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

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ABSTRACT

Background: Diabetic retinopathy (DR) is the most common microvascular complication of diabetes. Retinol and α-tocopherol of diabetic models prevent the damage of photoreceptor and retinal ganglion cells (RGC) caused by hyperglycemia. **Objective:** This study aims to examine the effect of retinol and α-tocopherol on photoreceptor and RGC densities and the expression of caspase-3 and -7 on the retinal layers of the diabetic rat model. **Methods:** Alloxan 150 mg/kg body weight single dose was used to develop animal models, which were separated into eight groups. These consist of one group without intervention (group 1), one positive control with only induced alloxan (group 2), and others receiving retinol (group 3 and 6), α-tocopherol (group 4 and 7), or their combination (group 5 and 8). Furthermore, histopathological examination was performed using Hematoxylin-Eosin staining to evaluate the photoreceptor and RGC densities, while immunohistochemistry staining evaluated the caspase-3 and -7 expressions. **Results:** In the treatment group, the highest and lowest densities were identified in diabetic rats given α-tocopherol (group 7) and retinol (group 3) respectively. The caspase-3 and -7 expression showed that the group given α-tocopherol (group 7) had the lowest value. **Conclusion:** In diabetic rats, retinol and α-tocopherol compounds maintained densities and prevented photoreceptor and RGC death. However, α-tocopherol was more promising than retinol or combinations in the prevention of retinal cells apoptosis.

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 worldwide (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 this complication. Neural apoptosis, response gliosis, glutamate excitotoxicity, the decline in neuroprotective components, and debilitation of the neurovascular coupling are depicted as causes of retinal neurodegeneration. One of the underlying pathomechanisms for DR found to precede visible vasculopathy is neurodegeneration (Jonsson et al., 2016). Previous study showed that the neuronal unit of the retina and DR are strongly related because retinal neurons and glial cells demonstrate biochemical defects and functional abnormalities. This involves rapid neuronal death, microglial cell activation, and enhanced oxidative stress generation by photoreceptors (Kowluru & Mishra, 2015).

The most often utilized diabetogenic drugs are alloxan and streptozotocin (Ighodaro et al., 2017). Alloxan is a highly potent diabetogenic cyclic-urea derivative that can generate reactive oxygen species (ROS) in a cyclic reaction with its reduction product, dialuric acid, in the presence of intracellular thiols, particularly glutathione. The beta cell toxicity is begins by the free radicals produced during the redox reaction. Autoxidation of dialuric acid produces superoxide radicals (O_2-), hydrogen peroxide (H_2O_2), and hydroxyl radicals in a final iron-catalyzed reaction step ($OH-$). These hydroxyl radicals ultimately cause beta cells to die due to their innately limited ability for antioxidative defense and the resulting state of insulin-dependent alloxan diabetes. As a thiol reagent, alloxan inhibits glucose-induced insulin secretion selectively by oxidizing important thiol groups in the glucokinase protein, disrupting oxidative metabolism and this beta-cell signaling enzyme (Lenzen et al., 1996). The dose used

66   to cause diabetes varies between 40-200 mg/kgBW through the intraperitoneal or intravascular

67   route (Sheriff et al., 2020).

68   Caspases involved in apoptosis have been subclassified by their mechanism of action and

69   are either initiator (caspase- 8 and -9) or executioner (caspase-3, -6, and -7) (McIlwain et al.,

70   2015). Caspase-3 with -7 are similar because the cysteine proteases share an optimal peptide

71   recognition sequence and have several endogenous protein substrates in common. In addition,

72   they are proteolytically activated by the initiator caspase-8 and -9 during death receptor- and

73   DNA-damage-induced apoptosis (Lamkanfi & Kanneganti, 2010). Caspase-3 and -7

74   expression as apoptotic markers might be used to investigate the alterations in the retina

75   following the diabetes condition. These are two of the essential caspase effectors in apoptotic

76   pathways, and the indicators can assess the level of tissue damage caused by the induction

77   agent (Kowluru & Koppolu, 2002; Lamkanfi & Kanneganti, 2010).

78   The amounts of damaged DNA and nitrosylated proteins are higher in the diabetic retina

79   due to increased oxidative stress (OS) and compromised antioxidant defense enzymes. Diabetic

80   experimental animals and humans have a lower level of antioxidant enzymes and vitamins

81   (Nita & Grzybowski, 2016). Antioxidants can be used to alleviate metabolic and functional

82   abnormalities as a result of the close relationship between OS and dysmetabolism associated

83   with the pathogenesis of DR. They can work on various levels, such as inhibiting the generation

84   of reactive oxygen species (ROS), lowering free radicals, or enhancing enzyme capacities. The

85   finding demonstrated that medicinal and aromatic plants' dietary or local bio factors could help

86   manage diabetes. OS triggers other unfavorable pathways to DR development and causes a

87   vicious circle of injury to macromolecules by magnifying additional ROS. Therefore, OS and

88   ROS are considered to have a role in DR by increasing glucose and significant metabolic

89   abnormalities (Bouterse & Kowluru, 2008; Silva et al., 2010).

