

Topical Potential Effect of Sodium Nitroprusside in Induced Oculohypertension in Rabbits

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Abstract:

Glaucoma is an optic neuropathy known by slow degenerative structural changes seen in retinal ganglion cells and the optic nerve head. Elevation in intraocular pressure caused by imbalance between the production of

aqueous humor and the drainage of this liquid through the trabecular meshwork. Nitric oxide donors being evaluated as a novel way to impact the progression of glaucoma by increasing the conventional outflow, hence lowering intraocular pressure. The aim of study was to evaluate the potential effect of topical sodium nitroprusside in glaucomatous rabbits. Thirty-two rabbits were used and distributed into four groups include: normal group, glaucoma induced group, sodium nitroprusside (SNP) group and timolol group. The treatment was given twice daily: at 9:00 a.m. and at 1:00 p.m. for 12 day. The intraocular pressure was recorded two times a day: at 9:00 a.m. and at 12:00 p.m. The intraocular pressure, nitrite level, endothelial nitric oxide synthase and the histology of ciliary body were measured. The results revealed a high significant decrease in intraocular pressure and no significant difference in nitrite level while a high significant elevation in endothelial nitric oxide synthase with improvement in the histology of ciliary bodies.

Key words: Glaucoma, intraocular pressure, Nitric oxide.

التأثيرات الموضوعية المحتملة لنتروبروسيد الصوديوم في علاج ضغط العين المرتفع المحفز في الأرانب

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الخلاصة:

الماء الأزرق في العين هو اعتلال عصبي بصري يُعرف بالتغيرات الهيكلية الانحلالية البطيئة التي تظهر في الخلايا العقدية الشبكية ورأس العصب البصري في العين. ان ارتفاع ضغط العين ينتج عن عدم التوازن بين كمية السائل المائي الذي تفرزه العين وبين قدرة الشبكة التريبينية على تصريفه. عرفت مانحات أكسيد النيتريك كطرق جديدة للتأثير على تطور الماء الأزرق في العين عن طريق تأثيرها في زيادة التدفق التقليدي للسائل المائي خلال الشبكة التريبينية وبالتالي خفض ضغط العين. ان الهدف من الدراسة هو تقييم التأثير المحتمل لمانح أكسيد النيتريك الموضوعي في الأرانب المصابة بالماء الأزرق. تم استخدام اثنين وثلاثين أرنب وتم توزيعها على أربع مجاميع و تشمل: ثمانية أرانب في المجموعة الطبيعية، وثمانية في المجموعة المستحثة لمرض الماء الأزرق، وثمانية في مجموعة النتروبروسيد و ثمانية في مجموعة

التيمولول. تم اعطاء العلاج مرتين يوميًا: الساعة 9:00 صباحًا والساعة 1:00 ظهرًا. خلال 12 يوم وُسجل ضغط العين مرتين في اليوم: الساعة 9:00 صباحًا والساعة 12:00 ظهرًا. تم قياس ضغط العين، مستوى النايتريت، انزيم أكسيد النيتريك البطاني ونسج الجسم الهدبي. اظهرت النتائج انخفاض كبير في ضغط العين وعدم وجود فرق معنوي في مستوى النايتريت في حين أن الارتفاع المعنوي العالي في انزيم أكسيد النيتريك البطاني مع تحسن في انسجة الأجسام الهدبية.

الكلمات المفتاحية: الماء الازرق ، ضغط العين المرتفع ،اكسيد النتريك

Introduction:

