TITLE PAGE

Title:
EFFECT OF RETINOL AND α-TOCOPHEROL SUPPLEMENTATION ON RETINAL PHOTORECEPTOR AND GANGLION CELL DENSITIES, INCLUDING THE EXPRESSION OF CASPASE-3 AND CASPASE-7 IN DIABETIC RATS MODEL

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COVER LETTER

Date: 15th April 2022

To
The Editor,
International Journal of Retina and Vitreous

I am enclosing herewith a **revised manuscript** entitled:

EFFECT OF RETINOL AND α-TOCOPHEROL SUPPLEMENTATION ON PHOTORCEPTOR AND RETINAL GANGLION CELL APOPTOSIS IN DIABETIC RATS MODEL

The aim of this paper is to examine the effect of retinol and α-tocopherol compounds on photoreceptor and ganglion cell density, as well as the caspase-3 and -7 expression (apoptotic marker) in the retinal layers of the diabetic rat model. The advantage of this manuscript is it has some informative data regarding biocompound effect on ocular disease especially on the microvascular complication of diabetes. We hope that these results also meet the paper scope that required in this journal and it could be published and disseminated for the benefit of science. We are looking for possible evaluation and also publication in International Journal of Retina and Vitreous.

Submitted manuscript is an original article. The corresponding author of this manuscript is Andi Muhammad Ichsan (am_ichsan@med.unhas.ac.id) and contribution of the authors as mentioned below:

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With the submission of this paper, I would like to undertake that:

1. All authors of this paper have directly participated in the planning, execution, or analysis of this study;
2. All authors of this paper have read and approved the final version submitted;
3. The contents of this manuscript have not been copyrighted or published previously;
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5. The contents of this manuscript will not be copyrighted, submitted, or published elsewhere.
6. The authors state there is no conflict of interest in writing this article.

Thank you very much your kind attention.

