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Original Research Article

Caffeine Reduces Intraocular Pressure via Downregulation of NLRP3 Expression in Hyaluronic Acid/Hypertonic Saline-Induced Glaucoma

Adeshina O. Adekeye[1]; Adedamola A. Fafure[1]; Faith S. Mellah[1]; Mahmud K. Zubairu[1]; Chinonso A. Chukwu[1]; Favour C. Kelechi[3]; Lawrence D. Adebayo[3]; James O. Oni[2]; Damilare A. Adekomi[4]; Linus A. Enye[1]

[1] *Department of Human Anatomy, Faculty of Basic Medical Sciences, College of Medicine and Health Sciences, Afe Babalola University, Ado-Ekiti, Nigeria*
[2] *Department of Pharmacology, Faculty of Basic Medical Sciences, College of Medicine and Health Sciences, Afe Babalola University, Ado-Ekiti, Nigeria*
[3] *Department of Human Physiology, Neurophysiology Research Unit, Federal University, Wukari, Nigeria*
[4] *Department of Human Anatomy, Neuroscience Unit, Osun State University, Osogbo, Nigeria*

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ABSTRACT

Glaucoma is a leading cause of permanent vision loss worldwide. It results from continuous disruption to the sensory cells of the retina and optic nerve tissue. Caffeine, a stimulant of the central nervous system, is extensively ingested for its ability to do away with drowsiness and fatigue. The present study investigated the roles of caffeine as an antioxidant and anti-inflammatory agent in relation to elevated intraocular pressure (IOP) subsequent to the administration of hyaluronic acid (H.A.) and hypertonic saline (H.S.) injections. 30 adult Male Long-Evans rats were allocated into six (n=6) groups. Control rats received 50 µL of dH_20. H.A. rats received 0.025 mL of hyaluronic acid at the corneoscleral junction. H.S. rats received 0.05 mL of hypertonic saline into the episcleral vein. Post-hyaluronic acid (PHA) and post-hypertonic saline (PHS) rats were administered 20 mg/kg of caffeine intraperitoneally. 20 mg/kg of caffeine was given to the caffeine group. IOP were recorded before and after the experiment. Oxidative stress was assessed through malondialdehyde (MDA) and superoxide dismutase (SOD). An immunohistochemistry procedure was performed to evaluate NLRP3 activity in the retina. Our result showed that. H.A. and H.S. injections caused oxidative stress by upregulating and downregulating MDA and SOD levels, respectively. H.A. and H.S. injection significantly elevated the IOP, and NLRP3 activation was seen in the retina. However, caffeine mitigated this inflammatory response. In summary, our study shows that caffeine may be beneficial in managing ocular health associated with elevated IOP, by lowering IOP, and mitigating inflammatory and oxidative stress responses.

Keywords: Glaucoma, Hyaluronic acid, Hypertonic Saline, Inflammasome, Oxidative Stress, Caffeine

Introduction

Caffeine (1,3,7-trimethylxanthine) is a xanthine alkaloid that is obtained from various plant sources.[1] It contains minimal amounts of theophylline but deprived of theobromine.[2,3] In some part of Africa, South America, and East Asia, caffeine is usually present in the seeds of coffee and cocoa, nuts of kola, and in leaves of some unique plants. As a widely consumed natural chemical, caffeine is classified as a CNS stimulant within the methyl/xanthine group, known for its global psychoactive effects.[4] This stimulant, is naturally found in coffee, tea leaves, cacao beans, and kola nuts, which acts by blocking adenosine receptors, a neurotransmitter that promotes sleep and relaxation.[5] The adenosine A1 together with A2A receptors exhibit high level of adenosine affinity and are accountable for endogenous adenosine's tonic activities.[6,3,7] Glaucoma, characterized by elevated intraocular pressure, irreversible disruption to the cells of the retina and optic nerve tissue, can result in vision loss and is in-turn the chief cause of complete vision loss throughout the world.[8]