The extensive investigation of vitamins A, C, E, and carotenoids are well-known antioxidants produced from food. Antioxidants can limit the generation of reactive oxygen species (ROS), scavenge free radicals, or boost the enzyme capabilities to reduce oxidative stress-induced damage to the retina (Silva et al., 2010).

Retinol, retinal, retinoic acid, and provitamin A carotenoids are unsaturated nutritional chemical molecules that make up 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. Furthermore, natural or synthetic animal models with known genetic lesions backed this claim with human studies of target genetic blinding diseases. Critical retinal enzymes and proteins as well as their substrates and ligands have been identified using structural and membrane biology in a cellular context (Kiser & Palczewski, 2016). In a reversible reaction catalyzed by the reduced nicotinamide adenine dinucleotide phosphate (NADPH) -dependent all-trans-retinol dehydrogenase, all-trans-retinal in the cytoplasm were degraded to all-trans-retinol. This product diffuses into the retinal pigment epithelium, which is esterified by lecithin retinol acyltransferase (LRAT) (Palczewski, 2010). Meanwhile, in vitro and in vivo studies showed a protective impact of α-tocopherol, a vitamin E derivative, on eye tissues. For up to 24 hours of exposure, a biomolecular compound of α-tocopherol can protect the retina against light damage (Ritch, 2007). Therefore, this study aimed to investigate the protective effect of retinol and α-tocopherol on the photoreceptor and retinal ganglion cells apoptosis in a diabetic rats model.

METHODS

Design

This study was conducted with a post-test group of forty animal subjects at the Animal and Pathology Laboratories of Hasanuddin University, Indonesia. This study received approval

from The Ethics Committee of Medical Research, Faculty of Medicine, Hasanuddin University, with number: 725/UN4.6.4.5.31/ PP36/2021.

Alloxan monohydrate (SIGMA USA, Cat. No. A7413) 150 mg/kgBW single dose intraperitoneally was used to induce the diabetic model. Supplementation was performed with retinol (SIGMA USA, Product No. R7632, CAS Number: 68-26-8) and α-tocopherol (SIGMA USA, Cat. No.258024) compounds. Furthermore, eight groups of animals were created, where group 1 was the negative control (wild type), group 2 was the positive control (alloxan induction without treatment), group 3 described diabetic rats on retinol for 1 week (after alloxan induction), group 4 represented the diabetic rats on α-tocopherol for 1 week (after alloxan induction), group 5 was the sample given a combination of retinol and α-tocopherol for 1 week (after alloxan induction), group 6 represented diabetic rats on retinol for 14 days (1 week each, before and after alloxan induction), group 7 described the samples given α-tocopherol for 14 days (1 week each, before and after alloxan induction), and group 8 was the combination of retinol and α-tocopherol for 14 days (1 week each, before and after alloxan induction).

Established animal experiment

Male Wistar rats (*Rattus norvegicus*) of 8-12 weeks old, weighing 160-200 g, were used for this study. All animals were given standard feed and access to *ad libitum* drinking water in a room with a 12-hour light-dark cycle. Each experimental animal in groups 2-8 received 150 mg/kg body weight an intraperitoneal injection of Alloxan monohydrate. Induction was considered successful where blood glucose levels were >200 mg/dl. Furthermore, blood sugar measurements were performed three times before alloxan injection, 3 days later, and a day before sacrifice. All samples in group 1 had a blood glucose level <200 mg/dl, while those in groups 2 to 8 had a blood glucose level >200 mg/d after being induced. Retinol compounds up to 900 mcg/day were administered to groups 3 and 6 (National Institues of Health, 2020). α-

140 tocopherol compounds up to 15 mg/day were also provided to groups 4 and 7 (Rasmussen &

141 Johnson, 2013), while 5 and 8 received both.

142

143 **Sample collection and processing**

144   The rats were sacrificed before enucleation by placing in a closed container filled with

145 cotton and ether for approximately ten minutes until there was no motoric reaction,

146 neurological reflexes, or heartbeat. Subsequently, the eye tissue was removed using the

147 enucleation approach, which involved pressing the eyeball on the base of the optic nerve,

148 cutting the optic nerve, and lifting the eyeball. Finally, all eyes were fixed with 10% formalin

149 and transported to the pathology laboratory.

150   Retinal tissue was cut using a microtome with a thickness of 5 µm and stained with

151 hematoxylin and eosin (HE) to calculate the density of ganglion and photoreceptor cells.

152 Caspase-3 (Cat No. C9598, Sigma USA) and -7 (Cat No. C1104, Sigma USA) expression in

153 the retinal layer was examined using immunohistochemistry (IHC). Quantitative approaches

154 were used to interpret cell density using an Olympus CX23 binocular microscope with 40-fold

155 objective magnification, and the results were expressed as a mean with standard deviation.

