

Ganglion cells apoptosis in diabetic rats as early prediction of glaucoma: a study of Brn3b gene expression and association with change of quantity of NO, caspase-3, NF-κB, and TNF-α

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Abstract

• **AIM:** To find a new concept to show whether or not apoptosis of retinal ganglion cells (RGCs) can be determined in the histology of acute hyperglycemia in the role of expressed Brn3b gene related to nitric oxide (NO), caspase-3, nuclear factor kappa-B (NF-κB), and tumor necrosis factor-α (TNF-α) as an early predictor of primary open angle glaucoma (POAG) eyes and their associations.

• **METHODS:** Experimental *in vivo* study was carried out using adult male, white Sprague-Dawley rats aged ≥2mo, weighing 150-200 g. The animals were divided into two groups, one group receiving intraperitoneal injection of streptozotocin 50 mg/kg in 0.01 mol/L citric buffer and pH 4.5 and a comparison made with the control group. Retinal tissue was divided into two parts (both experimental and control groups respectively): a) right retina for immunohistochemistry (IHC; caspase-3 and TNF-α); b) left retina was divided into two parts for the purpose of real-time polymerase chain reaction (PCR) test (RNA extraction for Brn3b gene expression analysis) and ELISA test (NO and NF-κB).

• **RESULTS:** The experimental group showed a decrease in Brn3b gene expression compared to the control group (1.3-fold lower in 2nd month; 1.1-fold lower in 4th month and 2.5-fold lower in 6th month). However, there was a decrease of NO, caspase-3, and an increase of NF-κB and TNF-α quantity.

• **CONCLUSION:** The expression of mRNA Brn3b gene is inversely proportional to apoptosis in RGCs. The quantity of NO, caspase-3, NF-κB and TNF-α is influential in expression of Brn3b in RGCs caused by hyperglycemia in diabetic rats.

• **KEYWORDS:** retinal ganglion cells; primary open angle glaucoma; Brn3b; apoptosis; nitric oxide; caspase-3; nuclear factor kappa-B; tumor necrosis factor-α

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INTRODUCTION

The worldwide prevalence of blindness due to glaucoma estimated at 60 million in 2010 and approximately 76 million individuals estimated as blind in 2020. In 2040, it is estimated that there will be 111.8 million individuals afflicted with blindness and 59 million individuals with open angle glaucoma^[1-2]. Women are under greater risk than men (3:1), and, in general have better life expectancy. In 1982, after cataracts, glaucoma was ranked as the third and in 1996 the second most prevalent cause of blindness in Indonesia^[3].

Glaucoma is a group of optic nerve diseases characterised by selective and progressive loss of retinal ganglion cells (RGCs). It is manifested clinically by thinning and loss of the neuroretinal rim and retinal nerve fibre layer, known as glaucomatous optic neuropathy, with corresponding visual field loss^[4]. Thinning retinal nerve fiber layer (RNFL) is shown by the diminishing of its presentation and texture attenuation due to disappearance of axon RGCs, along with supporting glia and its vascularization^[5].

Primary open angle glaucoma (POAG) with intraocular pressure (IOP) more than 21 mm Hg (normal range 10-21 mm Hg) is commonly found in the elderly population. The risk factors POAG include history of hereditary glaucoma, diabetes

mellitus, systemic hypertension, and long term usage of topical/systemic corticosteroid drugs, increment of IOP, high myopia, migraine and anemia. The exact pathogenesis that causes POAG is still unknown and despite normal range of IOP, progressivity of this disease remains persistent. The independent IOP factors related to hypoxia include, formation of reactive oxygen intermediates (ROIs) through growth factors pathways, genetic, or accompanied by underlying diseases, such as diabetic mellitus and blood circulation disorders^[6].

Hyperglycemia in diabetic retinopathy may lead to damage of the RNFL through four pathways. The first pathway, through the formation of ROIs and advanced glycation end products (AGEs) trigger vasoactive factor release, such as nitric oxide (NO), as nuclear factor kappa-B (NF- κ B), thus causing damage in the retinal cells and initiating apoptosis RGCs. The second pathway is the polyol pathway, which impairs the retinal vascular endothelium. The other two pathways activate signal transduction leading to growth and hexosamine biosynthetic factors. All these pathways induce circulatory problems, hypoxia, and inflammation in retinal vascular endothelium^[6-9].