*****Corresponding author. E mail: adedamolaf@abuad.edu.ng
Tel: +2348069501996

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When compared to cataracts, it is considered the second leading cause of visual impairment.Globally, over sixty-seven (67) million individuals are affected by glaucoma, with projections indicating a prevalence of 111.8 million by 2040.[9,10] In the United States, approximately 2 million people, predominantly older individuals have been reported to suffer from glaucoma[10]. A presentation in Africa revealed that over 50% of glaucoma patients have already lost sight in one or both eyes.[11] In Nigeria, statistics shows that glaucoma accounts for more than 16% of visual impairment among those aged 40 years and more.[12] Additionally, in Ebonyi State, over 53% of glaucoma patients were reported to be already blind. Timely detection of glaucoma can prevent blindness and other adverse effects associated with the condition. The prompt identification and management of glaucoma can be effectively achieved through consistent ocular assessments.[13,14] The categories of glaucoma encompass closed-angle glaucoma, which exhibits a higher prevalence among females, normal-tension glaucoma, and the prevalence type, known as open-angle glaucoma.[9] The progression of open-angle glaucoma is insidious and asymptomatic over an extended period. Central and peripheral vision may gradually diminish, culminating in visual impairment if intervention is not pursued. Closed-angle glaucoma may manifest either insidiously or abruptly, presenting symptoms such as intense ocular discomfort, visual distortion, mid-dilated pupils, conjunctival hyperemia, and nausea. [15] The factors or threat that is associated with glaucoma disease may encompass elevated intraocular pressure, familial predisposition to glaucoma, and hypertension.[16,17] The normative intraocular pressure is

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usually between 18-21 mmHg (unit: millimeters of mercury) or 2.8 kPa (unit: Kilopascal), with elevated pressures correlating with an increased risk. [18] In the pathophysiology of glaucoma, retinal ganglion cells (RGCs), responsible for conveying ocular stimuli aiming at central nervous system, undergo degeneration; once damaged, these cells exhibit no regenerative capacity. [15] However, research have shown that a spike in intraocular pressure does not always refers to optic nerve damage.[8,13] The mode of action of open-angle glaucoma is believed to involve obstruction of aqueous humor flow through the trabecular meshwork, as oppose to closed-angle glaucoma where there is sudden block of the trabecular meshwork.[19] Research has suggested that injury or assault to the optic nerve activates the NLRP3 inflammasome (which is NOD-like receptors or NLRs) within microglial cells found in the retina. Among these groups of proteins (22 NLRs) in mouse, NLRP3 is most effective in forming the inflammatory protein platform and complex. Initial retinal damage has been shown to trigger pro-inflammatory responses in glial cells in case of danger. Hyaluronic acid (HA) constitutes a principal element of the extracellular matrix of the trabecular meshwork, which plays a critical role in the aqueous humor outflow resistance mechanism. Injection of hypertonic saline into the episcleral veins increases the resistance of aqueous outflow channels to raise the IOP, and excessive accumulation of hyaluronic acid may obstruct the physiological drainage of aqueous humor by diminishing the diameter of corneoscleral intertrabecular spaces and/or modulating the aqueous flow via the juxtacanalicular basement membrane.[20] In this study, we induced retinal injury through consecutive hyaluronic and hypertonic saline injections to optimize an elevated intraocular pressure model and to review the role of NLRP3 inflammasome antibody and antioxidant parameters in retinal ganglion cell death in ocular diseases.

Materials and Methods

Experimental Animals

Thirty Long-Evans rats weighing 150-200 g were obtained from Ekiti State University's Animal House in Ado-Ekiti, Nigeria. The rats were housed and acclimated for fourteen days at Afe Babalola University's Animal House Unit in Ado-Ekiti. They were fed ADEHEZ Rodent Feed® and provided water ad libitum. The rats were sporadically assigned to six groups (n = 6). The rats in the control group were given a single dose of double-distilled water (0.05 mL) into the episcleral vein. The rats in H.A group was given a one-time administration (0.025 mL) of hyaluronic acid solution directly in the corneoscleral junction. H.S. rats (hypertonic saline) were given a one-time administration of 0.05 mL hypertonic saline solution into the *venae episclerales* (episclera vein). The post-hyaluronic acid rats (PHA) were given 0.025 mL injection of hyaluronic acid intraperitoneally and subsequently, 20 mg/kg of caffeine. The post-hypertonic saline rats (PHS) received 0.05 mL injection of hypertonic saline intraperitoneally, then 20 mg/kg of caffeine. Caffeine rats (CAF) were given 20 mg/kg of caffeine intraperitoneally for 7 days. [21,15,22]

Ethical Consideration

The guidelines of Institutional Animal Care and Use Committee (IACUC) protocol, United States were put into consideration when handling the experimental animals. Also, the Animal Ethics Committee of Afe Babalola University approved and monitored this research with approval number AB/EC/20/02/89.