156 Immunohistochemistry staining was conducted using a primary and secondary antibody (Cat.

157 No. UCS015-IFU, ScyTek USA) to identify caspase-3 and -7. The intensity of expression in

158 photoreceptor cells was categorized qualitatively using the Immunoreactive Scoring System

159 (IRS) modification method. The three categories are negative (caspase expression shows <5%),

160 low (5-20% expression), and high (>20% expression). Meanwhile, the intensity of caspase

161 expression in retinal ganglion cells was calculated quantitatively by counting the number of

162 cells and apoptotic bodies.

163

164 **Data Analysis**

165 Statistical analysis used an Independent T-test and Kruskal Wallis for the quantitative and

166 qualitative data (sig. $p < 0.05$).

167

168 **RESULTS**

169 According to Table 1, the blood sugar level of the negative control was 82 ± 2 mg/dl compared

170 to the diabetic groups (276 ± 15 to 426 ± 45 mg/dl). This showed that the experimental animal

171 could be used as a model for type 1 diabetes rats because they have hyperglycemic conditions.

172   The photoreceptor cell density showed the highest and lowest value in groups 1 and 2,

173 respectively. In the treatment group, the most effective value for approaching the normal group

174 of mice was in group 7, which received α-tocopherol supplementation for 14 days (pre and

175 post-alloxan induction). The statistical test results showed a significant difference in the

176 photoreceptor cell density among groups ($p = 0.002$), as shown in Figure 1.a. This result is in

177 line with the measurement of retinal ganglion cell density, where the highest and lowest value

178 was also obtained in groups 1 and 2, respectively. For the treatment group, the most effective

179 supplementation was shown in group 7 (Figure 1.b). In addition, the statistical test result

180 showed that there was a significant difference in the RGC density among groups ($p = 0.010$).

181   In calculating the expression of caspase-3, apoptosis in photoreceptor cells showed the

182 lowest and highest expression in groups 1 and 2, respectively. Values close to the normal were

183 shown by groups 4 and 7 (α-tocopherol supplementation groups), as presented in figure 2.a.

184 Statistical analysis showed a significant difference in the difference caspase-3 expression in

185 photoreceptor cell among groups ($p = 0.016$).

186   The above results are in line with the those of expression in retinal ganglion cells, where

187 the lowest and highest value was found in groups 1 and 2, respectively. The observation results

188 in the treatment group were found to be the most effective in the group given α-tocopherol

189 supplementation (groups 4 and 7), as shown in Figure 2.b. Statistically, these results showed a

190 significant caspase-3 expression in RGC among groups (p=0.010).

191 The expression value of caspase-7, apoptosis in photoreceptor cells showed the lowest in

192 group 1, while the highest was found in 5, 6, and 8. Observations in the treatment group

193 indicated that groups 4 and 7 (supplementation of α-tocopherol) showed the lowest expression.

194 Based on these results, it was obtained that this result did not significantly affect caspase-7

195 expression in photoreceptor cell among groups (p = 0.069). The value of the expression in

196 retinal ganglion cells showed the lowest and highest value in groups 1 and 2, respectively. In

197 the treatment group, the expression values close to the standard were samples in 4 and 7,

198 respectively. Figures 2.c and d indicated a significant difference between caspase-7 expression

199 in RGC among groups (p=0.010).

200

201 **DISCUSSION**

202 The retina is a weak and thin layer of tissue that originates from the neuroectoderm,

203 comprising of nine layers of sensory neurons in the visual pathway (Gupta et al., 2015).

204 Photoreceptors are visual system sensors that transform photon capture into a nerve signal

205 through a process known as phototransduction. Photoreceptor terminals interrelate with

206 surrounding photoreceptors and interneurons of horizontal and bipolar cells. They are required

207 for transmitting visual information and early processing in the retina (Fielder & Alistair, 2011).

208 Photoreceptors in the healthy retina are among the most active oxygen consumers in the

209 body, and the choroidal circulation supplies the majority of the oxygen to photoreceptors. As

210 a result, oxygen tension drops quickly from the Bruch's membrane to the retina's outer nuclear

211 layer, where it reaches the lowest values. This reduces oxygen reserve in photoreceptors, and

212 even a minor disruption of oxygen flow in diabetes can result in severe hypoxia. The creation

213   of acellular capillaries, capillary blockage, and capillary dropout can contribute to retinal

214   hypoxia, hence, the vascular pathology of DR (Becker et al., 2020).

215   On the other layer, retinal ganglion cells process and convey information from the retina

216   to visual centers in the brain. These output neurons comprise subpopulations with distinct

217   structures and functions (Sernagor et al., 2001). As a result, there is a remarkable diversity of

218   RGCs. The various subtypes have unique morphological features and pathways linking the

219   inner retina to the relevant brain areas (Kim et al., 2021). Retinal ganglion cells carry visual

220   signals from the eye to the brain but do not make chemical synapses with other neurons.