Hyperglycemia creates excessive production of reactive oxygen species (ROS) and thus, causes increase in lipid DNA, and protein modification in various tissues. Molecular modifications within these tissues result in imbalance between protective antioxidants and the free radicals, which in turn, initiates oxidative stress. Oxidative stress activity in triggering of mitochondrial dysfunction. NO plays an important role in glaucoma and may, lead to RGC death by apoptosis. It has been reported that excessive NO is produced in the retina and optic disk in glaucoma eyes. Hypoxia may also leads to excessive angiogenic factor expression, that triggers neovascularization in impaired basal membrane, tight junction deficiency between endothelial cells, and lack of pericyte cells. In hyperglycemia, NF- κ B, as an important transcription factor in signaling neuronal program to code protein, will induce a pro-apoptosis program^[10-11]. Hyperglycemia eyes have been proven to have the structural RNFL defect due to apoptosis, thus result in POAG.

Brn3b gene expressed in the RGCs, or also known as POU3b gene, plays an important role in the retinogenesis process. The individual presenting with Brn3b gene may indeed not have the RNFL defect, and vice versa^[12]. Apoptosis is regulated by a protein, termed as caspase, which occurs through the extrinsic and intrinsic pathways. Caspase-3 is more prominent than other types of caspase and is related to apoptosis through the extrinsic and intrinsic pathways^[13]. The extrinsic pathway of apoptosis is initiated by protein-ligand binding between tumor necrosis factor- α (TNF- α) and Fas and their receptors. Ligations of receptor transmitting in the absence of Brn3b

gene is the key factor in the signal transduction pathway, leading to NF- κ B activation and cytoprotective gene inductions^[14].

Previous clinical studies reported a structural connection between RGC loss in rats with Brn3b gene disorder^[15]. This study stressed, particular, the thickness of RNFL, inner plexiform layer (IPL) and number of cells in RGC layers, either histologically or with immunofluorescence examination, and showed that the phenotype with Brn3b^{+/+} gene in IPL has thick RNFL, IPL and more cell numbers of RGCs than the phenotype with Brn3b^{-/-} gene. The spectral domain optical coherence tomography (SD-OCT) machine, specifically designed to examine small animals, was used *in vivo* for retinal imaging in rats. Qualitative difference of RNFL thickness and number of cells in Brn3b^{-/-} retina and other phenotypes were also measured by this machine, showing the IPL layer with Brn3b^{-/-} phenotype to be thinner, due to loss of dendrite projection on RGCs and RNFL. The result revealed that eyes with natural phenotype (Brn3b^{+/-} and Brn3b^{+/+}) have thicker RGCs compared to retinas without Brn3b (Brn3b^{-/-})^[15]. However, to date, no research has yet clarified whether or not hyperglycemia could in fact lead to apoptosis of RGCs, especially related to phenotype Brn3b gene and the quantity of NO, caspase-3, NF- κ B, and TNF- α .

This animal study aimed to find a new concept to demonstrate whether or not any changes in RGCs take place in the early process of acute hyperglycemia with focus on. The role of expressed Brn3b gene related to NO, caspase-3, NF- κ B, and TNF- α .

MATERIALS AND METHODS

Ethical Approval This research has been awarded ethical approval from Standing Committee of Ethical Health Medical Research of Universitas Indonesia (No.641/UN2.F1/ETIK/20). Animal care and treatment complied with the guidelines of animal research standard protocol of Indonesia.

Experimental Design This is an experimental designed study using adult male, white Sprague Dawley rats, weighing between 150 to 200 g and conducted in an animal laboratory at the Faculty of Medicine, Universitas Indonesia from November 2015 to July 2016. All rats were fed with standard laboratory rat chow and water ad libitum and kept in a temperature-controlled environment (20°C-22°C) alternating cycle of 12-hour light and dark.

A total of 24 Sprague Dawley rats were included in this study with 15 in experimental groups composed of 5 rats in each subgroup, while the remaining 9 rats were reserved for the control group with 3 rats within each subgroup. There was no intervention in the control group. In order to produce hyperglycemia in the experimental group, all rats were given an intraperitoneal injection of 50 mg/kg streptozotocin (STZ) in 0.01 mmol/L citric buffer. After 3d, fasting blood glucose was

measured. Hyperglycemia, defined as fasting blood glucose, was ≥ 16.7 mmol/L or ≥ 250 mg/dL. If hyperglycemia was not achieved within 3d, another intra-peritoneal injection STZ was given as booster. Glucose blood was measured each morning and, if fasting glucose blood was higher than 250 mg/dL, then a lantus (insulin-like) injection was given. Then animals were followed up in the 2nd, 4th, and 6th months in the subgroups.