Glaucoma is defined as an optic neuropathy known by slow degenerative structural alterations seen in retinal ganglion cells (RGC) and the optic nerve head. Advanced neuronal cell loss leads to characteristic changes that are detected clinically and measured functionally and structurally [1-3]. Intraocular pressure elevation is a well-known risk factor for glaucoma. It is caused by an imbalance between the production of aqueous humor and the drainage of this liquid through the trabecular meshwork lining the anterior chamber angle [4, 5]. Outflow resistance in this region is lowered through the relaxation of contractile myofibroblast-like cells in trabecular meshwork and the adjacent scleral spur, or the narrowing of the ciliary muscle [6]. Sodium nitroprusside (SNP) is an inorganic compound with the formula $\text{Na}_2[\text{Fe}(\text{CN})_5\text{NO}]$, generally encountered as the dihydrate, $\text{Na}_2[\text{Fe}(\text{CN})_5\text{NO}] \cdot 2\text{H}_2\text{O}$. Own sodium salt is water-soluble comprised of Fe^{2+} that form complex with NO in addition with five cyanide anions. It acts in the body as prodrug, rejoining with sulfhydryl groups on erythrocytes, albumin, and additional proteins and discharge NO [7]. Nitric oxide (NO) is a vital signaling molecule responsible for different physiological functions in the body. It is formed by a family of enzymes, the nitric oxide synthases (NOS) through the conversion of L-Arginine to NO and L-citrulline [8]. Nitric oxide can diffuse into neighboring cells, like the vascular smooth muscle cells in the blood vessel and trabecular meshwork cells in the ocular tissue, where it linked to and stimulates the soluble guanylyl cyclase (sGC) enzyme. Once bound to sGC, it causes a conversion of

guanosine 5'-triphosphate (GTP) to 3'-5'-cyclic guanosine monophosphate (cGMP). Cyclic guanosine monophosphate then will go on to interact with different, protein kinases, cyclic-nucleotide gated channels and protein phosphodiesterases to yield physiological effects [9].

In the eye, nitric oxide plays an important role in physiological intraocular pressure IOP regulation. Its therapeutic effect depends strongly upon the position and concentration that transported [10]. Most drugs of glaucoma now unsuccessful to achieve sufficient IOP reduction, such because they do not target the conventional outflow pathway that controls IOP, and since endogenous NO plays a key role in conventional outflow pathway, exogenous NO donors may have therapeutic value as IOP lowering agents [11].

Materials and Method

Animals and Treatment

Thirty-two albino male rabbits were used in this approach weighing (2.5-3.5 kg). Rabbits were kept in the animal house of the National Center for Drug Control and Research, and before starting study protocol rabbits were maintained healthy for 3 weeks under consistent conditions (12 hrs light-dark cycles at room temperature) in the animal house which was provided with an air vacuum to be modified with the environment and were fed commercial pellets and water. All selected animals were examined before beginning of the study and were determined to be normal on ophthalmic and general examinations. Rabbits were divided in to four groups. Apparently normal group include eight rabbits were received distilled water as negative control, glaucoma induced group include eight

rabbit were received distilled water as positive control, timolol group include eight rabbits which received timolol eye drops (0.5%) after glaucoma induction ,and the fourth group include test group which include eight rabbits that received sodium nitroprusside SNP(0.08% w/v) after glaucoma induction. The treatment was applied two times a day: at 9:00 a.m. and at 1:00 p.m for twelve days. The IOP was measured every day (Day 1 to Day 12), the IOP was always recorded two times a day: at 9:00 a.m. before the first instillation and at 12:00 p.m. to avoid errors due to the diurnal pressure variations.

Chronic Ocularhypertension Model

The glaucoma model was produced by intravitreal injection of 50 unit of α -Chymotrypsin in 0.1 mL of sterile saline [12] by needle of gauge (31G, 0.23×9.5 mm) into the posterior chamber of the right eye only. The tip of the needle was swept across in the purpose of homogeneously distribute the enzyme into the posterior chamber, and the needle stayed in the posterior chamber for at least 1 min before being carefully removed to avoid any contact of the enzyme to the corneal endothelium [13]. After one-week IOP was measured by Shiotz Tonometer (5.5 g weight) then every week until IOP reach above 30 mmHg and induced acute glaucoma. Rabbits with increased IOP more than 30 mmHg were used to define the influence of drugs on IOP, while the rabbits with IOP lower than 30 mmHg were excluded from study [14].

Tested Drug Preparation

sodium nitroprusside (SNP) 0.08% w/v eye drops, were prepared by dissolving 0.08 grams mg of SNP powder and 1% (w /v) of benzalkonium chloride in 100 ml of isotonic buffer solution and the eye drops were freshly prepared each day, then bacteriological filter paper 0.45 μ m was used to filter the solutions to be ready for administration. The eye drops formulated

in sterile condition then packed into sterile containers [15].

