Caffeine Improves Elevated IOP by modulating Oxido-inflammatory responses in Rat Models of Glaucoma

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Abstract

Glaucoma, a neurodegenerative disease caused by continuous damage to the retina and optic nerve, is the leading cause of irreversible blindness globally. Caffeine, a central nervous system stimulant, is widely consumed for its psychoactive effects. This study aimed to determine caffeine's anti-oxidative and anti-inflammatory role on elevated intraocular pressure (IOP) following hyaluronic acid and hypertonic saline injections. Thirty (30) adult Long-Evans rats were distributed randomly into six (n=6) groups. The Control group received 50 µL of dH₂O. H.A. received 25 µL of hyaluronic acid into the corneosclera junction. H.S., 50 µL of hypertonic saline into the episcleral vein. PHA and PHS were treated with an intraperitoneal injection of 20mg/kg caffeine. Group CAF received an intraperitoneal injection of 20 mg/kg of caffeine. IOP measurement was taken, and markers of oxidative stress, malondialdehyde (MDA), superoxide dismutase (SOD) were assayed. The inflammasome immunoreactivity was evaluated. We observed severe inflammasome activation in the H.S. model of elevated IOP. H.A. and H.S. injections induced antioxidant imbalance by increased and decreased levels of MDA and SOD, respectively. In addition, an increase in the IOP and retina damage was observed following H.A. and H.S. injection, while caffeine demonstrated an ameliorative role in reviving the RGC damage. Caffeine demonstrated an ameliorative role in reducing the intraocular pressure and rejuvenating effect on the RGC, which was more evident in the hypertonic saline model of elevated IOP.

Introduction

Caffeine is present in coffee and found in tea. Coffee contains trace amounts of theophylline but no theobromine (Fredholm et al., 1999; Ferr'e et al., 2008). It can be found in the seeds and nuts or leaves of several plants in Africa, East Asia, and South America (Caballero et al., 2015). Caffeine is a natural chemical considered a central nervous system stimulant of methyl/xanthine class, which is widely consumed for its psychoactive effects globally. Caffeine usually acts by opposing the action of activated adenosine receptors (A.R.s). The adenosine A1 and A2A receptors have a high affinity for adenosine and are responsible for the tonic actions of endogenous adenosine (Fredholm et al., 1999; Daly et al., 2007; Ferr'e et al., 2008).

Glaucoma is the irreversible damage to the retina and optic nerve, which can lead to loss of vision, and it is the leading cause of irreversible blindness worldwide (Mantravadi et al., 2015). Glaucoma takes a lead as the second-leading cause of blindness after cataracts (Vadhar et al., 2015). Approximately six (6) to sixty-seven (67) million people have glaucoma globally, and its prevalence worldwide is expected to reach 80 million by 2020 (Mantravadi et al., 2015; Vadhar et al., 2015; Bogdanova, 2020). Quigley and Broman also suggest an increase in the burden of the disease worldwide between the years 2010 and 2020 (Bowman et al., 2006; Resnikoff et al., 2004). The condition affects about 2 million people in the United States and is predominant among older people. Altangerel et al., during a presentation in Africa, estimated that more than 50% of patients suffering from glaucoma are already blind in at least one eye. In Nigeria, glaucoma is responsible for 16% of blindness among people aged 40 years and above (Allison et al., 2020). It was also reported in Ebonyi State of Nigeria that more than 53% of patients living with
glaucoma were already blind. If detected timely, blindness and other adverse effects from glaucoma can be prevented. The early detection and treatment of glaucoma can be successfully attained by routine eye examinations (Altangerel et al., 2009; Ogbonnaya et al., 2012). The types of glaucoma include closed-angle glaucoma, which is more common in women, closed-angle glaucoma, and normal-tension glaucoma (Bogdanova, 2020). The development of open-angle glaucoma is slow and painless over time without pain (Ogbonnaya et al., 2012). The Central and peripheral vision may begin to wane, resulting in blindness if not treated. Closed-angle glaucoma occurs gradually or suddenly with symptoms like severe eye pain, blurred vision, mid-dilated pupil, redness of the eye, and nausea. In glaucoma, once vision loss occurs, it is permanent (Mantravadi et al., 2015; Vadhar et al., 2015). The risk factors of glaucoma may include increased intraocular pressure, a family history of glaucoma, and high blood pressure. The average eye pressures are about 21 mmHg (millimeters of mercury) or 2.8 kPa (Kilopascal), and higher pressures lead to greater risk (Ferri et al., 2010; Mantravadi et al., 2015; Vadhar et al., 2015). The retinal ganglion cells (RGCs), which transmit visual information to the brain, degenerate in glaucoma; once damaged, they do not regenerate (Fafure AA et al., 2021). However, in some cases, some may have elevated ocular pressure for years and never develop optic nerve damage (Mantravadi et al., 2015; Vadhar et al., 2015). The mechanism of primary open-angle glaucoma is believed to be the unhurried exit of aqueous humour through the trabecular meshwork, while the iris blocks the trabecular meshwork in closed-angle glaucoma (Mantravadi et al., 2015; Vadhar et al., 2015). Research has suggested that the retina's optic nerve injury or assault activated the NLRP3 inflammasome (NOD-like receptors) in retinal microglial cells. Among the 22 members of NOD-like receptors in mice, NLRP3 best forms the inflammatory multi-protein platform and complex. It has been reported that initial damage of the retina layers enhanced glial cells to activate pro-inflammatory responses in case of danger. In the present study, we induce retina damage by making sequential injections of hyaluronic acid, and hypertonic saline to better model elevated intraocular pressure and assess the role of the NLRP3 inflammasome and oxidative stress in RGC death in eye diseases.