Intraocular Pressure

Before the surgery began, a local anesthetic was applied to both eyes topically, and after the surgery, an antibiotic was applied to prevent infection. With the aid of a glass micro-needle, 2 molars of hypertonic saline (116 g in 1000 mL of dH_20) and 5.27 M of hyaluronic acid were injected directly into the episcleral vein and corneoscleral junction, respectively. An eye ointment known as Betadrone-N was applied to the site of injection after the ocular surgery, and the animals were kept in a suitable environment until they were all responsive. A TVGD-02 Tonometer for animals was used to measure the intraocular pressure for the experimental animals. Baseline intraocular pressure was established before the administration begins (Day 0), and at day 3, the IOP was measured after inducing the animals with glaucoma through the injection of hypertonic saline and hyaluronic acid respectively. Lastly, the IOP was taken at day 8 after the whole administration. Ensuring the experimental rats were conscious, all the intraocular pressure measurements were taking and documented three times, the mean was then recorded as the measured intraocular pressure. [23,15] The conscious rats were restrained with gentle pressure on the shoulders and the top of the head to avoid movements and restlessness. Prior to this, the animals were accustomed acclimatized to the handler to reduce anxiety and stress in the rats**.**

Biochemical and Immunohistochemical Analysis

After euthanizing the rats, the whole eye tissue was harvested, homogenized, and then spun at 4000 rpm for 5 minutes. The supernatant from the homogenate was collected to assess different oxidative stress parameters, such as the activities of superoxide dismutase (SOD) and malondialdehyde (MDA). The rats for histological analysis were anesthetized with ketamine hydrochloride, followed by intracardiac perfusion fixation with phosphate-buffered solution and 4% paraformaldehyde. After removing the eye, it was fixed in Davidson fixative for four hours or not more than 24 hours in preparation for the immunohistochemistry procedure. The samples were processed histologically, dehydrated through descending grades of ethanol, cleared in two changes of xylene, infiltrated in molten paraffin wax, embedded, trimmed, sectioned at 5 µm, and mounted on a charged slide. The sections were immuno-stained with antibodies designed for mapping NLRP3 inflammasome (E-AB-93112: dilution 1:100, Elabscience, China). To ascertain diaminobenzidine visualization of inflammasome protein (NLRP3), the secondary antibody used was Poly-HRP Anti Mouse/Rabbit IgG Detection System (with DAB solution), also gotten from Elabscience (E-IR-R217).[22]

Cell Count and Staining Intensity Determination

An industrial microscope with a digital camera (OPTO-Edu), together with a computer set, as well as Image-J software (version 1.53) was utilized for systematic analysis of the photomicrographs. Image J software (version 1.53) was employed to identify and quantify NLRP3 inflammasome-positive cells. Within a circular view section, positive cells were enumerated in a specified square area. Analysis was conducted on five retinal sections (5-µm widths) per animal. To prevent double-counting, a grid of lines that is not destructive was implemented on the ImageJ software.*[15]*

Statistical analysis

The Intraocular pressure (IOP) was studied and analyzed using Two-way analysis of variance (ANOVA) as a statistical tool. Oxidative stress was evaluated using One-way analysis of variance, later by Tukey's post hoc test. The entire results are presented as mean ± SEM, with statistical significance set at $p<0.05$.

Results and Discussion

The study confirmed that 2M concentration injections of hyaluronic acid (HA) and hypertonic saline (HS) into episcleral veins led to significant intraocular pressure elevation and excessive eye inflammation in Long-Evans rats. Intraocular pressure, or the eye pressure, is maintained by the fluid (aqueous humor) present in the eye. The improper drainage or obstruction in the drainage of this fluid could lead to compression or pressure on the structures of the eye including the blood vessels, thereby obstructing the flow of nutrients to the eye.[18] Measurements of intraocular pressure were carried out in experimental animals before the induction of glaucoma to obtain baseline IOP, after glaucoma induction, and at the end of caffeine administration. IOP was seen to be elevated in HA (36.00±2.00 mm Hg) and HS (30.00±2.00 mm Hg) rats compared to the control group (17.00±2.00 mm Hg). Caffeine treatment (CAF) significantly lowered IOP in the PHS (26.00±2.00 mm Hg) rats more than PHA (34.00±2.00 mmHg) rats. CAF group shows a lower IOP (18.00±2.00 mm Hg) than the HA and HS groups. (*P<0.05; *P<0.01). (Figure 1). Caffeine effectively

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decreased intraocular pressure and revitalized retina ganglionic cells in the hypertonic saline paradigm. In line with prior studies by Fafure et al. and Blanco et al., rats injected with hypertonic saline showed higher IOP levels than control rats (Figure 1). Significant differences between control, HA, HS, and CAF groups were observed after eight days of administration. Chandrasekaran et al. reported that increased in IOP is a major threat or risk factor associated with open-angle glaucoma (OAG).[25] It was noticed from this study that 50 µL of hypertonic saline was able to effectively increase the intraocular pressure and cause more damage than the hyaluronic acid. This could be as a result of the ability of the saline solution to induce inflammatory response, and obstruct the outflow of the aqueous humor, by forming a debris that blocked the canal angle of schlemm's.[26]