Intraocular Pressure Measurement

Intraocular pressure was measured by Schiotz Tonometer. Calibration of the tonometer was made first by putting the footplate of the instrument on the curved metallic portion (the artificial cornea) provided with the storage box. Following calibration, the footplate was sterilized with ether alcohol, then the animals were put in supine position, and the cornea was anesthetized by local anesthetic (2% lidocaine hydrochloride). A small weight was applied to a central plunger, causing the part of the cornea beneath the plunger to displace inward [16]. Since the Schiotz Tonometer does not measure pressure directly, a conversion table supplied with the instrument is used to translate scale readings into estimates of IOP in mmHg [17].

Measurement of Nitrite (NO₂-) Concentration in Aqueous Humor

One drop of 2% lidocaine hydrochloride was applied as a local anesthetic in the right eyes only of all rabbits at the end of study and 200 μ l of aqueous humor were drawn from the anterior in the last day of treatment after anesthetized by ketamine and xylazine. Nitrite concentration was measured in rabbit's aqueous humor by spectrophotometric method using Griess reagent system. [18]

Measurement of Endothelial Nitric Oxide Synthase (eNOS) Level in Ciliary Body Tissue Homogenate:

All rabbits were sacrificed by ether inhalation, then the right eyes of rabbits were taken off, sectioned and ciliary body was isolated. The preparation of the ciliary body homogenate was done primarily by removing the extra blood from ciliary body and was weighed by sensitive balance before homogenization. Then the tissues had milled to small pieces and homogenized in small porcelain mortar with a certain amount of phosphate buffer

saline (PBS) (usually each 10mg of ciliary body tissue 0.1ml phosphate buffer saline). Later the homogenates were centrifuged at 5000 rpm for 15 minutes, then the supernatants taken carefully by micropipette and stored in sterile eppendorfs at (- 40°C). Enzyme linked immunosorbent assay (ELISA) kit was used for measuring eNOS in tissue homogenate [19].

Histopathological Examination of Ciliary Body Tissue

A. Ciliary body tissue preparation and staining

The traditional processing procedure by paraffin embedded method was followed, since its widely used and its reagent readily available [20]. Paraffin method steps according to Bancroft and Steven 2008 are:

1. Fixation of rabbit's eyes in formaldehyde (10%), to harden the tissue.
2. Sectioning (tissue dissection): Cross sectional cut were done; the ciliary bodies of the rabbits' eyes were placed in to the plastic tissue cassettes for each group and were labeled by pencil.
3. Dehydration and infiltration of the tissue by using histokinette Shandon device. Dehydration was done to remove any water from the tissue block. Dehydration is done gradually at several steps to prevent shrinkage of the tissue. Then infiltration process was done to support the tissue during sectioning step by filling the tissue with paraffin. The plastic cassette that contain the tissue were putting in histokinette device baskets, were they should be embedded in the following sequence: Distilled water for washing, 70% alcohol for 2 hrs, 80% alcohol for 2 hrs, 90% alcohol for 2 hrs, 95% alcohol for 2 hrs, 100% alcohol for 2hrs, Xylene for 1 hr, Xylene for 1 hour, Paraffin for 2 hrs, Paraffin for 2 hrs.
4. Embedding and solidification of the tissue in to hard paraffin cube, by placing the tissue in metal mold and putting label for each piece, then pouring melted paraffin over the tissue in each mold, any

excess wax was removed, then allow the paraffin to solidify at room temperature first and then in refrigerator.

5. Section the tissue using microtome.
6. Slide preparation: Labeled slides which were used, with a diamond pen for labeling.
7. Placing the sections on slides: This was done using water bath with clean water of 45 °C, then thin section of paraffin was taken after cutting by microtome and gently placed it on the warm water in the bath and observe when the wax is melted and the wrinkles disappeared, a clean glass slide was dipped in water under the section and then pulled the slide out so that the section is on slide. The slides were left to dry for about 24 hrs, then the slides were arranged in special baskets that fit in the clearing and staining procedures jars.
8. Slides clearing: The paraffin was removed prior to staining so that only the tissue remains adhered on the slide because if paraffin present, it would prevent the stain from reaching the tissue. This done by socking the baskets few times in xylene jar, then transferred in to oven which was preheated to 65°C for 15 minutes, after that the process mentioned above repeated more times.
9. Slide staining: Two different dyes (Eosin and Hematoxylin) for slides staining which allow clear observation to the histological features. Staining g involves dipping the slides in the following materials for certain times: Absolute alcohol for 2 min., 80% alcohol for 2 min., 70% alcohol for 2min., wash by tap water 2-3 times, Hematoxylin for 1min., wash by tap water 2-3 times, Eosin for 2 min., 95% alcohol for 1 min., Absolute alcohol for 2 min., Absolute alcohol for 2 min. , Xylene for 2min.,and Absolute alcohol for 2 min
10. Mounting permanent cover slide: After staining, few drops of DPX (a mixture of distyrene, a plasticizer, and Xylene) a synthetic resin used for histology were added. It dries quickly and preserves stain, after that cover the slide with slide cover.