In the 2nd months of follow-up (FU) subgroup, 3 rats from the control subgroup and 5 rats from the experimental subgroup were sacrificed and enucleated and the eye balls placed in 10% formalin buffer solution. Similar procedures were conducted in 3rd and 6th months FU in the sub-groups. Retinal tissue was divided into two parts (both experimental and control groups respectively): a) right retina for immunohistochemistry (IHC) to measure caspase-3 and TNF- α : b) left retina was divided into two parts for the purpose of real time reverse transcription polymerase chain reaction (RT-PCR) test to analyze RNA extraction for Brn3b gene expression and enzyme-linked immunosorbent assay (ELISA) test to measure NO and NF- κ B.

Immunohistochemistry Procedure of Caspase-3 and TNF- α Examination Using Labeled-Streptavidin Biotin Method IHC staining commenced with the processing of histopathological slides. After deparaffinization and rehydration, the specimen was rinsed with running tap water for 10min. It was later immersed into distilled water for 5min and then into 1 mL H202 10% with methanol as solvent for 10min. It was then rinsed with distilled water for 5min and dipped into PBS three times, each time for 5min. Thereafter, the specimen was submerged in Sniper background solution (Biocare Medical[®], USA) and incubated at a temperature of 37°C for 15min. It was then dipped again in PBS three times, each time for 5min, followed by immersion of specimen into trypsin solution with CaCl₂ 0.2% as solvent with 1:1 ratio for 60min. Later, caspase-3 and TNF- α detection was performed by applying anti-caspase-3: HPA002643 (Sigma-Aldrich, Germany) and anti-TNF- α : ab1793 (Abcam, Germany) with 1:100 concentration and PBS as solvent, incubating the specimen overnight at temperature of 4°C, and rinsing with PBS. Secondary antibody, labeled with biotin secondary antibody Trekkie Universal Link (Biocare Medical[®], USA), was applied onto the specimen, followed by incubation at temperature of 37°C and rinsing with PBS. Next, streptavidin peroxidase Trekkavidin (Biocare Medical[®], USA) was applied at temperature of 37°C and the specimen rinsed with PBS. Visualization of antigen on the tissue was performed using chromogen 3.3 Diaminobenzidin (DAB; Biocare Medical[®], USA) with addition of 4 μ L DAB in 1000 μ L substrate solution^[16]. Thereafter, the specimen was rinsed with distilled water and submerged into hematoxylin solution as counterstain,

the specimen protected with cover glass and adhesive agent, Entellan[®] (mounting). The specimen was then labeled and observed under light microscope with 40 \times objective lens magnification. Positive result (+) was determined with presence of intracytoplasmic dark brown material. Negative result (-) was determined with absence of intracytoplasmic dark brown material. Microscopic examination on IHC staining was performed through observing specimen using light microscope and photomicroscopy with camera-equipped light microscope Eclipse 80i, DS Fi1 (Nikon, Japan) to determine the location and distribution of caspase-3 and TNF- α . Specimen analysis with IHC staining was performed in a semi-quantitative manner with Image J software. Counting was carried out on 10 viewing fields of each specimen with 100 \times objective lens magnification.

Procedure of RNA Extraction and RT-PCR cDNA Amplification To prepare retinal tissue sample, a tissue sample of 15 mg was separated for RNA extraction and RLT Buffer added. Sample disruption and homogenization were carried out using ultrasonic disruptor-type UD201 (Tomy, Germany) for 20s. Sample lysate was centrifuged at 17 000 \times g for 3min. Supernatant was taken for RNA extraction process. Lysate and 70% ethanol of similar volume with lysate, were mixed and homogenized with micropipette. A sample of 700 μ L (precipitate included) was transferred into QIAamp spin column in 2 mL collection tube and, centrifuged at ≥ 8000 \times g for 1min. Supernatant was then removed from the sample. The sample was again transferred into 2 mL collection tube with QIAamp spin column. Repetition of the third step was performed until all lysate including the precipitate had been processed. Supernatant and 2 mL collection tube were removed. QIAamp spin column was transferred into new 2 mL collection tube. RW1 buffer of 700 μ L was added into QIAamp spin column and centrifuged ≥ 8000 \times g for 1min. Concentration and purity of the RNA sample was evaluated using nanodrop 2000. Later, cDNA amplification was performed using real time Mx-3000 p (Agilent, Germany) with KAPA[®]FAST One Step qPCR Kit Universal-KAPPA Biosystem (Sigma, Germany). The outcome of Brn3b gene expression was valued and its fold calculation conducted by dividing controlled expression by average experimental expression.