Sincerely,

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FIGURE

A

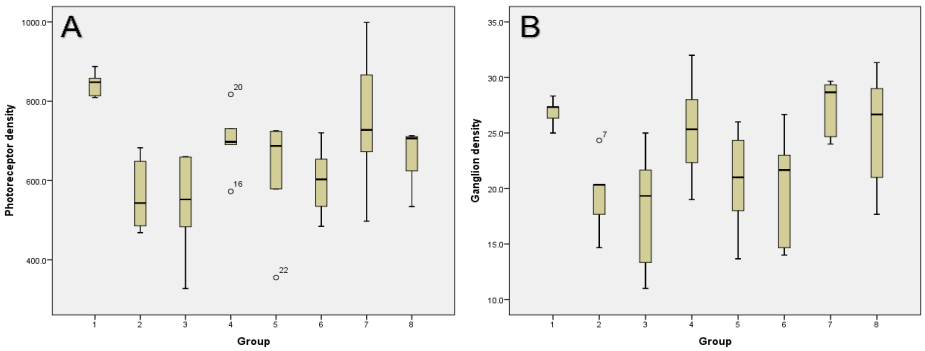


Figure. 1. A. Photoreceptor cells density; B. Ganglion cells density

B

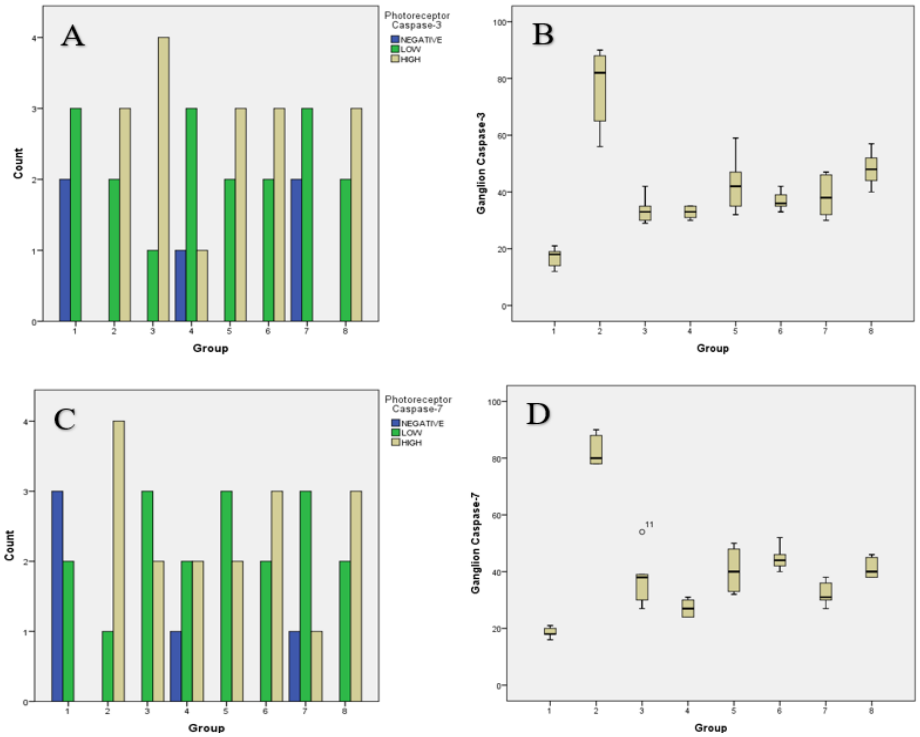
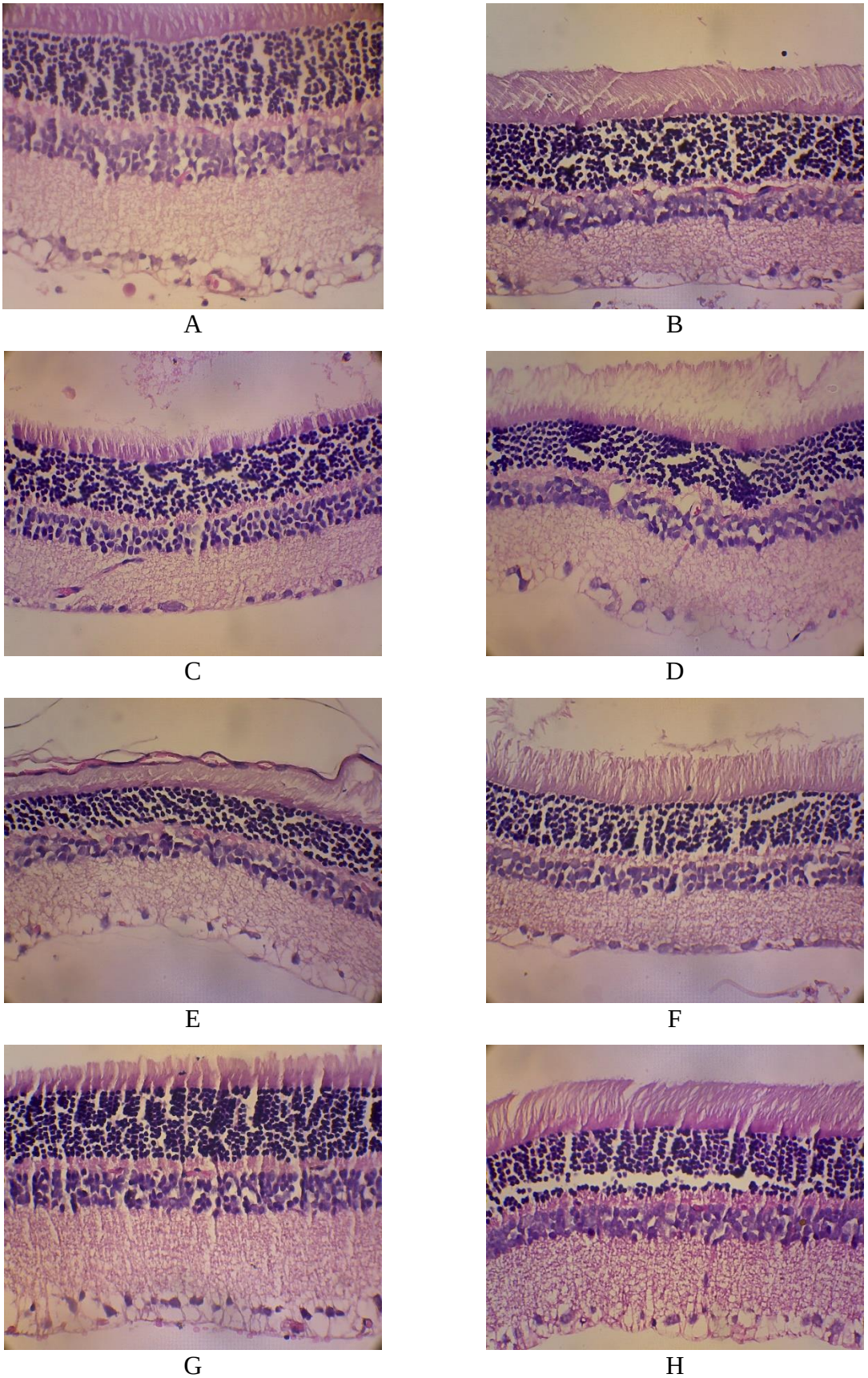


Figure.2. A. Caspase-3-expression on photoreceptor cells B. Caspase-3-expression on ganglion cells; C. Caspase-7-expression on photoreceptor cells D. Caspase-7-expression on ganglion cells.

12 **C**



13 **Figure. 3.** Hematoxylin and eosin staining for cell density**.** A = group 1, B = group 2, C =
14 group 3, D = group 4, E = group 5, F = group 6, G = group 7, H = group 8