B- Microscopically study

A digital microscope system with Leica DM4000 B LED was utilized to capture five regions of a slide (corners and the center) which randomly at x40 magnification power were selected [20].

Result

Intraocular pressure

The mean IOP of SNP 0.08% treated group at 9a.m. compared with the IOP at 12 p.m. of the same group (SNP group), and no significant difference ($P>0.05$) was

revealed along the study period except in the day1, there was a high significant difference ($P<0.01$) in IOP. When the IOP of SNP group at 9 a.m. and 12 p.m. were compared with IOP of normal group, the results showed no significant difference ($P>0.05$) between them, excluded from that, the IOP in day 1 and day 2, while there was a high significant decrease ($P<0.01$) in the IOP of SNP group at different time interval when compared with glaucoma group along the study days, as presented in figure (1).

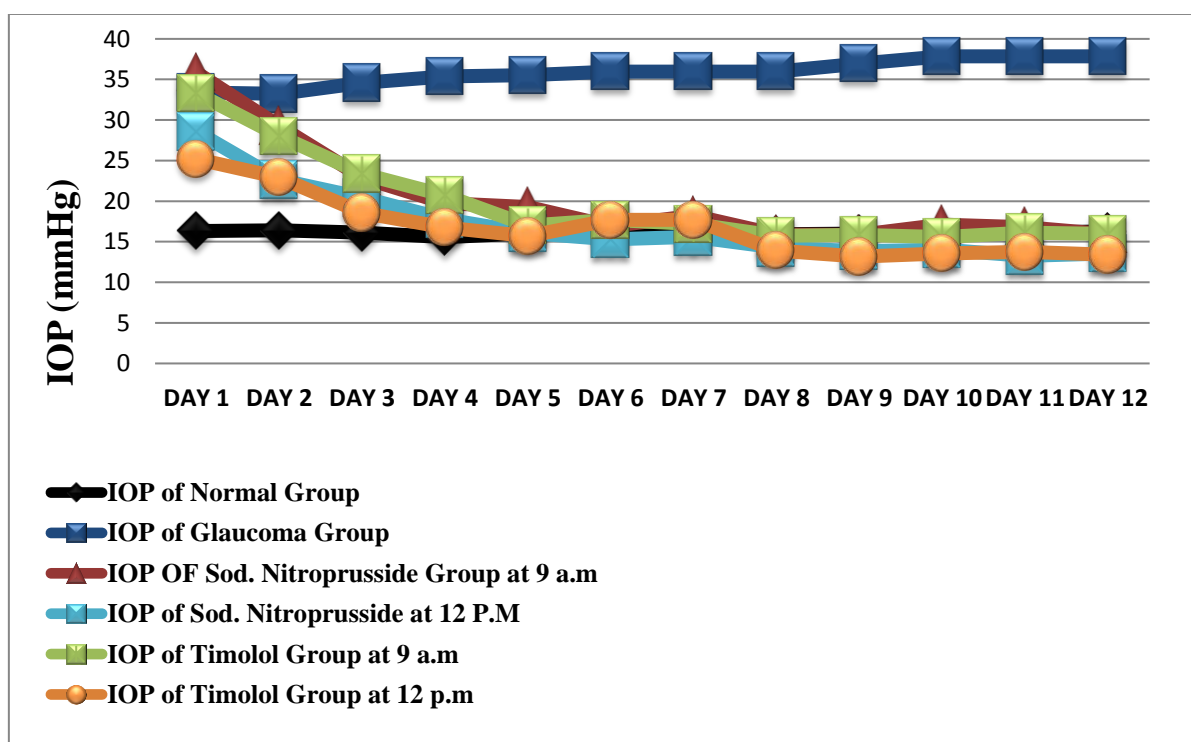


Figure (1): Mean Value of Intraocular Pressure of Sodium Nitroprusside Group at 9a.m and at 12p.m of Eye Drops Administration Compared to Normal, Glaucoma and Timolol Group. The Results Represented as mean± SEM