Procedure of NF- κ B Examination Using ELISA Method Left retinal sample, weighing 15-18 mg, was homogenized, frozen at -80°C, and left outside for a few minutes to defrost. Later, the sample was weighed and stored in 1.5 mL Eppendorf tube. The sample was then rinsed in ice cold water with PBS (0.02 mol/L, pH 7-7.2), homogenized by 500 μ L adding cold PBS (4°C-8°C) using ultrasonic disruptor. Homogenate was centrifuged at 1500 \times g (5000 rpm) for 15min at 4°C. Supernatant was extracted and then examined as sample. For

the next step, the microplate, an antibody-coated well, was prepared. The microplate used was coated with NF- κ B antibody. Supernatant of 100 μ L was added to each calibrator. In a concentration that was in accordance with the concentration of the given standard calibrator. The remaining well was used for sample and quality control (QC). Balance solution of 10 μ L was then added to sampel and QC well, 50 μ L conjugate was then added to all wells, apart from the blank well. All wells were later covered with adhesive paper and, incubated at a temperature of 37°C for 1h. All solutions in the wells were removed and rinsed 5 times with wash solution. Substrate A and substrate B were added to all wells with adhesive paper, wrapped with alumunium foil to avoid light, and incubated for 15min at 37°C. Stop solution 50 μ L was added to all wells. Finally, optical density (OD) was read using microplate reader (spectrophotometer) at wavelength of 450 nm.

Procedure of NO Examination Using Chemical Reaction

Method Left retinal sample, weighing 15-18 mg, was homogenized, frozen at -80°C, and left outside for a few minutes to defrost. Later, the sample was weighed in 1.5 mL Eppendorf tube. The sample was then rinsed in ice-cold water with PBS (0.02 mol/L, pH 7-7.2) and homogenized using ultrasonic disruptor. Homogenate was centrifuged at 1500 \times g (5000 rpm) for 15min at 4°C.

Reagents were prepared as follows: R1=100 mL (solution), stored at temperature of 4°C, R2=100 mL (solution), stored at temperature of 4°C, R3=powder, dissolved in 50 mL distilled water in bottle with shading light attribute, stored at temperature of 4°C, and R5=12 mL solution, stored at room temperature. Chromogenic reagents (CA solution) were prepared as follows: R3 (V): R4 (V)=2.5:1:1 stored at temperature of 4°C, and if the solution changed into a darker shade, it was not discarded. To prepare standard solution, sodium nitrate solution (2 mmol/L) was diluted 100 times with distilled water (1 mL sodium nitrate solution +99 mL distilled water) into 20 μ mol/L and stored at 4°C. To process these solutions, three different tubes were prepared: A) blank tube; B) standard tube; C) sample tube. Distilled water (200 μ L) was added into tube A. Sodium nitrate solution (20 μ mol/L; 200 μ L) was added to tube B. Sample (200 μ L) was added tube C. R1 (800 μ L) was later added to all tubes. Next, R2 (400 μ L) was added to all tubes and mixed thoroughly. The tubes were later incubated for 10min at 37°C and centrifuged at 3700 rpm for 10min. Supernatant up to 800 μ L from each tube was extracted. Thereafter, chromogenic reagent (400 μ L) was added to all tubes, mixed thoroughly, and incubated for 15min at 37°C. OD was read with 0.5 cm diameter cuvette using spectrophotometer multiscan at wavelength of 550 nm. Protein concentration was then read using spectrophotometer nanodrop 2000.