D

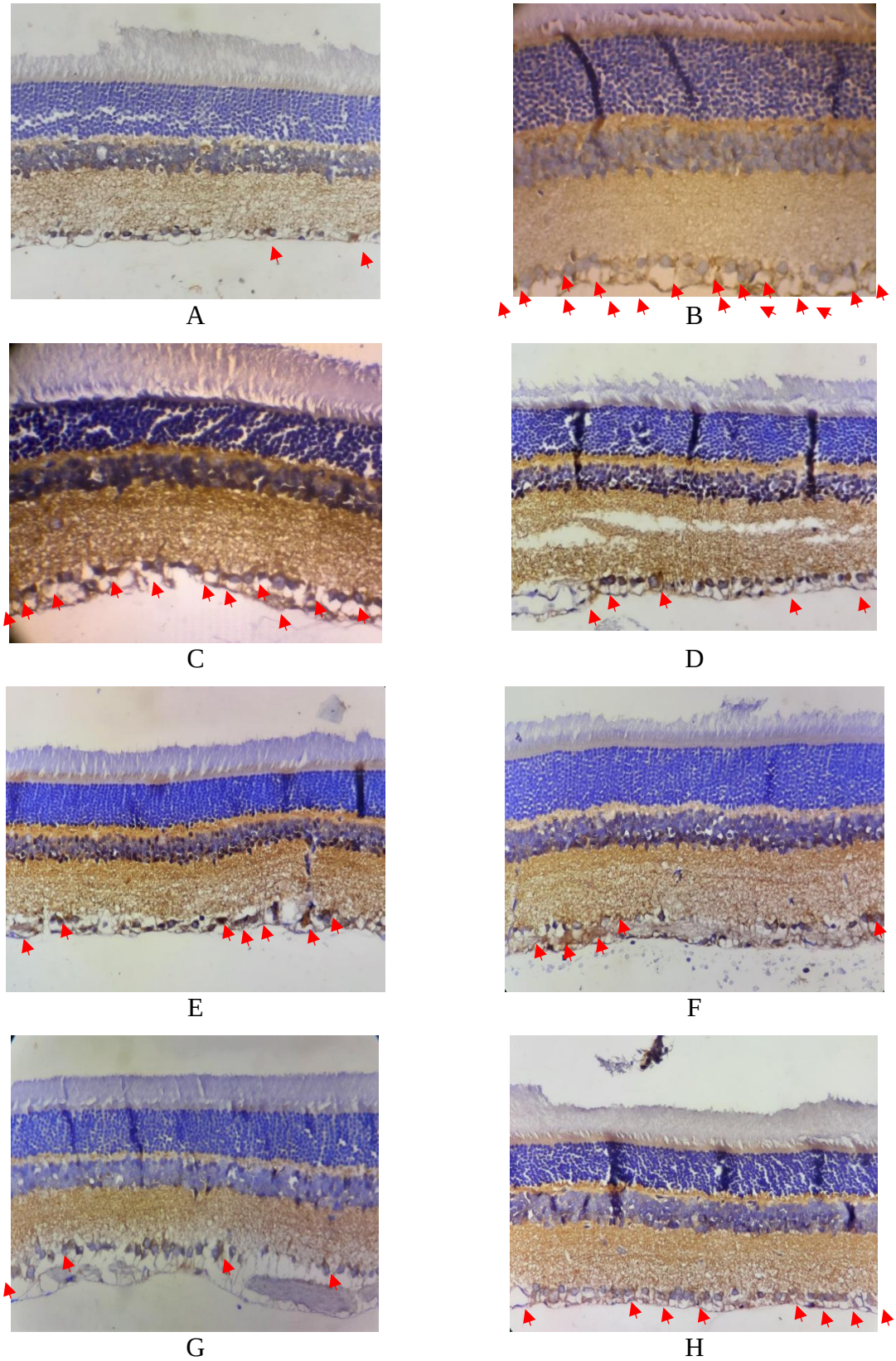


Figure. 4. Immunohistochemistry staining for Caspase-3 expression. A = group 1, B = group 2, C = group 3, D = group 4, E = group 5, F = group 6, G = group 7, H = group 8. The red arrow shows caspase 3

E

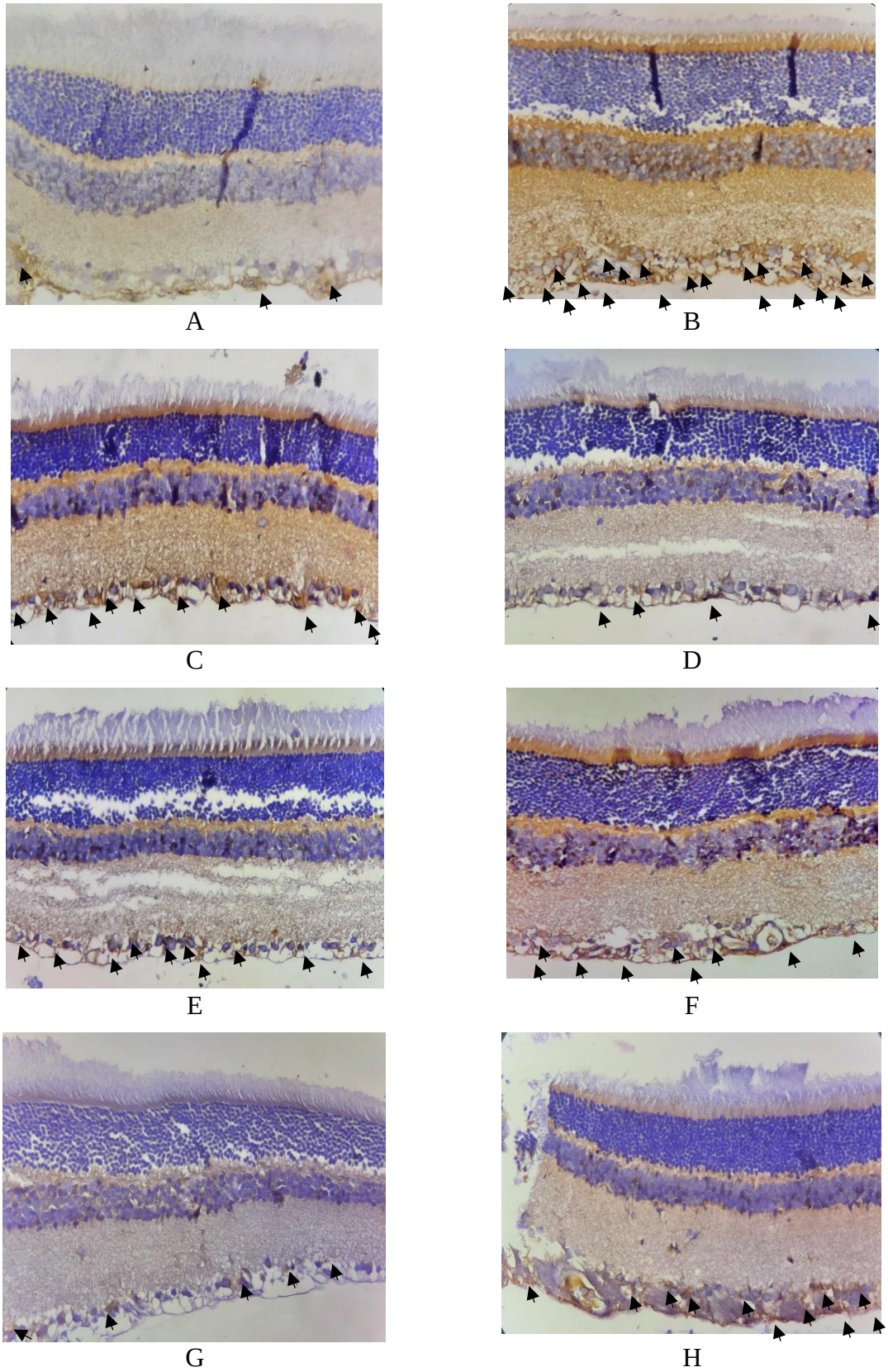


Figure. 5. Immunohistochemistry staining for Caspase-7 expression. A = group 1, B = group 2, C = group 3, D = group 4, E = group 5, F = group 6, G = group 7, H = group 8. The black arrow shows caspase 7

