).

RESULTS AND DISCUSSION

IOP measurements: VH and NGF groups showed significant increases in IOP measurements at t3, t4 and t5 ($p<0.05$ in all comparisons for both groups). No significant differences were observed between t5 and t6 or between the measurements prior to the MC injection ($p>0.05$ in all comparisons for both groups). Control group IOP measurements remained comparable to t0 throughout the experiment ($p>0.05$ in all cases).

At baseline (t0) no significant differences in IOP were observed among the three experimental groups ($p>0.05$ in all cases). All group measurements remained comparable until t3 when the two hypertonic groups exhibited significantly elevated IOP levels compared to controls ($p<0.001$ in all cases). VH and NGF IOP remained significantly higher than controls until the end of the experiment ($p<0.001$ in all cases). On the other hand, IOP levels of NGF and VH groups were similar throughout the study. Only at t4, NGF group members exhibited a slightly increased IOP which was not observed at previous nor following measurements (Table 1 and Fig. 1).

Histopathological study (RGCs): NGF group members exhibited significantly higher RGC numbers in digitally examined and quantified immunohistochemistry samples compared to VH group members ($p<0.001$) and lower than controls ($p<0.05$) (Fig. 2). VH group had the lowest RGC counts, significantly less than NGF ($p<0.001$) and controls ($p<0.001$) (Table 2, Fig. 2 and 3).

Table 1: IOP mean values (SD) of NGF, VH and control groups during the experimental period. t0: 12 days, t1: 8 days, t2: 0 day before the injection of 2% Methylcellulose solution (MC). t3: 2, t4: 5, t5: 10 and t6: 20 days after the MC injection and the induction of elevated intraocular hypertension

Times							
Groups	t0	t1	t2	t3	t4	t5	t6
NGF	11 (1.6)	10 (1.8)	9 (0.6)	30 (5.7)	42 (3.55)	53 (3.4)	57 (3.05)
VH	12 (2.7)	11 (1.62)	10 (1.07)	28 (8.8)	38 (2.7)	54 (4)	59 (1.89)
Control	11 (1.85)	11 (1.84)	10 (1.2)	11 (1.15)	10 (0.73)	12 (1.94)	12 (1.94)

Table 2: Mean number (SD) of Retinal Ganglion Cell (RGCs) density (cells/mm²) in the three groups (NGF, VH and control)

Groups	RGCs
NGF	1.285 (235)
VH	765.000 (165)
Control	1.580 (206)

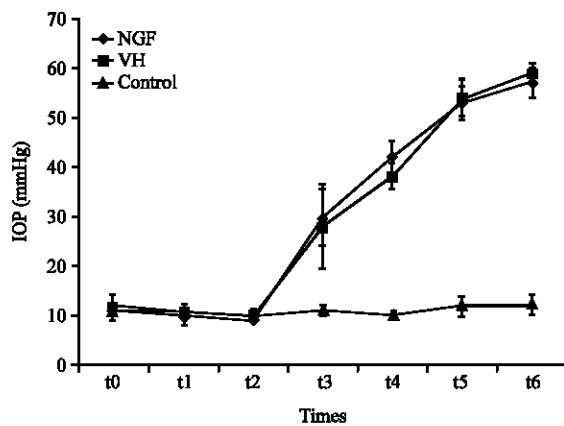


Fig. 1: Intraocular pressure measurements. Intraocular Pressure (IOP; mean±SD) pre-post intra-anterior chamber 2% methylcellulose injection in NGF, VH and control groups

Glaucoma caused by elevated intraocular pressure is characterized by RGC loss in lamina's cribrosas level, nerve fiber layer thickness and by a general disturbance of the RGC layer histology (Manni *et al.*, 1996). The mechanism implicated in this apoptotic procedure constitutes the main concern of ophthalmological society.

The present study focused on the potent neuroprotective role of NGF intraocular administration in a rabbit model with elevated IOP-induced glaucoma. According to the study by Lambiase *et al.* (1997), the endogenous NGF highest levels are detected in the aqueous humor in a period of 4-15 days after the induction of experimental ocular hypertension in rabbits. Furthermore, Dicou *et al.* (1994) have shown that the retinal cells production of NGF is increased in an Experimental Uveitis Model, probably playing a critical role in the prognosis of retinal cell damage occurring during the inflammatory course. Based on these results, the administration of exogenous NGF on the 15th and 10th days after the induction of elevated IOP was

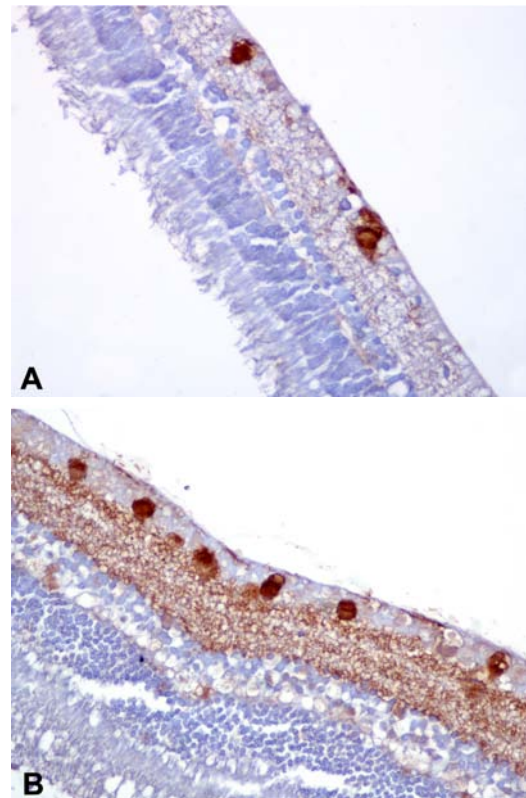


Fig. 2: Histopathological evaluation of the retinal damage. Immunostaining with antibody against PGP-9.5, diaminobenzidine as chromogen (brown color), 400x original magnification. Immunoreaction is localized in Ganglion Cell Layer (GCL) and in the Inner Plexiform Layer (IPL): A) there is noticeable loss of ganglion cells in group VH; B) compared to group NGF

examined in order to investigate whether the maintenance of increased NGF levels due to the increased endogenous NGF levels at these times in combination to the parallel exogenous administration could alleviate retinal damage.