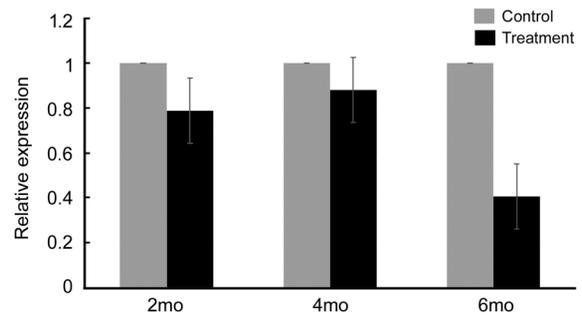


Figure 1 Brn3b gene expression relative ratio Method of Livak.

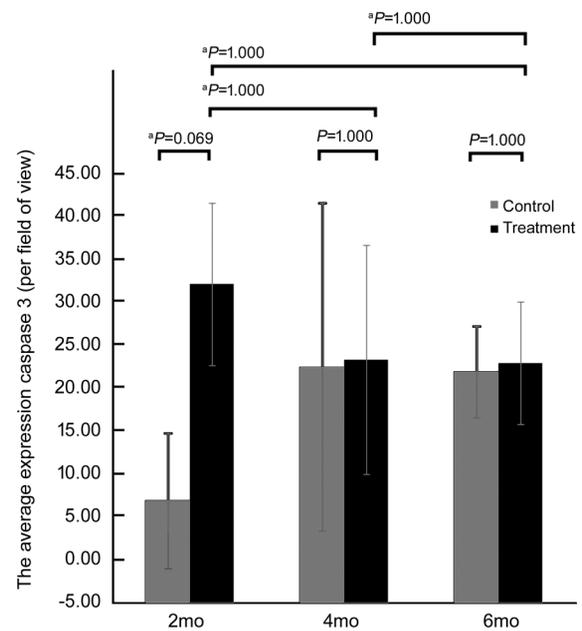


Figure 2 Caspase-3 comparison among 2nd, 4th, and 6th month groups *Post hoc Bonferroni from one way ANOVA test.

Statistical Analysis Statistical analysis was analyzed using SPSS version 20.00 software. One way ANOVA/Welch with post hoc Bonferroni was used according to the distribution of data. $P < 0.05$ with 95% confidence interval was used to determine the statistical significance of differences.

RESULTS

Brn3b Gene Expression Relative Ratio Calculation Graph

Figure 1 reveal the comparison of thr result of Brn3b gene expression relative ratio calculation between the experimental group and the control groups. Following normalization by gen Hprt-1 (reference gene), a decrease was determined in Brn3b gene expression in the experimental group when compared to the control groups. The decrease was reported as 1.3 times in 2mo, 1.1 times in 4mo and 2.5 times in 6mo. Fold calculation was carried out by dividing the sum total of expression in the control groups with the mean sum total of expression in the experimental group.

Comparison of the mean quantity of caspase-3 and TNF- α expression among the groups be seen in Figures 2 and 3. There is no significant difference in the mean sum total of caspase-3 level among the 2nd, 4th and 6th months of FU within

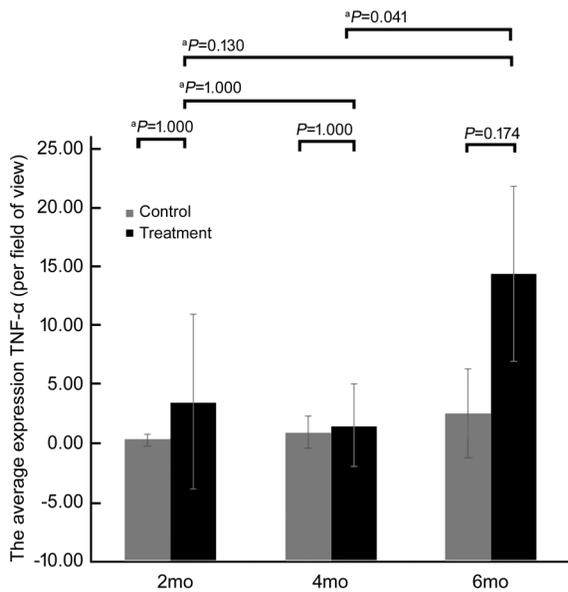


Figure 3 TNF-α expression comparison among 2nd, 4th, and 6th month groups ^aPost hoc Bonferroni from one way ANOVA test.