EFFECT OF RETINOL AND α-TOCOPHEROL SUPPLEMENTATION ON PHOTORCEPTOR AND RETINAL GANGLION CELL APOPTOSIS IN DIABETIC RATS MODEL

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ABSTRACT

Background: Diabetic retinopathy which is caused by oxidative stress is the most common microvascular complication of diabetes. Supplementing with retinol and tocopherol is thought to save photoreceptor cells from neural apoptosis and retinal ganglion cell damage caused by hyperglycemia. **Objective:** This study aims to examine the effect of retinol and α-tocopherol compounds on photoreceptor and ganglion cell density, as well as the caspase-3 and -7 expression in the retinal layers of the diabetic rat model. **Methods:** Alloxan 150mg/kg BW was used to develop animal models, which were then separated into eight groups and treated with retinol, α-tocopherol, or a combination of both. Histopathological examination on retinal layers was performed using Hematoxylin-Eosin (HE) and Immunohistochemistry (IHC) staining. **Results:** In the treatment group, the maximum density of photoreceptor cells and ganglion cells was identified in diabetic rats given tocopherol for 14 days while the lowest value was discovered in the group given retinol for 7 days. Statistical analysis on the caspase-3 and caspase-7 expression revealed that the group given α-tocopherol for 7 days had the lowest value ($p=0.010$). **Conclusion:** In diabetic rats, α-tocopherol and retinol compounds help to maintain densities and prevent the death of retinal photoreceptor and ganglion cells. However, in diabetic rats, α-tocopherol was more beneficial than retinol or combinations in terms of cell number and caspase expression.

Keywords: *Diabetic retinopathy, retinol, α-tocopherol, photoreceptor cell, retinal ganglion cell, apoptosis.*

BACKGROUND

Diabetic retinopathy (DR) is one of the typical causes of visual impairment in the productive-age class around the world (Song & Wong, 2014). Based on the abnormalities of the retinal microvasculature, DR is a microvascular complication of diabetes. However, a recent pathophysiological model has highlighted that neurodegeneration is a crucial and early component of retinopathy. Neural apoptosis, response gliosis, glutamate excitotoxicity, the decline in neuroprotective components, and debilitation of the neurovascular coupling are all depicted as causes of retinal neurodegeneration. Neurodegeneration is a common early symptom of DR and often precedes visible vasculopathy (Jonsson *et al*., 2016). Previous research showed that the neuronal unit of the retina and diabetic retinopathy are strongly related because biochemical defects and functional abnormalities are demonstrated by retinal neurons and glial cells. This involves fast neuronal death, microglial cell activation, and enhanced oxidative stress generation by photoreceptors (Kowluru & Mishra, 2015).

The amounts of oxidatively damaged DNA and nitrosylated proteins are higher in the diabetic retina due to increased oxidative stress (OS), and antioxidant defense enzymes are compromised. Diabetic experimental animals and humans have a lower level of antioxidant enzymes and potential antioxidant vitamins (Nita & Grzybowski, 2016). As a result of the close relationship between OS and dysmetabolism associated with the pathogenesis of DR, suitable antioxidants can be used to alleviate metabolic and functional abnormalities. Antioxidants can work on various levels, such as inhibiting the generation of reactive oxygen species (ROS), lowering free radicals, or enhancing antioxidants enzyme capacities. The finding demonstrated that using dietary or local bio factors of medicinal and aromatic plants could help manage diabetes. OS triggers other pathways that are unfavorable to DR development and causes a vicious circle of injury to macromolecules by magnifying additional ROS. Therefore, OS and

ROS are considered to have a role in DR, by increasing glucose and significant metabolic abnormalities (Silva *et al*., 2010).

Vitamins A, C, E, and carotenoids, which have been extensively investigated, are well-known antioxidants produced from food. Additionally, antioxidants can limit the generation of reactive oxygen species (ROS), scavenge free radicals, or boost the antioxidant enzyme capabilities. This supplementation has proven to reduce oxidative stress-induced damage to the retina (Silva *et al*., 2010).

Retinol, retinal, retinoic acid, and provitamin A carotenoids are all unsaturated nutritional chemical molecules that makeup vitamin A (Zhong *et al*., 2012). Painstaking biochemical reconstitution experiments have enabled recent improvements in the molecular knowledge of the retinoid cycle in the mammalian retina. Natural or synthetic animal models with known genetic lesions backed up this claim, as well as human studies of target genetic blinding diseases. Critical retinal enzymes and proteins and their substrates and ligands have been identified using structural and membrane biology and placed in a cellular context (Kiser & Palczewski, 2016). In a reversible reaction catalyzed by the NADPH-dependent all-trans-retinol dehydrogenase, all-trans-retinal in the cytoplasm are degraded to all-trans-retinol (RDH). After that, al-trans-retinol diffuses into the RPE where it is esterified by lecithin: retinol acyl transferase (LRAT) (Palczewski, 2010). Meanwhile, in vitro, and in vivo studies showed a protective impact of α-tocopherol (derivative of vitamin E) on practically all eye tissues. For up to 24 hours of exposure, a biomolecular compound of α-tocopherol can protect the retina against light damage (Engin, 2009a). In addition, α-tocopherol has been shown to protect against retinal phototoxicity and central nervous system ischemia injury (Ritch, 2007).