221   However, they form gap junctions with other RGCs and amacrine cells, allowing RGC signals

222   to feedback into the inner retina (Vlasiuk & Asari, 2021).

223   A pathogenic disease, such as diabetic retinopathy causes a decrease in the electrical

224   activity of neurotransmitters from photoreceptors and RGC cells to the nerve fiber layer

225   (Antonetti, 2012). DR is a duration-dependent disease infrequently discovered during the early

226   years of diabetes. However, it substantially develops with time, nearly 90% of patients showing

227   retinopathy after 20–25 years of diabetes (Kowluru & Mishra, 2015). After cellular membranes

228   are damaged, and intracellular components are released, oxygen-derived free radicals mediate

229   tissue injury (Nur Azlina & Nafeeza, 2008).

230   Antioxidants have the potential of preventing retinopathy development in diabetic rats

231   and the implicated retinal metabolic abnormalities (Silva et al., 2010). Therefore, to protect the

232   retina and choroid, optimal combinations of vitamins B1, B2, B6, L-methylfolate,

233   methylcobalamin (B12), C, D, natural α-tocopherol complex, lutein, zeaxanthin, α-lipoic acid,

234   and n-acetylcysteine are necessary (Rasmussen & Johnson, 2013).

235   This study showed a substantial difference in cell density between diabetic and non-

236   diabetic rats after alloxan induction as well as supplementation with retinol and α-tocopherol

237   substances. Retinol supplementation appeared to affect maintaining the retinal cell densities

positively. However, it was not better than the α-tocopherol and combination supplementation groups. The higher density values proved this in groups 3 and 6 compared with 2. The study by Zhong et al. (2012) reported that retinoids might create cation radicals due to interactions with different radicals or photoexcitation with light. Furthermore, 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).

In the retinoid cycle, retinol is an excellent substrate for LRAT and quickly converted into fatty acid esters. Their propensity to form oil droplets excludes fatty acid esters from circulation (Kiser & Palczewski, 2016). The mechanism of vitamin A transport is mediated by the plasma retinol-binding protein (RBP), a specific and sole carrier in the blood. The specific membrane receptor stimulated by retinoic acid 6 (STRA6) mediates cellular vitamin A uptake. (Zhong et al., 2012) Structural and membrane biology have been used to detect critical retinal enzymes and proteins as well as their substrates and ligands, placing them in a cellular context. The most presently accepted modulators of the retinoid cycle have demonstrated promising results in animal models of retinal degeneration (Kiser & Palczewski, 2016).

The α-tocopherol supplementation group was closest to the normal values for photoreceptor and retinal ganglion cell densities. A similar result was found in Ritch (2007), which stated that the α-tocopherol had been suggested to protect against retinal phototoxicity and central nervous system ischemia (Ritch, 2007). 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. By inhibiting the peroxidation of membrane lipids and scavenging lipid peroxyl radicals, it 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).

263   Vitamin E refers to eight naturally occurring compounds (α-, β-, γ-, δ-tocopherol, and α-

264   , β-, γ-, δ-tocotrienol). α-tocopherol is the most common form retained in human plasma out of

265   the eight forms (Gagné et al., 2009). Vitamin E is crucial for erythrocytes' stability as well as

266   central and peripheral nerves conductivity. Therefore, several countries have established

267   dietary vitamin E recommendations (Péter et al., 2015).

268   The loss of photoreceptors in the diabetic retina is still debatable, and various optical

269   coherence tomography (OCT) studies in diabetic patients show that the thickness of the inner

270   retina, including the nerve fiber, retinal ganglion cell, and inner plexiform layers, decreases

271   with the duration of diabetes (Becker et al., 2020). In a diabetic animal model study, the outer

272   nuclear layer thickness is frequently reduced, specifically in models of type 1 disease with

273   early-onset. Furthermore, various studies supported the notion that photoreceptor loss increases

274   with disease duration (Kern & Berkowitz, 2015). A similar condition was found, where cell

275   densities significantly decreased in group 2 compared to others in photoreceptor and retinal

276   ganglion cells (Figure 3).

277   A high dose of α-tocopherol following positive results in a diabetic rat model to prevent

278   diabetes-related vascular damage was examined in the clinic for administration. This was

279   performed to restore retinal blood flow in diabetic type I patients, which was discovered to

280   control levels. Furthermore, the α-tocopherol is useful in DR by the nonenzymatic free radical

281   scavenging action outside the cell. Antioxidant therapy with α-tocopherol has been shown in

282   humans to improve retinal vascular hemodynamics (Silva et al., 2010).