the experiment group ($P=0.109$). The level of TNF-α, shows there are at least two groups with significant differences. Post hoc Bonferroni test showed a significant differences in the mean quantity of TNF-α level between the 4th and 6th month experimental groups ($P=0.041$). The comparison quantity among NO and NF-κB levels can be seen in Figures 4 and 5. It observed that, there is a significant difference in the mean of NO level among the groups ($P=0.002$). Further post hoc Games Howel test showed a significant difference in the mean quantity of NO level between the 2nd and 4th month control groups ($P=0.037$), however, there was no significant differences in the mean of NF-κB level among 2nd, 4th and 6th month groups ($P=0.194$). Correlation analysis revealed that Brn3b gene expression was correlated with caspase-3 expression ($r=0.439$, $P=0.116$), level of NO ($r=0.235$, $P=0.418$), and TNF-α ($r=0.38$, $P=0.181$). More importantly, Brn3b gene expression was significantly correlated with the level of NF-κB ($r=0.565$, $P=0.035$; Figure 6).

DISCUSSION

This study demonstrated an increased glucose level in hyperglycemia rats with a decrease in the relative ratio value in Brn3b gene expression when compared to the control groups. This finding indicates that no increment of Brn3b gene occurs in RGCs due prolonged hyperglycemia stress, *i.e.* apoptosis low expression of Brn3b gene might lead to apoptosis in RGCs in hyperglycemia eyes. The role of Brn3b gene was established as a key factor in maintaining the existence of RGCs structure^[15]. In connection with retinogenesis, the function of Brn3b gene plays a critical role as a precursor in the development of RGCs populations^[17]. However, molecular mechanism of Brn3b gene activation in RGCs

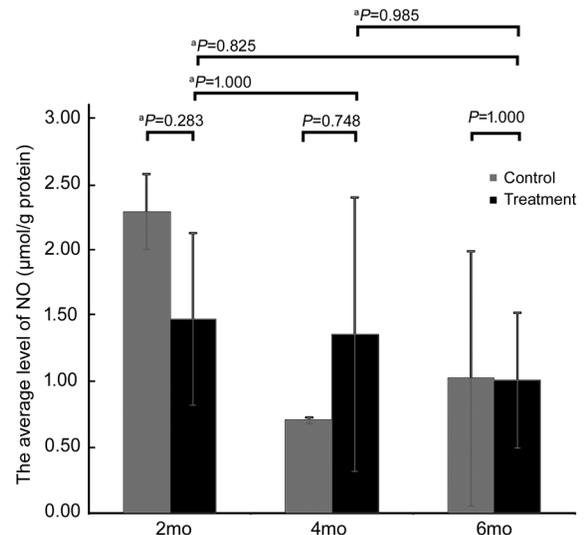


Figure 4 NO level comparison among 2nd, 4th, and 6th month groups ^aPost hoc Games Howell from one way ANOVA Welch test.

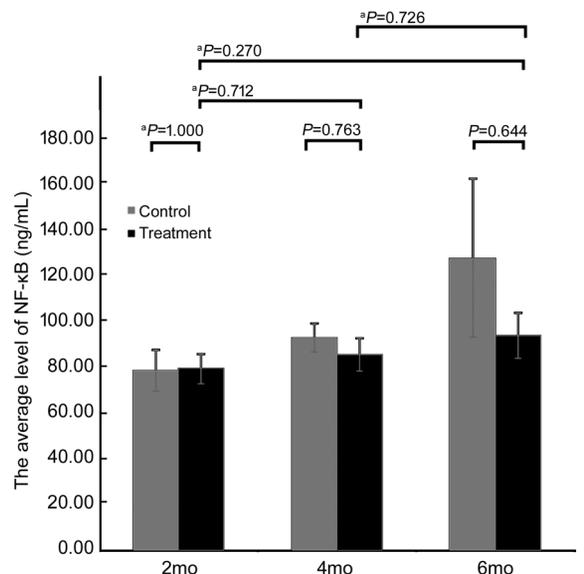


Figure 5 NF-κB level comparison among 2nd, 4th, and 6th month groups ^aPost hoc Bonferroni from one way ANOVA test.