METHODS

This study was further aimed at investigating the effect of retinol and α-tocopherol on photoreceptor and ganglion cell densities, as well as apoptosis by evaluating caspase-3 and -7 expression.

Design

This was a true experimental with only a post-test group which made use of forty animal subjects. Alloxan monohydrate (SIGMA USA, Cat. No. A7413) was then used to induce the diabetic model, while supplementation was performed with retinol (SIGMA USA, Product No. R7632, CAS Number: 68-26-8) and α-tocopherol (SIGMA USA, Cat. No.258024) compounds. Eight groups of animals were created, namely: 1 presented as a negative control (wild type), 2 presented as a positive control (alloxan induction without treatment), 3 was diabetic rats on retinol for 1 week (after alloxan induction), 4 was diabetic rats on α-tocopherol for 1 week (after alloxan induction), 5 was diabetic rats given a combination of retinol and tocopherol for 1 week (after alloxan induction), 6 was diabetic rats on retinol for 14 days (1 week each, before and after alloxan induction), 7 was diabetic rats given tocopherol for 14 days (1 week each, before and after alloxan induction), and 8 was diabetic rats given combination of retinol and tocopherol for 14 days (1 week each, before and after alloxan induction).

Established of animal experiment

Male Wistar rats (*Rattus norvegicus*), 8-12 weeks old, weighing 160-200 grams were used for this study. All animals were given standard feed and provided access to *ad libitum* drinking water in a room with a 12-hour light-dark cycle. Each experimental animal in groups 2-8 received 150mg/kg body weight an intraperitoneal injection of Alloxan monohydrate. In cases where blood glucose levels were >200 mg/dl, induction was considered successful.

Furthermore, blood sugar measurements were performed three times namely, before injection of alloxan, three days later, and a day before sacrifice. All samples in group 1 had a blood glucose level <200 mg/dl until the termination procedure was completed, while samples in groups 2 to 8 had a blood glucose level >200 mg/d after being induced until termination. Retinol compounds up to 900 mcg/day were administered to groups 3 and 6 (National Institutes of Health,2020), α-tocopherol compounds up to 15mg/day were provided to groups 4 and 7 (Rasmussen & Johnson, 2013), while groups 5 and 8 received a combination of both.

Sample collection and processing

The rats were killed before enucleation, by placing them in a closed container filled with cotton and ether. Subsequently, the animals were placed for ten minutes until there was no motor reaction, neurological reflexes, or heartbeat. The eye tissue was removed using the enucleation approach, which involved pressing the eyeball on the base of the optic nerve, cutting the optic nerve, and lifting the eyeball. All eyes were fixed with 10% formalin and transported to the pathology lab.

To calculate the density of retinal ganglion and photoreceptor cells, retinal tissue was cut using a microtome with a thickness of 5 m and stained with hematoxylin and eosin (HE). Caspase-3 (Cat No. C9598, Sigma USA) and caspase-7 (Cat No. C1104, Sigma USA) expression in the retinal layer was examined using immunohistochemistry (IHC). Quantitative approaches were used to interpret cell density using an Olympus CX23 binocular microscope with 40-fold objective magnification, and the results were expressed as a mean with standard deviation. To identify caspase-3 and caspase-7, Immunohistochemistry staining was conducted using primary and secondary antibody (Cat. No. UCS015-IFU, ScyTek USA). The intensity of expression in photoreceptor cells was categorized qualitatively using the Immunoreactive Scoring System (IRS) modification method. There are 3 categories namely, negative (once

caspase expression shows <5% of the total field of view), Low (5-20% expression), and high (>20% expression). Meanwhile, the intensity of caspase expression in ganglion cells was performed quantitatively by counting the number of cells and apoptotic bodies that express binding colour.

Data Analysis

Statistical analysis of the data were using an Independent T-test for the quantitative data and Kruskall Wallis test for qualitative data (sig. $p<0.05$).

RESULTS

According to table 1, the blood sugar level of the negative control group was 82±2 mg/dl compared to the other groups (276±15 mg/dl to 426±45 mg/dl). This revealed that the experimental animal can be used as a model for type 1 diabetes rats because they have been in a hyperglycaemic condition. Quantitative method was used to identify cell densities with normally distributed and this showed a statistically significant difference ($p <0,05$), with groups 4 and 7 being the closest result to the normal value (Table 1 and Fig. 1)

The data was divided into three categories namely, negative, low, and high, Kruskal-Wallis analysis was used to test the interpretation of photoreceptor cell expression. In addition, T-test analysis was used to determine the expression value in ganglion cells. Table 1 shows the findings of statistical calculations (p-value) for immunohistochemical evaluation of caspase-3 and -7 expression in photoreceptor and retinal ganglion cells, with the groups 4 and 7 showing the most similar caspase-3 and -7 expression to the healthy group (fig.2).

DISCUSSION

The retina is a fragile thin layer of tissue that originates from the neuroectoderm. It is made up of nine layers of sensory neurons that begin the visual pathway (Gupta *et al*., 2015). Photoreceptors are visual system sensors that transform photon capture into a nerve signal through a process known as phototransduction. Photopigment, mitochondria, endoplasmic reticulum, nucleus, inner fiber, and synaptic terminals are all components of a photoreceptor. Photoreceptor terminal ends interrelate with surrounding photoreceptors and interneurons of horizontal and bipolar cells, and they are required for visual information transmitting and early processing in the retina (Fielder & R., 2011). A pathogenic disease, such as diabetic retinopathy, caused a decrease in electrical activity and nerve fiber changes. Therefore, even though the retinal neuronal structure is different from the peripheral sensory system, diabetic retinopathy is similar to diabetic peripheral sensory neuropathy (Antonetti, 2012).