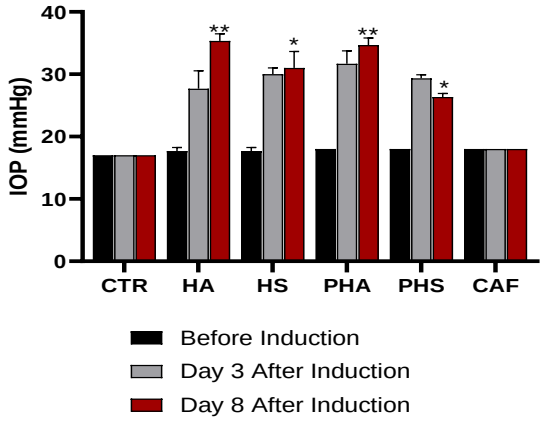

Figure 1: Intraocular pressure evaluation in the experimental rats administered with hyaluronic acid, hypertonic saline solution, and caffeine. Control (CTR); HA represents hyaluronic acid; HS represents hypertonic saline group; PHA represents post hyaluronic acid group; PHS represents post hypertonic saline group; while CAF stand for caffeine only (**P<0.05; P<0.01*). Legend: * = significant difference when compared to the baseline (CTR)

Redox imbalance or oxidative stress as a result of unstable molecules, contributed to the pathogenesis of several chronic conditions, including neurodegenerative, inflammatory, and retinal degenerative diseases.[27] In this study, we evaluated oxidative stress markers by examining superoxide dismutase (SOD) and malondialdehyde (MDA) levels in ocular homogenates of glaucomatous rats. Our findings, as depicted in Figure 2, revealed significant difference in MDA concentrations when comparing control rats to hyaluronic acid, hypertonic saline solution, post-treatment, and caffeine-treated rats (*P<0.05; *P<0.01).

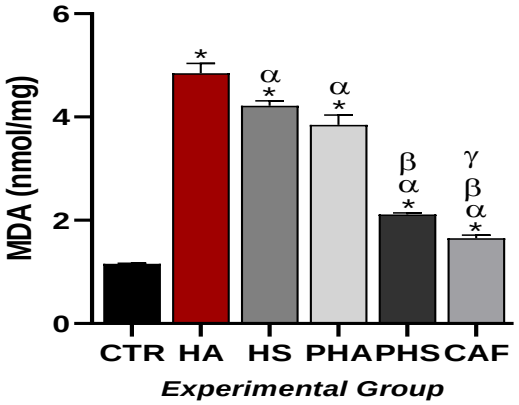

Figure 2: Bar chart showing malondialdehyde concentration (nmol/mg) in the entire ocular tissue. Control (CTR); HA represents hyaluronic acid; HS represents hypertonic saline group; PHA represents post hyaluronic acid group; PHS represents post hypertonic saline group; while CAF stand for caffeine only. MDA stands for malondialdehyde (**P<0.05; P<0.01*).

Rats treated with hyaluronic acid (HA) and hypertonic saline (HS) exhibited notably higher MDA levels than control rats. Post-hyaluronic acid (PHA) rats showed a marked decrease in MDA compared to HA only rats, while post-hypertonic saline (PHS) rats demonstrated a substantial decrease in MDA relative to HS rats. Caffeine-treated rats displayed elevated MDA levels compared to controls but significantly lower levels than other treatment groups. This research further emphasizes the importance of oxidative damage in experimental models of elevated intraocular pressure. Previous research by Ko et al., has also demonstrated that increased MDA levels in subjects with elevated IOP result from polyunsaturated fatty acids peroxidation, leading to the production of various aldehydes, including 4-HNE, which can induce neuronal apoptosis.[28] The high MDA concentrations observed underscore the significance of lipid peroxidation in higher intraocular pressure. Our results indicate that injection of both hyaluronic acid and hypertonic saline solution have a significant impact by increasing the MDA concentrations compared to the control rats (Figure 2).
Furthermore, the superoxide dismutase assay results showed significant differences in SOD levels between the control group and all treatment groups (*P<0.05). HA and HS rats exhibited significantly lower SOD levels compared to controls. PHA and PHS rats showed increased SOD levels relative to HA and HS rats. Caffeine-treated rats (PHA and PSA) demonstrated decreased SOD levels compared to controls but significantly higher levels than HA and HS rats. Figure 3 illustrates that caffeine treatment significantly increased SOD levels in PHA and PHS groups. These findings align with the observed decrease in serum SOD levels in glaucoma patients, potentially resulting from lipids and phospholipids which is an oxidized cellular constituent generated by free radicals. This can in-turn cause lipid peroxidation and there after lead to retinopathy and optic neuropathy.[27,29]