is yet un-known. Other studies have suggested that there is another protein molecule in-volved, which activates Brn3b gene in the cell nucleus^[18]. In patients with a hyperglycemia condition, Brn3b gene expression was seen to play a role in insulin receptor inhibition by reducing caspase-3 and TNF-α, including triggering of NF-κB and NO signaling, thus resulting in protection against apoptosis in RGCs. Hence, it could be suggested that Brn3b is a cytoprotective gene, offering a further research target in the development of a therapeutic strategy in the management of early glaucoma patients, especially POAG, frequently diagnosed in diabetic patients. This study also statistically comparable results when observing the mean quantity of caspase-3 expression among the subgroups. However, but in the hyperglycemia group, caspase-3 expression showed a significant rise in the 2nd month, but then gradually dropped in the other subsequent subgroups. Caspase-3 is a

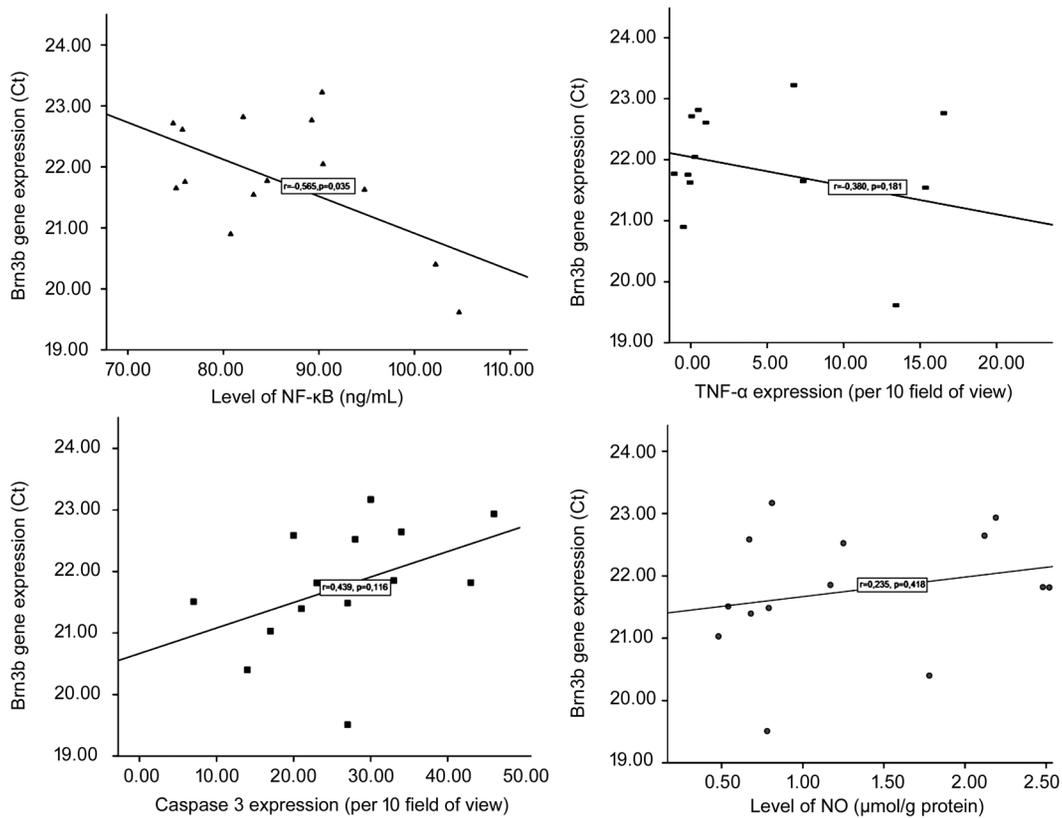


Figure 6 Correlation of NO, caspase-3, NF-κB, and TNF-α with expression of Brn3b in treatment 2nd, 4th, and 6th month groups.

protein plays an important role in the pro-apoptosis cascade in RGCs cycle through the extrinsic and intrinsic pathways. In theory, the amount of caspase-3 should increase in order to signal the proapoptosis process. However, Kowluru and Koppolu^[19] described that an absence of caspase-3 activation in RGCs up to 6 to 8mo might lead to apoptosis of capillary cells the diabetic rat. This condition occurs due to the primary antibody in the cytoplasm before lysis of the cell, where by caspase-3 remains in the cell nucleus. The cell nucleus then begins to degenerate until caspase-3 expression significantly drops. This study might explain why caspase-3 expression at 4th and 6th months was low in hyperglycemia rats and initiated apoptosis of RGCs.