Materials And Method

Experimental Animals

Thirty (30) animals (Long-Evans rats) with an average weight of 150 g were procured from the Animal House of Ekiti State University, Ado-Ekiti, Ekiti State, Nigeria. The animals were housed and acclimatized for two weeks in the Animal House Unit of Afe Babalola University, Ado-Ekiti, Ekiti State, and were fed with rodent meal (ADEHEZ Rodent Feed®, Ado-Ekiti, Nigeria) and access to drinking water were provided ad libitum. The rats were distributed randomly into six (n = 6) groups. The Control group received a single dose (50 µL) of double-distilled water orally. Group H.A. received a single dose (25 µL) of hyaluronic acid into the corneoscleral junction. Group H.S. (hypertonic saline) received a single dose (50 µL) of hypertonic saline into the episclera vein. Group PHA (post hyaluronic acid) received 25µL injection of hyaluronic acid followed by an intraperitoneal injection of 20 mg/kg of caffeine. Group PHS (post hypertonic saline) received 50µL injection of hypertonic saline, followed by an intraperitoneal injection of 20 mg/kg of...
caffeine. Group CAF (Caffeine) received an intraperitoneal injection of 20 mg/kg of caffeine (Jing et al., 2017; Fafure et al., 2021; Adekeye et al., 2021).

**Ethical Consideration**

All experimental animals were handled with care following the Institutional Animal Care and Use Committee (IACUC) protocol of the National Institute of Health, United States. This study was approved by the Animal Ethics Committee of Afe Babalola University (AB/EC/20/02/89).

**Intraocular Pressure**

The eyes were treated topically with local anesthesia prior to the surgery and an antibiotic after the surgery. Hypertonic saline (2 M) (116 g in 1L of water) and hyaluronic acid were injected into the episcleral vein (Gossman et al., 2016; Fafure AA et al., 2021) and corneosclera junction of the right eye through a glass micro-needle. After administration, Betadrone-N eye ointment was applied, and the rats were placed on a warm platform until they regained consciousness. Intraocular pressure (IOP) was measured using TVGD-02 Tonometer. Baseline IOP was obtained prior to the first hypertonic saline injection (Day 0) and after (Day 3 and day 8) administration of H.S. and H.A. The IOP of the animals was taken and recorded three times, after which the average mean served as the measured IOP. All IOP measurements were performed in conscious rats as described by Fafure AA et al (2021).

**Biochemical and Immunohistochemical Analysis**

Biochemical analyses were done to assay oxidative stress following Malohydehyde (MDA) activities and Superoxide Dismutase (SOD). The remaining eye from each group was harvested and post-fixed in Davidson fixative for immunohistochemistry studies. Sections of the eye (5 µm thick) were coronally cut on a microtome and immunoreacted with primary antibodies directed against the NLRP3 inflammasome. The eye sections were mounted on gelatin-coated slides, dehydrated and cover-slipped. For diaminobenzidine (DAB) visualization of NLRP3, the proper biotinylated secondary antibody (goat anti-mouse IgG for NLRP3 from Elabscience, Wuhan, China) (Adekeye et al., 2021).

**Cell Count And Staining Intensity Determination**

Photomicrographs were taken and analysed systematically using an OPTO-Edu industrial camera light microscope, a computer, and an image-processing and analysis software Image-J (Version 1.53). The NLRP3 inflammasome positive cells were ascertained and estimated with Image J software (version 1.53). In a section of the circular view, the number of positive cells was counted within a given square area. Five sections (5-µm widths) of the retina in each animal were analyzed. A non-destructive grid of lines was set to avoid counting the same cells twice (Fafure et al., 2021).