283   In this study, the administration of the combination of retinol and α-tocopherol did not

284   show any more effective results to maintain the retinal cell densities than the single

285   supplementation of α-tocopherol. A previous study reported that supplementation with

286   substantial doses of retinol was demonstrated to reduce the bioavailability in growing pigs and

287   calves (Hymoller et al., 2016). Due to its abundance in human and animal tissues, α-tocopherol

288 is a significant contributor to dietary lipid peroxidation in vivo. As a result, there have been

289 various investigations about its effects on lipid peroxidation and combinations with other

290 antioxidants (Wang & Quinn, 1999).

291 A resonance-stabilized phenoxyl radical is created during the α-tocopherol donation of

292 electrons. This has a reduced reaction compared to lipid-derived peroxyl radicals and does not

293 quickly reproduce the radical chain in lipid peroxidation. Subsequently, certain biological

294 reductants, such as ascorbate (vitamin C), ubiquinol, or dihydrolipoic acid, convert the

295 tocopherol radical back to tocopherol. Retinoids' interaction with hydroxyl radicals, peroxyl

296 radicals, such as trichloromethyl peroxyl radical, or the photoionization of retinoids by

297 exposure to ultraviolet light is responsible for the cation radicals production (El-Agamey et al.,

298 2017).

299 The apoptosis can be characterized by the expression of biochemical markers called

300 caspases (Lavrik et al., 2005; Nuñez et al., 1998). The expression of caspase-3 and -7 (Figures

301 4 and 5) was also conducted in this study. Apoptosis, which called programmed cell death, is

302 a morphologically unique process that includes cell shrinkage, cytoplasm condensation, plasma

303 membrane blebbing, and fragmentation of chromatin and DNA into oligonucleosomes (Park et

304 al., 2020).

305 The caspases are a family of genes essential for maintaining homeostasis through

306 regulating cell death and inflammation. This biomarker produces active signaling molecules

307 that aid in apoptosis and are divided into two types based on their modes of action, including

308 initiator (-8 and -9) and executioner caspases (-3, -6, and -7) (McIlwain et al., 2015).

309 Caspase-3, a key effector caspase in apoptotic pathways, is 32-kDa proenzyme that is not

310 active. This is broken at the aspartate residue to form the p12 and p17 subunits necessary for

311 producing the active caspase-3 enzyme. Furthermore, this is in charge of morphological and

312 biochemical alterations during apoptosis and can be used in computing the apoptotic index

(Huang et al., 2017). Caspase-7 is also an executioner caspase that plays a critical role in optic nerve injury and retinal ganglion cell death. The inhibition might be a novel therapeutic strategy for some neurodegenerative diseases of the retina (Choudhury et al., 2015).

This study showed that the percentage of cell 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 classified Huang, et al. (2017) method into negative = <5%, low = 5-20%, and high = >20% (Huang et al., 2017). Moreover, quantitative measurement was obtained for the expression of the caspase in the retinal ganglion cells.

The statistical analysis found a significant difference in caspase-3 and -7 expressions among groups, and α-tocopherol groups showed a better effect on retinal cell apoptosis prevention than others. Antioxidants prevented the progression of retinopathy in diabetic rats' retinas, which showed elevated oxidative stress. According to previous studies, apoptosis of retinal neuronal cells is increased in experimental diabetes in rats and humans. The apoptosis-induced cell death leads to ongoing neurodegeneration, where neurons are destroyed before another histopathology occurs (Abu El-Asrar et al., 2007). The significant results of the α-tocopherol groups could be due to its biochemistry compound (2,7,8-trimethyl-2- (2'-carboxyethyl)-6-hydroxychroman (Ɣ-CEHC)) that suppresses cyclo-oxygenase activity with an anti-inflammatory effect (Gagné et al., 2009).

This study did not show a significant effect in combination of retinol and α-tocopherol supplementation to prevent retinal cell apoptosis. It could occur because the apparent 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 vitamin A (El-Agamey & Fukuzumi, 2011; Li et al., 2013).

337   The findings are comparable to the study conducted by Salerno (2007) on the effects of

338   α-tocopherol consumption on apoptosis. It was stated that α-tocopherol (10, 20, 50, or 100 μM

339   in 0.25 M MetOH) was the only agent capable of inducing a slight statistically significant

340   reduction in intracellular caspase-3 activity (P<0.05). The combinations of α-tocopherol and

341   carotenoid cleavage products (13 μg/ml) showed a high up-regulation of intracellular caspase-

342   3 activity, and the treatment had more significant effect than carotenoid derivatives (Salerno et

343   al., 2007). The different results were shown because they used a combination of α-tocopherol

344   and carotenoid cleavage products. In contrast, a combination of α-tocopherol and retinol, which

345   is pure forms of vitamin A was used. The administration of vitamins C and E reduced

346   superoxide generation in the retina, and diabetic mice given this combination experienced a

347   partial reduction in retinal neovascularization. The benefits of retinal cell survival become

348   increasingly well-known once antioxidants such as ascorbic acid, acetate, α-tocopherol, Trolox

349   cysteine, β-carotene, and selenium are consumed. The same components can also minimize

350   lipid peroxides and prevent superoxide dismutation with catalase reduction. Therefore, it is

351   suggested to increase the application or consumption of a broader range of antioxidants as an

352   effective strategy to prevent retinopathy (Silva et al., 2010).