DR is a duration-dependent disease, that is infrequently discovered during the early years of diabetes but substantially develops with time, with nearly 90% of patients showing indications of retinopathy after 20–25 years of diabetes (Kowluru & Mishra, 2015). After cellular membranes are damaged and intracellular components are released, oxygen-derived free radicals mediate tissue injury (Nur Azlina & Nafeeza, 2008). Antioxidants have the potential of preventing retinopathy development in diabetic rats and the implicated retinal metabolic abnormalities (Silva *et al*., 2010). For the protection of the retina and choroid, optimal combinations of vitamins B1, B2, B6, L-methylfolate, methylcobalamin (B12), C, D, natural α-tocopherol complex, lutein, zeaxanthin, α-lipoic acid, and n-acetylcysteine are necessary (Rasmussen & Johnson, 2013**).**

There is a substantial difference in cell density between diabetic and non-diabetic rats after alloxan induction and supplementation with retinol and α-tocopherol substances in this

study. Meanwhile, in terms of retinal cell density, α-tocopherol outperforms retinol. Subsequently, the vitamin E refers to eight naturally occurring compounds (α-, β-, γ-, δ-tocopherol, and α-, β-, γ-, δ-tocotrienol). α-tocopherol is the most common form retained in human plasma, out of the eight forms (Gagné *et al*., 2009). Vitamin E is crucial for erythrocytes' stability and central and peripheral nerves conductivity. Several countries have established vitamin E dietary consumption recommendations. For example, based on the Recommended Daily Allowance (RDA) in the US for α-tocopherol in adults men and women is 15 mg (Péter *et al*., 2015).

The loss of photoreceptors in the diabetic retina is still being topic of debate. Various OCT studies in diabetic patients show that the thickness of the inner retina, including the nerve fibre, ganglion cell, and inner plexiform layers, decreases with the duration of diabetes (Becker *et al*., 2020). In diabetic animal models, the outer nuclear layer thickness is frequently reduced, especially models of type 1 disease with early onset. Furthermore, various studies support the notion that photoreceptor loss increases with disease duration (Kern & Berkowitz, 2015).

Once the fat is oxidized and free radical reactions propagate, α-tocopherol is a powerful chain-breaking antioxidant that counteracts reactive oxygen species molecules creation. The α-tocopherol has been suggested to protect against retinal phototoxicity and central nervous system ischemia injury (Ritch, 2007). By inhibiting the peroxidation of membrane lipids and scavenging lipid peroxyl radicals, α-tocopherol protects essential cellular structures from damage produced by oxygen free radicals and reactive products of lipid peroxidation (Kanter *et al*., 2009). This also protects the polyunsaturated fatty acids found in membrane phospholipids and plasma lipoproteins, because of its peroxyl radical scavenging activity (Rizvi *et al*., 2014).

Concerning the pharmacodynamics of tocopherols, studies on the human eye have shown that vitamin E retinal levels are greater than those of the choroid or vitreous and are linked to

vitamin E serum level. Protein kinase C (PKC) is a path taken by α-tocopherol. According to current studies, tocopherol decreases free-radical creation and tyrosine kinase activity in the Tissue Plasminogen Activator (TPA)-induced in the primary human fibroblasts or HL-60 cells. The antiproliferative action of α-tocopherol through the PKC pathway in the vascular smooth muscle cell model is revealed by numerous studies published in vivo and in vitro joint investigations. However, α-tocopherol administration has reduced the linkage of Activator Protein 1 (AP-1) to DNA in para-methoxyamphetamine (PMA)-stimulated cells, although this is yet to be seen in phase G0 cells (Engin, 2009b).

At the inner blood-retinal membrane, scavenger receptor class B type I is responsible for the absorption of -tocopherol from the circulating blood and is required for the maintenance of -tocopherol in the neural retina. -tocopherol can protect the retina for up to 24 hours after being exposed to light. Vitamin E is a vital preventative against a variety of dangerous light-induced eye illnesses and disorders (cataractogenesis and retinal photodeterioration) (Tachikawa *et al*., 2007). There has also been evidence of a link between α-tocopherol and several eye disorders in the past. Retinitis pigmentosa, for example, has been linked to an H101Q mutation in the α-tocopherol transfer protein gene (Pang *et al*., 2001). Cryotherapy combined with vitamin E prophylaxis seems to reduce the severity and complications of threshold retinopathy of prematurity (Johnson *et al*., 1995). Persons with exudative macular degeneration have lower average levels of α-tocopherol (Mares-perlman *et al*., 1995).

Following positive results in a diabetic rat model for the prevention of diabetes-related vascular damage, high dosages of α-tocopherol, the predominant antioxidant in the lipid phase, were examined in the clinic for administration. This was performed to restore retinal blood flow in diabetic type I patients as this was discovered to control levels. Furthermore, the α-tocopherol is useful in DR by the nonenzymatic free radical scavenging action outside the cell.

Antioxidant therapy with α-tocopherol has been shown in humans to improve diabetic retinal hemodynamics (Silva *et al*., 2010).

Retinoids may create retinoid cation radicals as a result of interactions with different radicals or photoexcitation with light. Also, from studies performed, there is an indication that semi-oxidized retinoids can oxidize certain amino acids and proteins, and that α-tocopherol can scavenge retinol and retinoic acid cation radicals (Zhong *et al*., 2012).

According to Hymøller (2016), supplementation with substantial doses of retinol was demonstrated to reduce the bioavailability of α-tocopherol in growing pigs and calves. In both humans and poultry, retinol supplementation has been demonstrated to impact vitamin D3 bioavailability. This showed a more relevant ratio of retinol to vitamin D3 than the actual vitamins concentrations in the diet (Hymoller *et al*., 2016).