**Statistical analysis**
The Intraocular pressure (IOP) of the treated group animals was compared with those of the control using Tonopen and two-way analysis of variance (ANOVA). Oxidative stress was compared with those of control using One-way analysis of variance (ANOVA) and analysed with Newman-Keuls for post hoc. All results are presented as mean ± SEM and are considered statistically significant at p < 0.05.

Results

BIOCHEMICAL ASSAY

Malondialdehyde (Mda)

Figure 1 below showed a significant difference in MDA level when the control group was compared with hyaluronic acid, hypertonic saline, post-treatment, and caffeine-treated group (*p < 0.05). H.A. and H.S. groups show a significant increase in MDA levels compared to the control group. The post hyaluronic acid group shows a significant decrease in MDA level compared to the H.A. group. Post hypertonic saline (PHS) reveals a significant reduction in MDA level when compared to the H.S. group. At the same time, Caffeine (CAF) shows an increase in MDA level compared to control but a significant decrease compared to other treated groups (see Fig. 1).

Superoxide Dismutase

The figure below shows a significant difference in the SOD level of the control group compared with all the treated groups (*p < 0.05). Hyaluronic acid and hypertonic saline (H.S.) groups show a significant decrease in SOD level compared to control. Post hyaluronic acid (H.A.) and post hypertonic saline (PHS) groups reveal an increase in SOD levels compared to H.A. and H.S. groups. The caffeine (CAF) group shows a decrease in SOD level compared to control but a significant increase compared to the H.A. and H.S. groups (see Fig. 2).

IOP MEASUREMENT

Figure 3 below showed a statistically significant difference (*p < 0.05) in IOP level when comparing the control (C) group, hypertonic saline (H.S.) group, and post hypertonic saline (PHS)/intervention, and caffeine (CAF) treated group (see Fig. 3).

IMMUNOHISTOCHEMISTRY

Immunohistochemical studies on the retina and optic nerve sections evaluating the ameliorative role of caffeine following hyaluronic acid (H.A.) and hypertonic saline (H.S.) induced elevated intraocular pressure.
**Inflammasome (Nlrp3)**

Sequential injection of hypertonic saline and hyaluronic acid promotes NLRP3 inflammasome activation. 

i) Control group reveals no expression of NLRP3 inflammasome.  
ii) Injection of hyaluronic acid significantly increases the RGC NLRP3 inflammasome expression.  
iii) Injection of hypertonic saline significantly leads to an increase in NLRP3 inflammasome.  
iv) Treatment with caffeine in the H.A. group (ii) revealed little expression of NLRP3 inflammasome.  
v) Treatment with caffeine in the H.S. group (iii) shows a significant reduction in the expression of NLRP3 in the RGC layer.  
vi) Caffeine group revealed little expression of NLRP3 in the RGC layer than the control group (see Fig. 4).

**Discussion**

During the study, we confirmed that hyaluronic acid and hypertonic saline injections of 2M concentration injected into episcleral veins produced a marked elevation of intraocular pressure in Long-Evans rats, with excessive anterior segment inflammation in some. However, we also demonstrate that caffeine effectively reduced intraocular pressure and rejuvenated retina ganglionic cells in the hypertonic saline model of elevated IOP. In line with previous research executed by Fafure A.A et al. (2021), Blanco et al. (2019), elevated IOP was observed in rats injected with hypertonic saline compared to the control group, as seen in Fig. 3. The difference between the control, H.A., H.S., and CAF groups was highly significant after eight (8) days of H.A. and H.S. injection. CAF treatment significantly reduced IOP in the H.S. group than in the H.A. group. There was also a minute increase in IOP in the caffeine group compared with the control group but not statistically significantly. Elevated IOP is a significant threat or risk factor for glaucoma, and a significantly higher mean IOP has been reported in previous studies by Chandrasekaran et al. (2005).