Meanwhile when the IOP of SNP group at 9 a.m. was compared with that of timolol group at 9 a.m., the results revealed no significant difference along the trial period. Furthermore, no significant change in the IOP of SNP 0.08% treated group at 12 p.m. when compared with IOP of timolol group at 12 p.m. except for day 2.

Nitrite Level

The statistical analysis revealed that there was no significant difference ($P>0.05$) in NO₂- level of SNP 0.08% treated group when compared with nitrite level of glaucoma group, timolol and normal group.

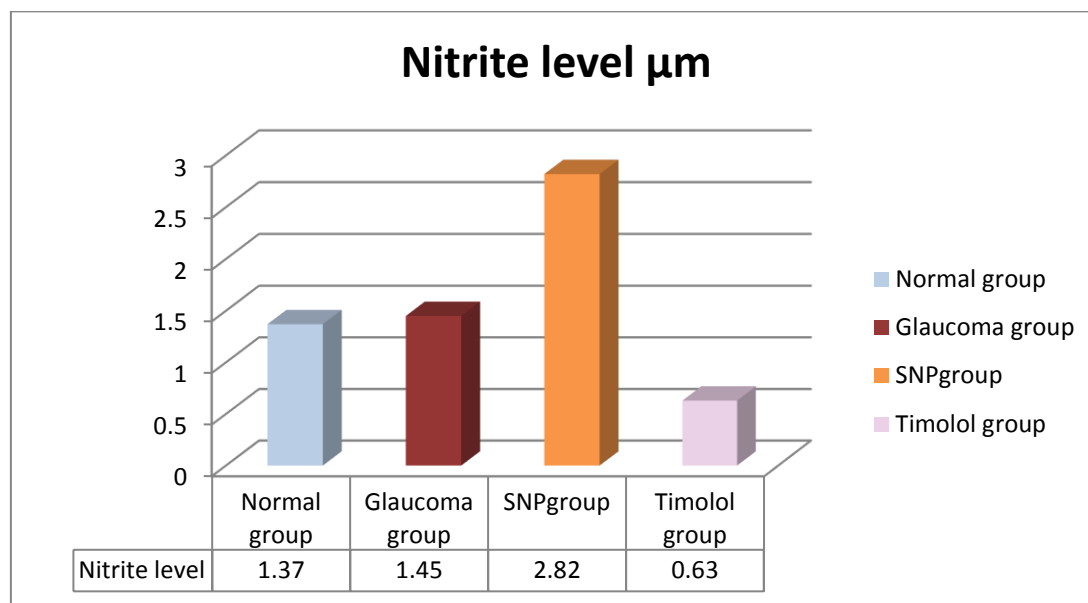


Figure (2): Bar Chart of the Mean Values of Nitrite Concentration (μm) in Aqueous Humor of the Study Groups. The Results Represented as Mean \pm SEM.

Endothelial Nitric Oxide Synthase Level in Ciliary Body Tissue:

The level of endothelial nitric oxide synthase eNOS in ciliary body tissue was measured when the study was finished, and the results were represented as mean \pm SEM. The results of current study revealed a high significant elevation ($P < 0.01$) in the eNOS concentration of SNP group when compared with glaucoma group (2.39 ± 0.05 vs. 1.97 ± 0.046 , respectively).

There was a high significant change between the concentration of eNOS in

ciliary body tissue of SNP group and in the tissue of normal group (2.39 ± 0.05 vs. 2.09 ± 0.06 , respectively; $P < 0.01$). Furthermore, a high significant elevation ($P < 0.01$) in the eNOS concentration of SNP group when compared with eNOS of timolol group (2.39 ± 0.05 vs. 2.103 ± 0.048 , respectively; $P < 0.01$) while there was no significant change ($P > 0.05$) in the eNOS concentration of in ciliary body tissue of glaucoma group when compared with normal group (1.97 ± 0.046 vs 2.09 ± 0.06 , respectively ($P < 0.01$) as revealed in the figure 3 .