The experimentally induced elevated IOP can be achieved by performing different techniques as episcleral veins injection (Tezel *et al.*, 2012) and laser trabeculoplasty (Yucel *et al.*, 1999). In this study, an intra anterior segment injection of MC which is considered an effective and reproducible way to increase IOP levels was

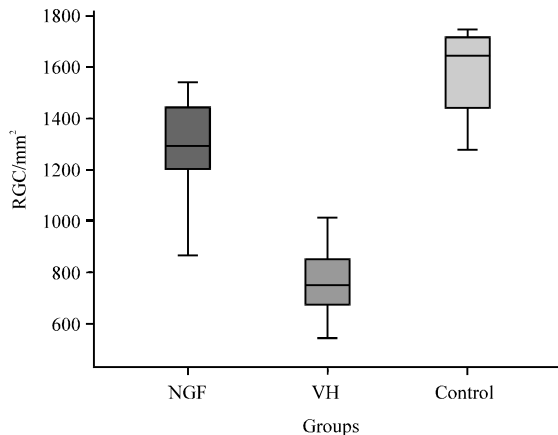


Fig. 3: Estimation of Retinal Ganglion Cells density. Retinal Ganglion Cells (RGCs) density (cells/mm²) in NGF, VH and Control Groups, 20 days post induction of intraocular pressure and after the intravitreal NGF administration

performed (Manni *et al.*, 1996). The values of the pressures recorded after this intervention were higher as compared with baseline levels suggesting an ischemic origin of retinal damage. However, according to other studies, higher pressure levels are required to induce ischemia (Grehn and Prost, 1983).

As expected, NGF intravitreal administration on the 5th and 10th days following the induction of elevated IOP ameliorated the RGC survival. In accordance with the findings, several studies in different animal models reached similar outcomes (Rudzinski *et al.*, 2004; Lambiasi *et al.*, 2009; Colafrancesco *et al.*, 2011a).

In the study, the evaluation of retinal damage was based on immunohistochemical estimation of RGC density with PGP 9.5 staining. Other methods for assessing the numbers of retinal ganglion cells in the retina of animals have been followed (Oyster *et al.*, 1981; Filippopoulos *et al.*, 2006). Immunohistochemical staining of apoptotic cells in the RGC layer has been also performed in other studies (Park *et al.*, 2011).

The increase of NGF endogenous production after any kind of neuronal damage (Linker *et al.*, 2009; Colafrancesco and Villoslada, 2011; Borlongan, 2012) is considered as an adaptive biological mechanism to resist this degeneration. More precisely (Lambiasi *et al.*, 1997) reported that the elevation of endogenous NGF after the induction of increased IOP represents a reparatory procedure in order to retard ganglion cell loss. NGF exhibits its neuroprotective role by blocking the apoptotic processes through the activation of TrKA receptors. This activation triggers a molecular pathway into cells, promoting their survival (Ichim *et al.*, 2012). According

to the aforementioned, the neuroprotective role of exogenous NGF administration may be attributed to the initiation of similar pathways (Lambert *et al.*, 2004). Coassin *et al.* (2008) showed that the endogenous increase in NGF levels in experimental glaucoma in rats is not sufficient to protect against RGCs apoptosis. The additional administration of NGF in the study probably supported the survival of RGCs by enhancing the endogenous NGF activity.

Similar to the findings by Colafrancesco *et al.* (2011b) in the study, the administration of NGF did not affect the IOP levels of the right eyes in the treated animals in almost all measurements as compared to the untreated rabbits. Literature research reveals that elevated IOP is not the only mechanism causing neuronal degradation. Other changes in the neuronal environment such as the neurotransmitter imbalance, the free radical generation and the elimination of growth factors may be implicated in RGC loss (Colafrancesco *et al.*, 2011b). It could be hypothesized that NGF may be involved in the above changes thus improving retinal histopathology without affecting IOP levels.

In spite of the significance of the findings, it would be important to further validate the experimental methodology in a larger animal sample in order to safely transfer the conclusions in clinical research. Besides, it would be important to investigate in future experiments whether NGF has a long enough half-life to alleviate the RGC's loss. For this reason, it would be essential to examine NGF neuroprotective role in RGC apoptotic procedures for a more extended period (>20 days) in order to have more sufficient evidence from the follow-up measurements and to also record any potential long-term adverse effects.

Although, PGP 9.5 staining revealed increased RGCs density in the study, no complementary methods investigating the survival effects of NGF on RGCs were performed. Furthermore, it would be interesting to evaluate the use of additional dose or dose duration schemes. The estimation of endogenous NGF and the investigation of the expression of NGF receptors could finally provide answers to some unclarified critical questions.

CONCLUSION

NGF can inhibit retinal damage and promote RGCs survival in an animal model of increased intraocular hypertension. It remains to extent the knowledge gained to future experimental but also clinical studies so as to elucidate whether NGF could be potentially used in combination with the already established glaucoma care and treatment in humans.

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