Due to its abundance in human and animal tissues, α-tocopherol is the considerable significant inhibitor of dietary lipid peroxidation in vivo. As a result, there have been various investigations about its effects on lipid peroxidation and combinations with other antioxidants than other tocopherols (Wang & Quinn, 1999). A resonance-stabilized phenoxyl radical (the tocopheroxyl radical) is created during the α-tocopherol donation of electrons. This has a reduced reaction compared to lipid-derived peroxyl radicals and does not reproduce the radical chain in lipid peroxidation easily. Subsequently, certain biological reductants, such as ascorbate (vitamin C), ubiquinol, or dihydrolipoic acid, then convert the tocopheroxyl radical back to tocopherol. The apparent antioxidant synergism between α-tocopherol and other antioxidants is based on recycling. Furthermore, α-tocopherol decreases and recycles other semi-oxidized forms such as cation radicals of the two forms of vitamin A namely, retinol and retinoic acid (El-Agamey & Fukuzumi, 2011; Li *et al*., 2013). Retinoids interaction with hydroxyl radicals, peroxyl radicals, such as trichloromethylperoxyl radical, or the

photoionization of retinoids by exposure to ultraviolet light is responsible for the cation radicals production (El-Agamey *et al*., 2017).

The study's second finding was that α-tocopherol had a substantial effect on retinal cell apoptosis prevention. This could be due to 2,7,8-trimethyl-2- (2'-carboxyethyl)-6-hydroxychroman (Ɣ-CEHC) compound, the metabolite of α-tocopherol that suppress cyclo-oxygenase activity, and have an anti-inflammatory effect (Gagné *et al*., 2009).

Apoptosis, commonly called as programmed cell death, is a morphologically unique process that includes cell shrinkage, cytoplasm condensation, plasma membrane blebbing, and fragmentation of chromatin and DNA into oligonucleosomes (Park *et al*., 2020). Caspases produce active signaling molecules that aid in apoptosis and are divided into two types based on their modes of action, which include initiator caspases (-8 and -9) and executioner caspases (-3, -6, and -7). Caspase-3, a key effector caspase in apoptotic pathways, is 32-kDa proenzyme that is not active. This is broken at the aspartate residue to form a p12 and p17 subunit necessary in the production of the active caspase-3 enzyme. Also, this is in charge of morphological and biochemical alterations during apoptosis and can be used in computing the apoptotic index (Huang *et al*., 2017).

The findings of this study are comparable to those of a study by Salerno (2007) on the effects of α-tocopherol consumption on apoptosis. In that study, α-tocopherol (10, 20, 50, or 100μM in 0.25 M MetOH) was the only agent that induced a slight statistically significant reduction in intracellular caspase-3 activity ($P<0.05$). Meanwhile, combinations in different amounts of α-tocopherol and carotenoid cleavage products (13 μg/ml), showed an elevated up-regulation of intracellular caspase-3 activity. The combination treatment had a far greater effect than carotenoid derivatives alone, as reported by the first column pair (Salerno *et al*., 2007).

Antioxidants prevented the development of retinopathy in diabetic rats' retinas, which revealed elevated oxidative stress. According to recent studies, apoptosis of neuronal retinal

cells is increased in experimental diabetes in rats and humans. The apoptosis-induced cell death leads to persistent neurodegeneration in diabetic retinas, where neurons are destroyed before another histopathology is seen (Abu El-Asrar *et al*., 2007).

This study results showed that the percentage of cells staining at each intensity level was used to grade the interpretation of caspase-3 and -7 expressions in photoreceptor cells. The degree of positivity using Immunoreactive Scoring System (IRS) modification, which was used by Huang, et.al (2017) method, we classified it into Negative = 0-<5% expression, Low = 5-25% expression, and High = >25% expression (Huang *et al*., 2017).

Oxidative stress and hypoxia may cause photoreceptor injury in diabetes. Due to diabetes-induced overproduction and deposition of reactive oxygen species (ROS) in the retina, elevated oxidative stress becomes a crucial component of DR pathogenesis. This is certainly relevant in photoreceptors because of defective mitochondrial electron transport and NADPH oxidase (Nox) activity. In diabetic retinas, oxidative stress is greatly higher in the dark, leading to higher oxygen demand throughout the dark cycle (Becker *et al*., 2020).

Many in vitro and in vivo investigations have shown close relation of hyperglycemia-induced oxidative stress in a range of cell types. In diabetic neurons, oxidative stress was related to mitochondrial malfunction and the programmed cell death caspase pathway activation. Many studies investigated mitochondrial disease and malfunctions in association with vitamin E and other antioxidants insufficiency. Meanwhile, fairly little evidence on the long term health and function benefits of antioxidant-rich mitochondria are available (Lauridsen & Jensen, 2012). There is compelling evidence that reactive oxygen species play a role in causing mitochondria to release numerous key apoptotic actors into the cytosol, including cytochrome c and AIF. The majority of studies showed that oxidative stress in the retina was elevated because of diabetes and this is crucial in diabetic retinopathy development (Abu El-Asrar *et al*., 2007).

One major pathophysiological cause of ectopic neovascularization in DR is retinal hypoxia. The most thoroughly analyzed factors in diabetic retinal neovascularization are Hypoxia-driven upregulation of Hypoxia-inducible factor 1-alpha (HIF1-α) and vascular endothelial growth factor (VEGF). Additionally, medicines that inhibit VEGF bioactivity are used regularly for the treatment of diabetic macula oedema and proliferative DR. Photoreceptors in the healthy retina are among the most active oxygen consumers in the body. The choroidal circulation, not the retinal blood vessels, supplies the majority of the oxygen to photoreceptors. As a result, oxygen tension drops quickly from the Bruch's membrane to the retina's outer nuclear layer, where it reaches its lowest values. This reduces oxygen reserve in Photoreceptors and even a minor disruption of oxygen flow in diabetes can result in severe hypoxia. The creation of acellular capillaries, capillary blockage, and capillary dropout are all thought to contribute to retinal hypoxia and hence, the retinal vascular pathology of DR (Becker *et al*., 2020).