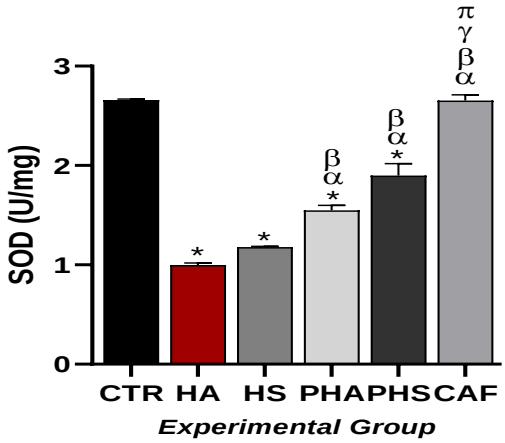

Figure 3: Bar chart showing superoxide dismutase concentration (U/mg) in the entire ocular tissue. Control (CTR); HA represents hyaluronic acid; HS represents hypertonic saline group; PHA represents post hyaluronic acid group; PHS represents post hypertonic saline group; while CAF stand for caffeine only. SOD stands for superoxide dismutase (**P<0.05; P<0.01*).

Neurodegenerative disorders are incurable conditions characterized by progressive neuronal loss. These disorders typically trigger neuroinflammatory responses in the microglial cells of the CNS.[30] Pyroptosis, is a form of cell death associated with inflammation, often initiated by intracellular pathogen infection.[31] It is considered a mechanism of retinal pigmented epithelium cell death. The basis of this inflammatory cell death involves the activation of an intracellular multi-protein complex known as the inflammasome. Studies have

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demonstrated that when the retina is disrupted, neuroglial cells often initiate pro-inflammatory responses to counter perceived threats. Our investigation revealed that hypertonic saline significantly enhances NLRP3 inflammasome expression in retinal ganglion cells (RGCs) compared to control and caffeine groups. While hyaluronic acid injection also notably activated the NLRP3 inflammasome in RGCs compared to the control, it appeared less harmful than hypertonic saline solution (*P<0.05; *P<0.01). Caffeine treatment in both hyaluronic acid (HA) and hypertonic saline (HS) groups showed a reduction in NLRP3 activation (Figure 4). These findings back up the idea that NLRP3 activation may be an early inflammatory initiator. [32] Our results also align with Roh *et al*.'s observations that increased intraocular pressure (IOP) caused by episcleral vein cauterization (EVC) resulted in optic nerve axon degeneration and eventual RGC loss[33].

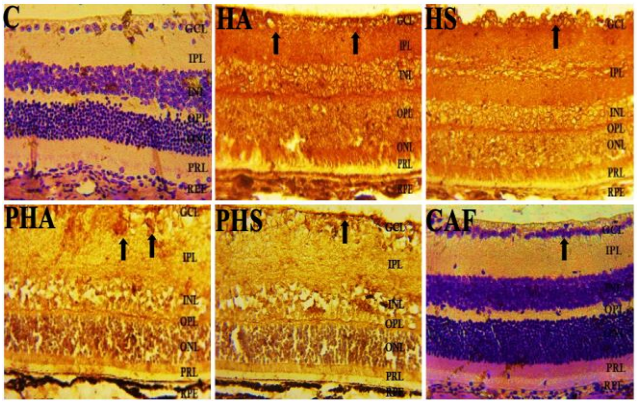

Figure 4: Immunohistochemistry results showing NLRP3 inflammasome activity in the retina of rats treated with hypertonic saline solution and hyaluronic acid as a model for glaucoma. Control (CTR); HA represents hyaluronic acid; HS represents hypertonic saline group; PHA represents post-hyaluronic acid group; PHS represents post-hypertonic saline group; while CAF stands for caffeine only. The expression of inflammasome-positive cells is indicated by a round brown precipitate and a black arrow (Mag. x800).

Conclusion

This study demonstrates that hyaluronic acid and hypertonic saline serve as effective glaucoma models, capable of significantly raising intraocular pressure, with hyaluronic acid acutely exacerbating this effect. The elevated IOP in both models in-turn leads to retinal oxidative imbalance and significant inflammasome activation in RGCs. Conversely, caffeine exhibited a mitigating effect on intraocular pressure and a restorative impact on the retina ganglionic cell layer. In conclusion, caffeine proved more effective in reducing IOP in the hypertonic saline paradigm of elevated IOP.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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