353

354   **CONCLUSIONS**

355   Retinol and α-tocopherol compounds have a protective effect of maintaining the retinal cells'

356   densities and preventing the cells from apoptotic process. Moreover, the α-tocopherol

357   compound showed better results compared to retinol compound or a combination of both.

358   Future studies including in humans are needed to demonstrate the better understanding of α-

359   tocopherol supplementation in preventing diabetic retinopathy progression.

360

361 **List of abbreviations**

362 DR: Diabetic retinopathy, OS: oxidative stress, ROS: reactive oxygen species, LRAT: retinol

363 acyltransferase, IRS: Immunoreactive Scoring System, RDA: Recommended Daily

364 Allowance, PKC: Protein kinase C, NADPH: Nicotinamide adenine dinucleotide phosphate

365 (NADPH) oxidase, VEGF: vascular endothelial growth factor., IHC: immunohistochemistry,

366 RBP: retinol-binding protein, STRA6: stimulated by retinoic acid 6 (STRA6), OCT: optical

367 coherence tomography.

368

369 **DECLARATION**

370 **Ethics approval and consent to participate**

371 This study received approval from The Ethics Committee of Medical Research, Faculty of

372 Medicine, Hasanuddin University, with number: 725/UN4.6.4.5.31/ PP36/2021.

373

374 **Consent for publication**

375 Not applicable

376

377 **Availability of data and materials**

378 The data supporting these findings are available from the corresponding author upon

379 reasonable request.

380

381 **Competing interests**

382 The authors declare that they have no competing interests.

383

Funding

387

Author Contribution

AMI: design of the work, medical procedure execution (alloxan injection, feeding retinol and α-tocopherol, sacrifice animal model), data analysis and interpretation, drafting the work for publication. **AB:** work conception, animal care, statistical data analysis, and interpretation. **SL:** work conception, treatment material selection, work drafting, and publication revision. **UAM:** performing the medical procedure (tissue preparation and interpretation) and 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. **HSM:** work conception and data analysis. All authors read and approved the final manuscript.

398

Acknowledgment

402

Authors' information

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

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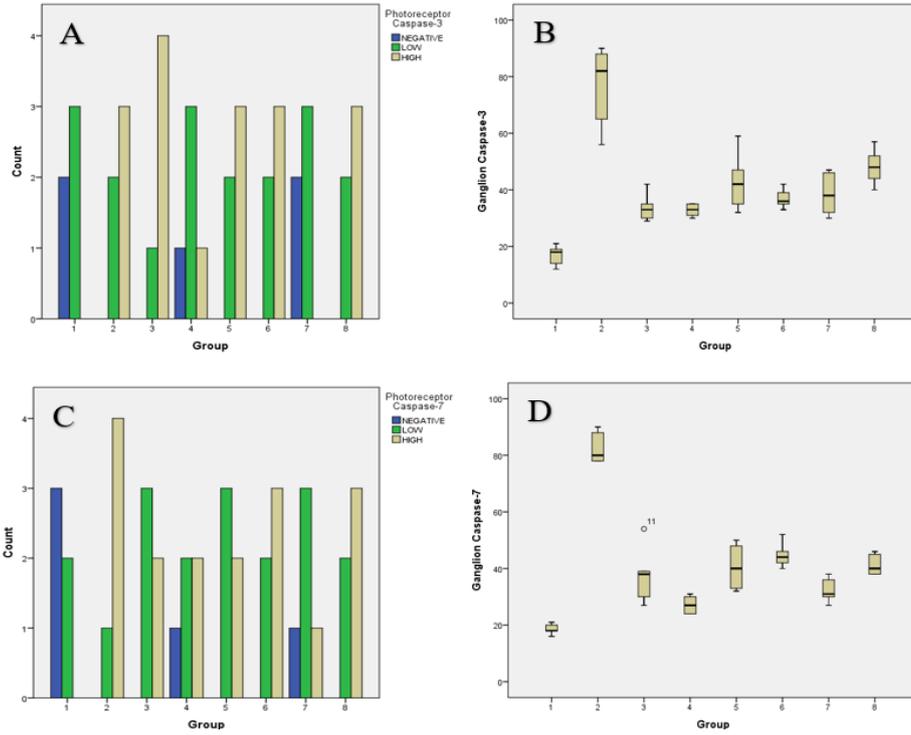534   natural history of vitamin A as a light sensor. *Nutrients*, *4*(12), 2069–2096.