The significant effect on α-tocopherol consumption is similar to Silva et.al (2010) and Engin (2019). A provision of vitamins C and E decreased superoxide generation in the retina, and diabetic mice given this vitamin combination showed partial reductions in retinal neovascularization. Once antioxidants such as ascorbic acid, acetate, α-tocopherol, Trolox cysteine, β-carotene, and selenium are consumed, the benefits related to retinal cell survival become increasingly well-known. The same components can also minimize lipid peroxides and prevent SOD and catalase reduction. Therefore, it is suggested to increase the application or consumption of a wider range of antioxidants as an effective strategy to avoid retinopathy (Silva *et al*., 2010)

CONCLUSIONS

In diabetic rats, tocopherol and retinol compounds alter cell density, caspase-3, and caspase-7 expression in photoreceptor cells and retinal ganglion cells. Additionally, once compared to retinol compounds and combination, tocopherol compounds were more effective in terms of cell density and caspase expression. Tocopherol therapy can be recommended as a supplemental therapy in the care of diabetic patients to reduce retinopathy progression.

List of abbreviations

DR: Diabetic retinopathy, OS: oxidative stress, ROS: reactive oxygen species, LRAT: retinol acyl transferase, IRS: Immunoreactive Scoring System, RDA: Recommended Daily Allowance, PKC: Protein kinase C, TPA: Tissue Plasminogen Activator, PMA: para-methoxyamphetamine, AP-1: Activator Protein 1, Nox: NADPH oxidase, HIF1-α: Hypoxia-inducible factor 1-alpha, VEGF: vascular endothelial growth factor.

DECLARATION

Ethics approval and consent to participate

This study was received approval from The Ethics Committee of Medical Research, Faculty of Medicine, Hasanuddin University with Approval number: 725/UN4.6.4.5.31/ PP36/2021.

Consent for publication

Not applicable

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This research project is funded by Hasanuddin University in term of "Dana Penelitian Dasar Universitas Hasanuddin 2021" program (Contract No.: 915/UN4.22/PT.01.03/2021).

Author Contribution

AMI: design of the work, medical procedure execution (alloxan injection, feeding retinol and tocopherol, sacrifice animal model), data analysis and interpretation, drafting the work for publication. **HSM:** work conception, data analysis. **AB:** work conception, animal care, data statistical analysis, and interpretation. **SB:** work conception, treatment material selection, work drafting, and publication revision. **UAM:** performing the medical procedure (tissue preparation and interpretation) drafting the work for publication. **AAD, ICI:** caring for the animal model, performing post-injection follow-up, sacrifice animal model, tissue processing and analysis, composing and critically revising the work for key intellectual content.

Acknowledgment

Gratitude goes to Mrs. Syamsiah, ST, Mrs. Mardiati, Amd., Ak and Mrs. Juniarsih Tande Padang, Amd., Ak for their technical assistance in material and histopathological preparation.

Authors' information

All named authors meet the International Committee of Medical Journal Editors (ICMJE) criteria for authorship for this article, take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.

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TABLE

Table 1. Descriptive data

Group	Treatment	Photoreceptor cells density (mean±SD)	p-value	Ganglion cells density (mean±SD)	p-value	Caspase-3 expression on Photoreceptor cells (n)	p-value	Caspase-3 expression on Ganglion cells (mean±SD)	p-value	Caspase-7 expression on Photoreceptor cells (n)	p-value	Caspase-7 expression on Ganglion cells (mean±SD)	p-value	Blood glucose level (mean±SD)
1	Negative control (Wild type)	843±32		26±1		High: 0 Low: 2 Neg: 3		16.80±3.701		High: 0 Low: 3 Neg: 2		18.60±1.94		82±2
2	Alloxan induction without treatment	565±95		19±3		High: 4 Low: 1 Neg: 0		76.20±14.97		High: 2 Low: 3 Neg: 0		77.20±10.82		276±15
3	Retinol for 7 days	536±138		18±5		High: 2 Low: 3 Neg: 0		33.80±5.16		High: 2 Low: 3 Neg: 0		44.80±25.78		349±63
4	α-tocopherol for 7 days	701±88		25±5		High: 1 Low: 4 Neg: 0		32.80±2.28		High: 2 Low: 3 Neg: 0		27.20±3.27		426±45
5	Combination of retinol and α-tocopherol for 7 days	614±156	**0.002***	20±4	**0.010***	High: 3 Low: 2 Neg: 0	**0.016**[#]	43.00±10.70	**0.010***	High: 3 Low: 2 Neg: 0	**0.069**[#]	40.60±8.29	**0.010***	400±83
6	Retinol for 14 days (pre and post induction)	599±93		20±5		High: 3 Low: 2 Neg: 0		37.00±3.53		High:3 Low: 2 Neg: 0		44.80±4.60		387±81
7	α -tocopherol for 14 days (pre and post alloxan induction)	752±190		27±2		High: 1 Low: 4 Neg: 0		38.60±7.79		High: 2 Low: 3 Neg: 0		32.40±4.50		404±66
8	Combination of retinol and α-tocopherol for 14 days (pre and post alloxan induction)	657±78		25±5		High: 3 Low: 2 Neg: 0		48.20±6.64		High: 3 Low: 2 Neg: 0		41.40±3.84		424±52

**One-way ANOVA test (sig<0.05)*

[#]Kruskal-Wallis test (sig<0.05)