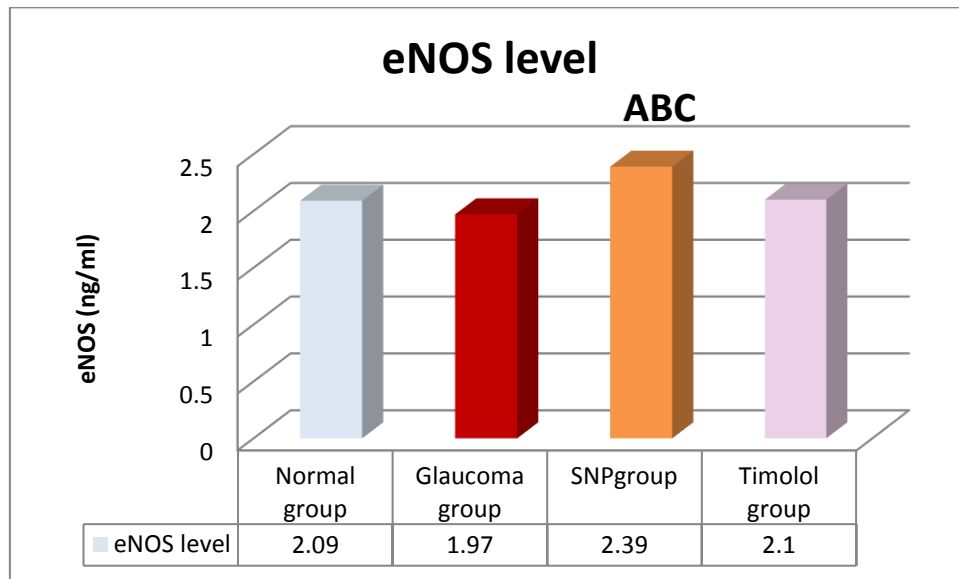


Figure (3): Bar Chart Represents the Mean Values of eNOS Concentration (ng/ml) in the Tissue of Different Groups. The Results represented as mean±SEM. Statistically high significant difference ($P<0.01$) when:

- A: Compared with normal group.**
- B: Compared with glaucoma group.**
- C: Compared with timolol group**

Histopathological Changes of Ciliary

Body:

Ciliary bodies examination by light microscopy presented a normal looking of ciliary body tissue with prominent brown pigmentation in the right rabbits' eyes of normal group as shown in figure (3-8 A, B). Meanwhile; a damage of epithelial cells, decrease in pigmentation and ciliary muscle thickening of right rabbits' eyes, were detected when ciliary body tissue of

glaucoma induced group was examined by light microscopy as shown in figure (3-8 C, D). Microscopic section of ciliary body tissue of rabbits' eyes of SNP treated group, revealed a nearly normal looking of ciliary body tissue with normal thickness and normal pigmentation as shown in figure (3-8 E, F). As well a nearly normal looking of ciliary body tissue was observed in the eyes of timolol treated group (figure 3-8 G, H).