Oxidative stress is a significant factor to be considered in the pathology of many chronic diseases such as neurodegeneration, inflammatory process, and retinal degenerative diseases (Ahmad & Ahsan, 2020). Markers of Oxidative stress were assessed by measuring Superoxide dismutase (SOD) and Malondialdehyde (MDA) concentration in the eye homogenate of the glaucomatous rat. This research again demonstrates the significance of oxidative stress in a model of experimental elevated intraocular pressure. As previously reported by Ko et al., 2005; Yücel et al., 2005, increased MDA levels of elevated IOP subjects were due to the peroxidation of polyunsaturated fatty acids resulting in the formation of multiple aldehydes, including 4-HNE, which is capable of inducing apoptosis in neuronal cells. The high concentrations of MDA validate the importance of lipid peroxidation in elevated IOP. The result from this study reveals that hyaluronic acid and hypertonic saline can significantly alter or increase the concentration of MDA compared to that of the control group (Fig. 1). Figure 2 revealed that caffeine treatment significantly reduced the level of SOD in the H.A. and H.S. groups. This finding agrees with a decrease in serum SOD level observed in increased IOP subjects (Aslan et al., 2008), which might be due to oxidized cellular components, such as lipids and phospholipids produced by free radicals, which can lead to lipid peroxidation and in turn trigger the onset of retinopathy and optic neuropathy (Ahmad & Ahsan, 2020).
Neurodegenerative disorders are incurable conditions or circumstances that lead to progressive degeneration or death of nerve cells. In this condition, neuroinflammatory responses are usually triggered in the central nervous system resident immune cells (Thomas et al., 2017). Pyroptosis or caspase 1-dependent apoptosis is an inflammatory form of cell death frequently triggered by infection with intracellular pathogens (Bedoui et al., 2020). It is regarded as one of the mechanisms of retina pigmented epithelium cell death (Suetomi et al., 2018). The foundation of this inflammatory cell death is enabling an intracellular multi-protein complex named the inflammasome. Research has shown that disruption of the neural retina often triggers neuroglial cells to activate pro-inflammatory responses against a threat. In this study, hypertonic saline significantly increases the expression of NLRP3 inflammasome in the RGC compared to the control and caffeine groups. Hyaluronic acid injection significantly activated the NLRP3 inflammasome in the RGC compared to the control but was less effective compared to the hypertonic saline. Treatment with caffeine in both the H.A. and H.S. groups revealed a reduction in the NLRP3 activation. Our results support the hypothesis that NLRP3 activation may be an early trigger of inflammation (Suetomi et al., 2018). These results also corroborate the findings of Roh et al. (2012), which stated that elevated IOP by episcleral vein cauterization (EVC) led to axon degeneration in the optic nerve and eventual loss of RGCs.

Conclusion

The present study has shown that hyaluronic acid and hypertonic saline are good glaucoma models with the capacity to significantly elevate intraocular pressure, which leads to an oxidative imbalance in the retina and robust inflammasome activation in the RGC. On the other hand, caffeine demonstrated an ameliorative role in reducing the intraocular pressure and rejuvenating effect on the RGC layer. Conclusively, caffeine was more effective in lowering IOP in the hypertonic saline model of elevated IOP.

Declarations

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Authors Contribution

Adedamola Adediran Fafure: Original draft, investigation, conceptualization, methodology. Adesina Oloruntoba Adekeye: Investigation, review, and editing. Faith Seember Mellah: Methodology, investigation, draft. Mahmud Kamaru Zubairu: Methodology, investigation. Linus Anderson Enye: Analysis, review, and editing. James Olukayode Oni: writing- review, and editing. All authors have thoroughly reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

Declaration of competing interest

The authors declare no competing interests.
References


Figure 1

Graph representing the concentration of MDA (nmol/mg) in the whole eye tissue. Control (C); hyaluronic acid (H.A.); hypertonic saline (H.S.); Post hyaluronic acid (PHA); Post hypertonic saline (PHS); Caffeine (CAF). Malondialdehyde (MDA) (*P<0.05)
Figure 2

Graph representing the concentration of SOD (U/mg) in the whole eye tissue. Control (C); hyaluronic acid (H.A.); hypertonic saline (H.S.); Post hyaluronic acid (PHA); Post hypertonic saline (PHS); Caffeine (CAF). Superoxide dismutase (SOD) (*P<0.05).
Measurements of IOP in animals with hyaluronic acid, hypertonic saline injection, and caffeine treatment. Elevated IOP was observed in H.A. and H.S. groups compared to the control group. CAF significantly reduced IOP in the H.A. and H.S. groups. CAF group shows a lower IOP than the H.A. and H.S. groups but slightly higher than the control group. Control (C); hyaluronic acid (HA); hypertonic saline (HS); Post hyaluronic acid; Post hypertonic saline (PHS); Caffeine (CAF); (*$P<0.05$: $P$ value=0.0001).
**Figure 4**

Photomicrographs showing immunoreactivity for the NLRP3 inflammasome in the retina of a glaucomatous rat. (i) Represents the control group; (ii) represents the hyaluronic acid group (H.A.); (iii) represents the hypertonic saline group; (iv) stands for the hyaluronic acid treated with caffeine (PHA); (v) represents hypertonic saline group treated with caffeine (PHS); (vi) represents the caffeine group; The round brown precipitates and black arrows indicate the expression of inflammasome positive cells. **Mag x800.**