This study also noted an increase in TNF-α in hyperglycemia eyes which mostly occurred in case of cell deterioration. Caspase-3 is one of the pro-apoptosis proteins that plays a role as main executor in the RGCs apoptosis process, as described. Thus, the less caspase-3 expressed in RGCs nuclei, the more protected RGCs are against apoptosis through Brn3b gene expression. In the study carried out by Luo *et al*^[20] demonstrated active caspase-3 was translocated from the cytoplasm into the nucleus cell through simple diffusion, following leakage formed in cytoplasm-cell nucleus barrier. However, it is still unknown how the molecular mechanism to activate caspase-3 enters into the nucleus cell. It has been suggested that another protein molecule is involved in

caspase-3 activation within the cell nucleus. Other studies have proposed that active caspase-3 enters the cell nucleus with the help of a substrate-like carrier protein, such as a-kinase anchoring protein 95 (AKAP95), the most probable protein carrier^[21]. AKAP95 is a protein in the cell nucleus, which has pseudo caspase-3. It is recognized by and binds with active caspase-3. This shows that active caspase-3 is brought into the nucleus cell through identification motive of pseudo caspase-3 from protein AKAP95. Apoptosis detection by assessing caspase-3 in the cell nucleus would be more specific with TUNEL assay^[22].

An interesting result observed in this study was the high TNF-α level after prolonged hyperglycemia eyes when compared to the control eyes. When NF-κB translocated into the cell nucleus, two events take place: 1) after NF-κB translocation, degradation occurs and thus the subsequent process ceases. Hence, ELISA antibody becomes undetected; 2) after NF-κB translocation, NF-κB is synthesized into TNF-α. Furthermore, in the case that the TNF-α expression is accumulated in hyperglycemia eyes. This would appear to result in NF-κB degradation.

Ideally, TNF-α should increase gradually during hyperglycemic conditions in RGCs. As a proinflammation cytokine, TNF-α causes neuronal cell death in RGCs, which triggers apoptosis signaling. This elevates pro-apoptosis proteins in order to activate caspase down-stream^[23].

NO is an instable neurotransmitter that acts as an activator of neurotoxic substances, which triggers the extrinsic signaling pathway of apoptosis^[24]. These substances can move through plasmic membrane or initiate apoptosis signal transduction. Considering the instability of NO level in hyperglycemia and control eyes, similar results were found between the groups in this study. However, this study showed when Brn3b gene is lower, the level of NO increases, whereas high NO will lead to damage of RGCs structure.

In accordance with Jousseaume *et al's* study^[25], who confirmed that NF-κB activation in the retina can be observed clearly in the early stages of diabetic retinopathy. The NF-κB level up to the 6th month experimental groups in this study could indeed represent the early stages of diabetic retinopathy with a tendency to increase. Even though its level is lower than in the control groups, this at least gives us a hint that there is an initial Brn3b gene repression. Nonetheless, Brn3b gene repression is unable to control an increase in TNF-α until apoptosis occurs. NF-κB is a protein that serves as a trigger of transcription factor or as a promoter induction, in particular, the genes involved in apoptosis. Naturally, NF-κB expression modulates proapoptosis through activated proinflammation (TNF-α) and increases free radical production (NO, super oxide)^[26-27]. In the case that the blood sugar level is high, activation of NF-κB triggers the promoter apoptosis in the endothel cell and retinal pericyte^[28]. Further studies on the role of Brn3b protein in the molecular mechanism of the formation and function of RGCs are needed for clarification. When Brn3b gene expression is repressed, NF-κB will be activated to initiate the apoptosis cycle, as shown in this study; additionally a negative correlation between Brn3b gene expression with NF-κB and TNF-α and positive correlation with NO and caspase-3.

The limitation of this study is a relatively small number of samples. Therefore, further research with larger number of samples is deemed necessary, but this study is able to explain the repression of Brn3b gen in hyperglycemia eyes and its association with pro-inflammatory molecules.

In conclusion, it can be concluded that the lower the expression of mRNA Brn3b gene, the higher the RGCs caused by hyperglycemia in diabetic rats. This study also demonstrated that apoptosis occurs even though, NO and caspase-3 cannot be classified as influential when compared to NF-κB and TNF-α in RGCs.

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