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536

537

1   **FIGURE**

2   **A**
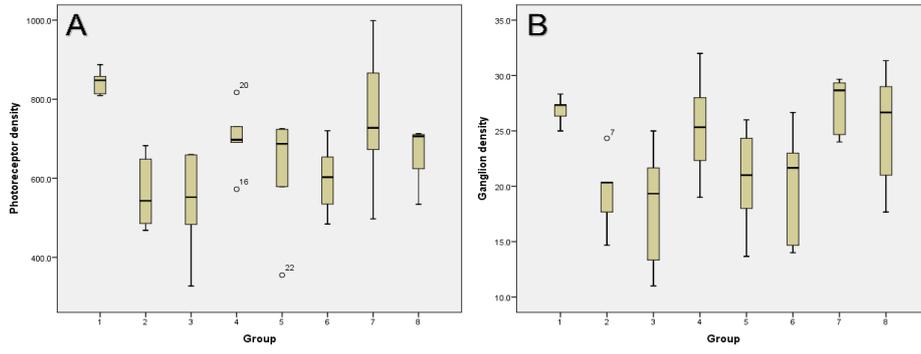
3
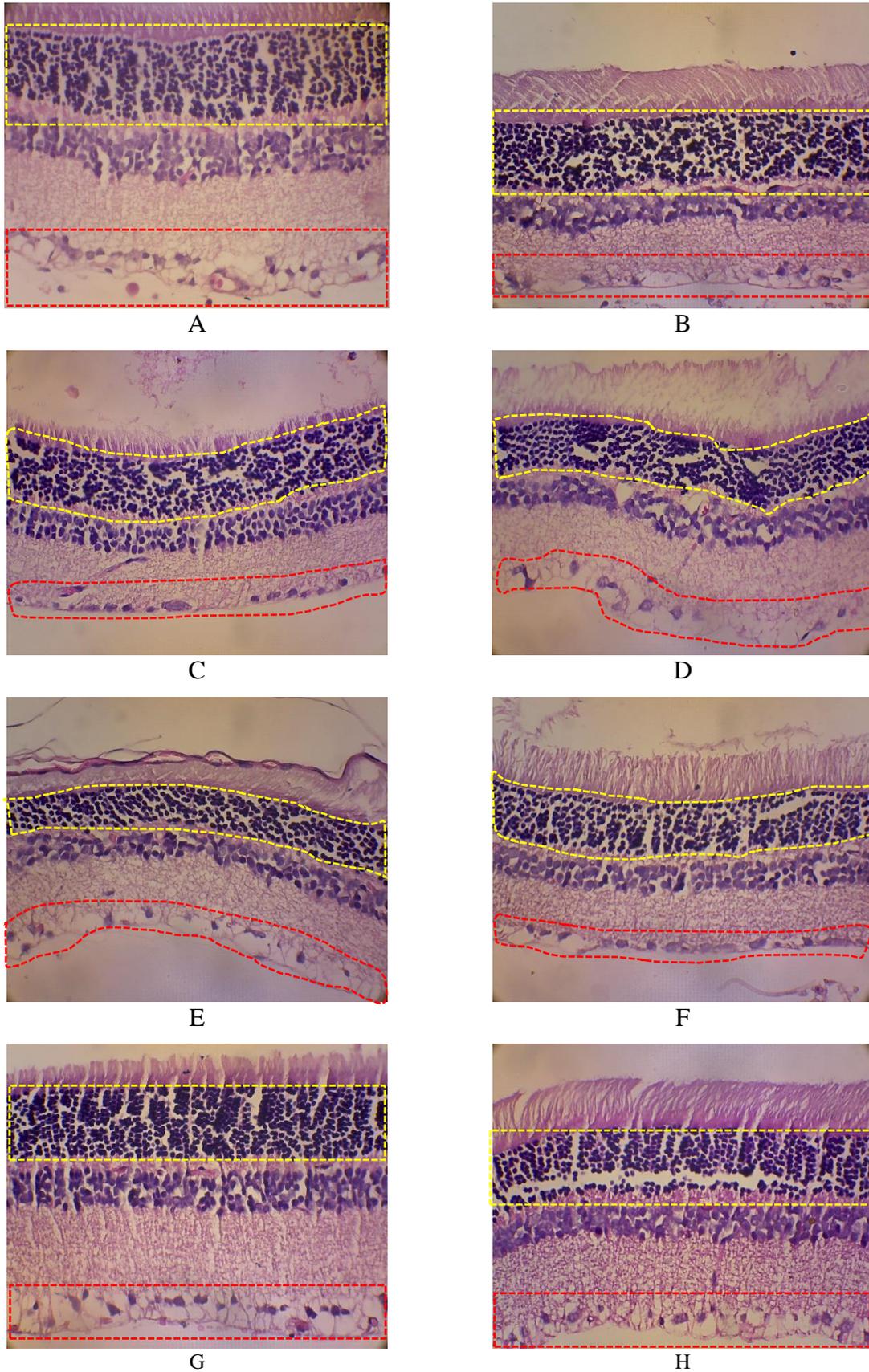4   **Figure. 1.** A. Photoreceptor cells density; B. Ganglion cells density.