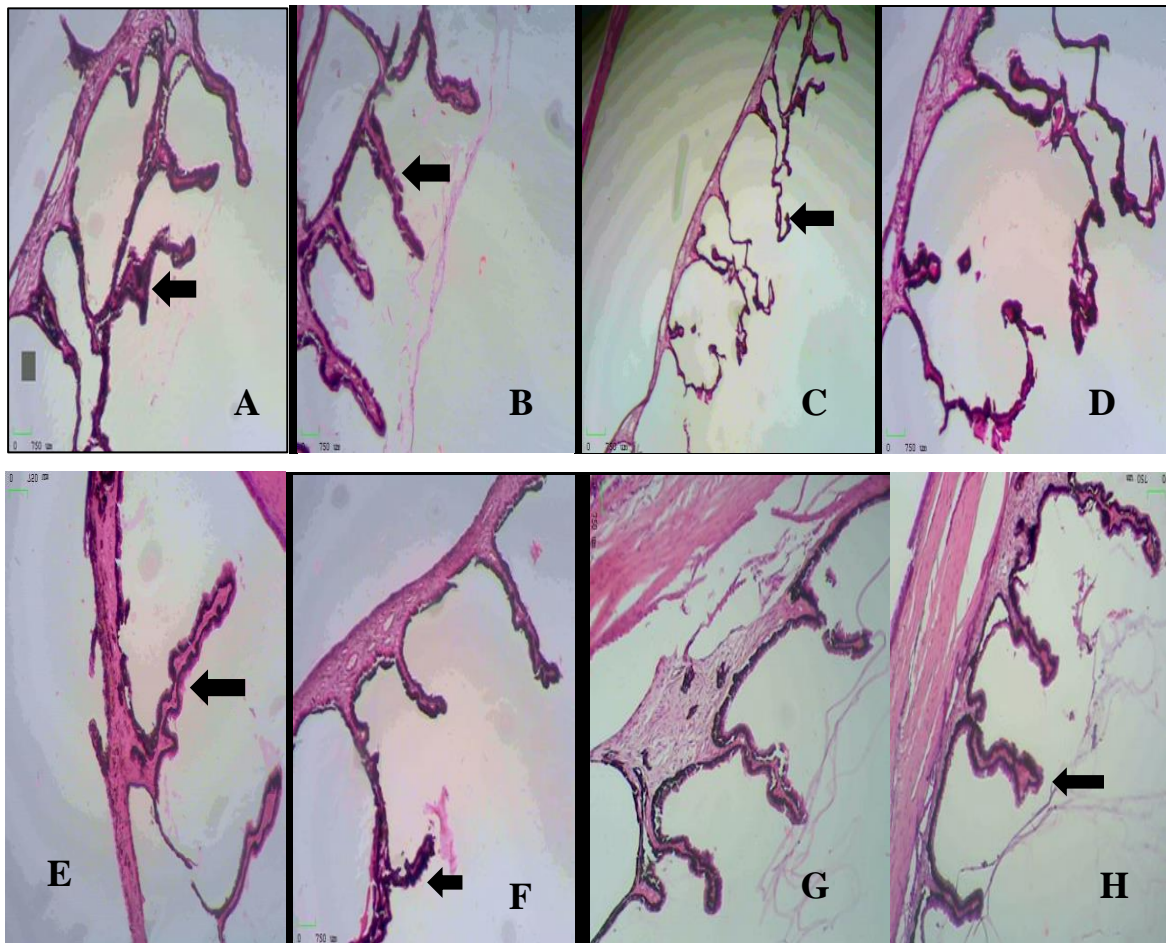


Figure 4: Microscopic Section of Rabbits Ciliarybody Tissue of H&E Stain (x40).

- (A, B) Isolated ciliary body of normal rabbit's eyes.
 (C, D) Isolated ciliary body of glaucomatous rabbit's eyes.
 (E, F) Isolated ciliary body of SNP treated eyes.
 (G, H) Isolated ciliary body of timolol treated eyes.

Discussion

The findings of the current study have revealed a high significant ($P < 0.01$) decrease in IOP of SNP (0.08%) treated group at 12 p.m. when compared with IOP at 9 a.m. in the first day of study, and a persistent decrease was continued daily with approximately 2-3 mmHg after three hours of the first instillation along the twelve days of treatment. In agreement with this study, Heyne et al. verified that multiple topical treatments of SNP significantly decreased IOP in normotensive primates from 2 to 6 hours with a maximal IOP reduction of 20% at 3 hours post dose [21]. Kotikoski et al. demonstrated that, the IOP lowering effect

of SNP, and other nitric oxide donor like, S-nitrosothiol, and spermine NONOate in normotensive rabbits after either topical or intravitreal dosing, reached a maximum at 2 to 5 hours [21]. Meanwhile; previous study revealed that administration of NO donors such as nitroglycerin, ISDN, sodium nitrite, and sodium nitroprusside (SNP), rapidly lowered IOP with a peak effect at 1 to 2 hours in a normotensive rabbit model [21]. In fact, the IOP reduction by sodium nitroprusside SNP (0.08%) may be explained by its role in the trabecular meshwork relaxation and perhaps an increase in the permeability of the schlemm's canal [23,24]. At that point

enhancement of aqueous humor out flow through the conventional pathway was predictable as a result from cGMP activation via exogenous NO that released from SNP in ocular tissues (where it serves as a marker for NO release). It is well known that, under physiological conditions AH outflow is primarily achieved through the trabecular meshwork and Schlemm's canal that accounts (60%–90% of outflow in humans and nonhuman primates), and a minor contribution is from the uveoscleral or nonconventional pathway [25]. While under pathological conditions of elevated IOP, the conventional pathway becomes the restrictive factor in AH outflow, with the uveoscleral pathway becoming a more significant contributor, this is due to increased stiffness in the TM and its extracellular matrix [26]. Trabecular meshwork cells are known to be highly contractile in nature, analogous to vascular smooth muscle cells (VSMC), in which the role of nitric oxide-cGMP signaling in endothelium dependent relaxation is well understood [27].

Nitric oxide can diffuse into VSMC in the blood vessel and TM cells in the eye, where it binds to and stimulates the sGC enzyme, resulting in increased production of cGMP and activation of protein kinase G (PKG) [21,22]. The activation of protein kinase G in turn, leads to inhibition of Rho A and thus Rho kinase, activation of K⁺ channels, inhibition of L-type Ca²⁺ channels, and increased uptake of calcium into the sarcoplasmic reticulum. These signaling pathways, together with the direct action of PKG, result in activation of myosin light chain phosphatase, while lower intracellular Ca²⁺ levels result in inhibition of myosin light chain kinase. Following dephosphorylation of the regulatory light chain of myosin, inhibits actin–myosin interaction, enhancing cell relaxation [22]. Ultimately, this leads to a widening of the intercellular spaces in the juxtacanalicular TM and schlemm's canal, thus facilitating conventional AH outflow [28, 29]. The nitrite represents the final

product of NO oxidation pathways. The statistical analysis in the present study have shown no significance difference ($P>0.05$) in nitrite concentration among the study groups, although the nitrite concentration tended to be higher in SNP group. This is in agreement with previous study in which SNP elevated the nitrite level in the ocular hypertensive rabbits [30], but also statistically no significant ($P>0.05$) and this may be related to sodium nitroprusside, which have a short half-life (1-2) minute [31], and the nitrite may be diffused systemically to the left eyes during 3 hours after the administration. The results of current study had shown a high significant ($P<0.01$) elevation in eNOS level in SNP group compared with glaucoma group. AMP stimulated protein kinase AMPK is a sensor of energy form in cells also a regulator of cellular homeostasis. The AMPK is activated in endothelial cells, through sheer stress or growth factors which aid in the discharge of nitric oxide [32]. The data of current study had presented no significant difference ($P>0.05$) in the eNOS concentration in glaucoma group compared with normal group and this may be related to the technique which had been used to measure eNOS level in tissue homogenate and due to samples size which consist of ciliary body tissue homogenate, it is small and sensitive tissue and may be not enough to produce more clear result.

Chen et al. demonstrated the association between nitric oxide and expression of AMPK in cells and concluded that, AMPK regulates NO production in cells and this relate to eNOS phosphorylation throughout AMPK at site ser-1177 [32]. Accordingly, a rise in activity of AMPK under several pathological and physiological cases, like a stimulation of vascular endothelial growth factor leading to elevate in synthesis of nitric oxide by eNOS [33,34].

The histology of ciliary bodies for all groups was examined by light microscopy and it was revealed that the ciliary body of right rabbit's eyes of SNP treated group

looked apparently normal. This may be explained by the fact that nitric oxide had anti-apoptotic activity.

A previous study presented that in endothelial cells, low concentrations of NO inhibit TNF α -induced apoptosis, while high concentrations of NO induce apoptosis [35]. The cGMP-dependent pathway has anti-apoptotic activity in endothelial cells and the NO anti apoptotic effect is partially mediated by this pathway [35]. While the histology of ciliary body of glaucoma group showed a loss of pigmentation and atrophy of ciliary processes. It was found that α -chymotrypsin causes a breakdown and destruction of zonular fibers, primarily close to the ciliary body, which then break loose and travel into the anterior chamber causing a temporary blockage of the filtering meshwork and subsequent glaucoma [36].

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