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.

10   **C**

A

B

C

D

E

F

G

H

11   **Figure. 3.** HE staining for cell density. A = group 1, B = group 2, C = group 3, D = group 4, E = group 5,
12   F = group 6, G = group 7, H = group 8; Yellow line: photoreceptor cell's nucleus; Red line: retinal ganglion
13   cells.

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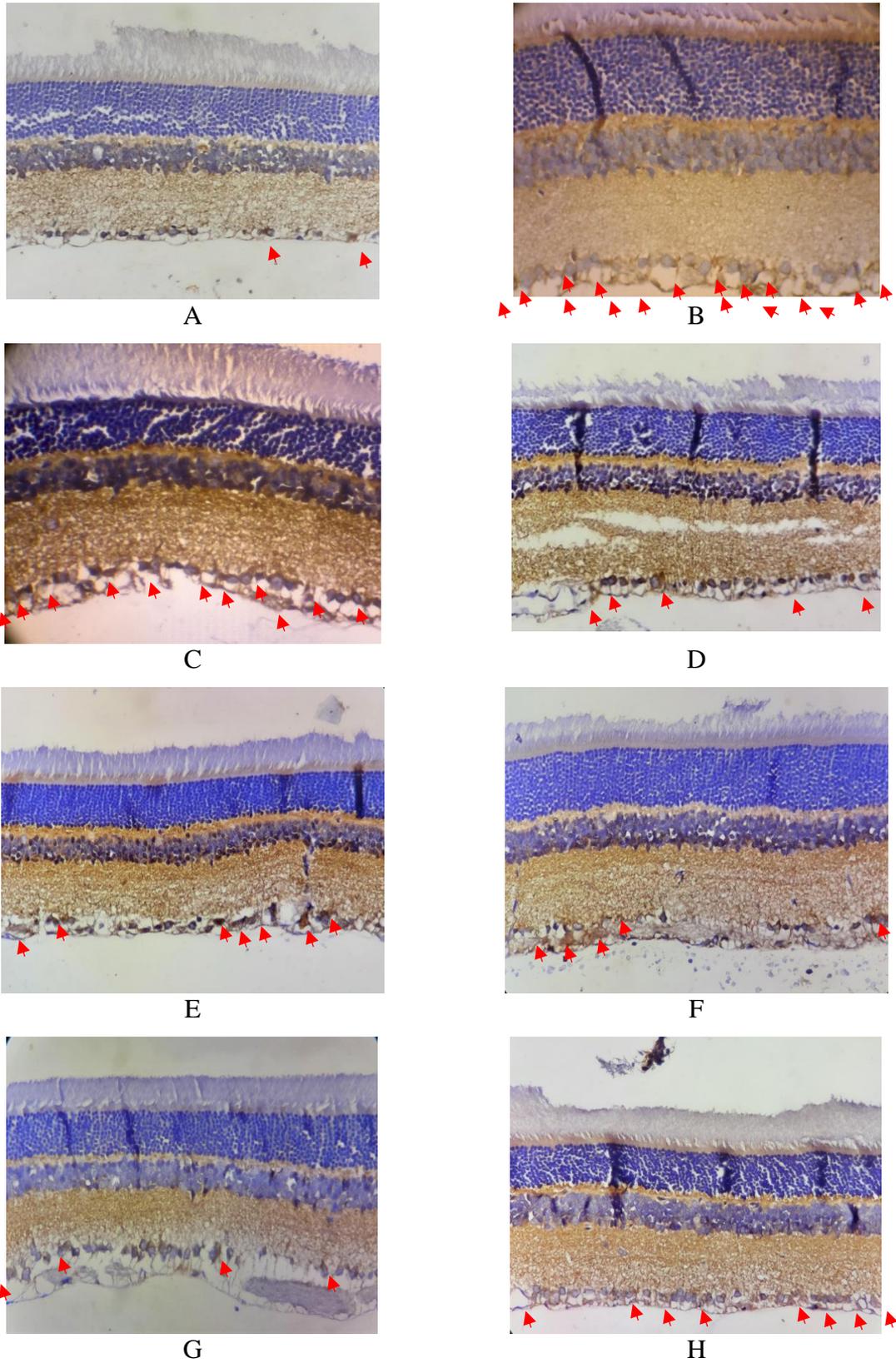
Figure. 4. Immunohistochemistry staining for Caspase-3 expression. A = group 1, B = group 2, C = group 3, D = group 4, E = group 5, F = group 6, G = group 7, H = group 8. The red arrow shows caspase 3

18   **E**

A

B

C

D

E

F

G

H
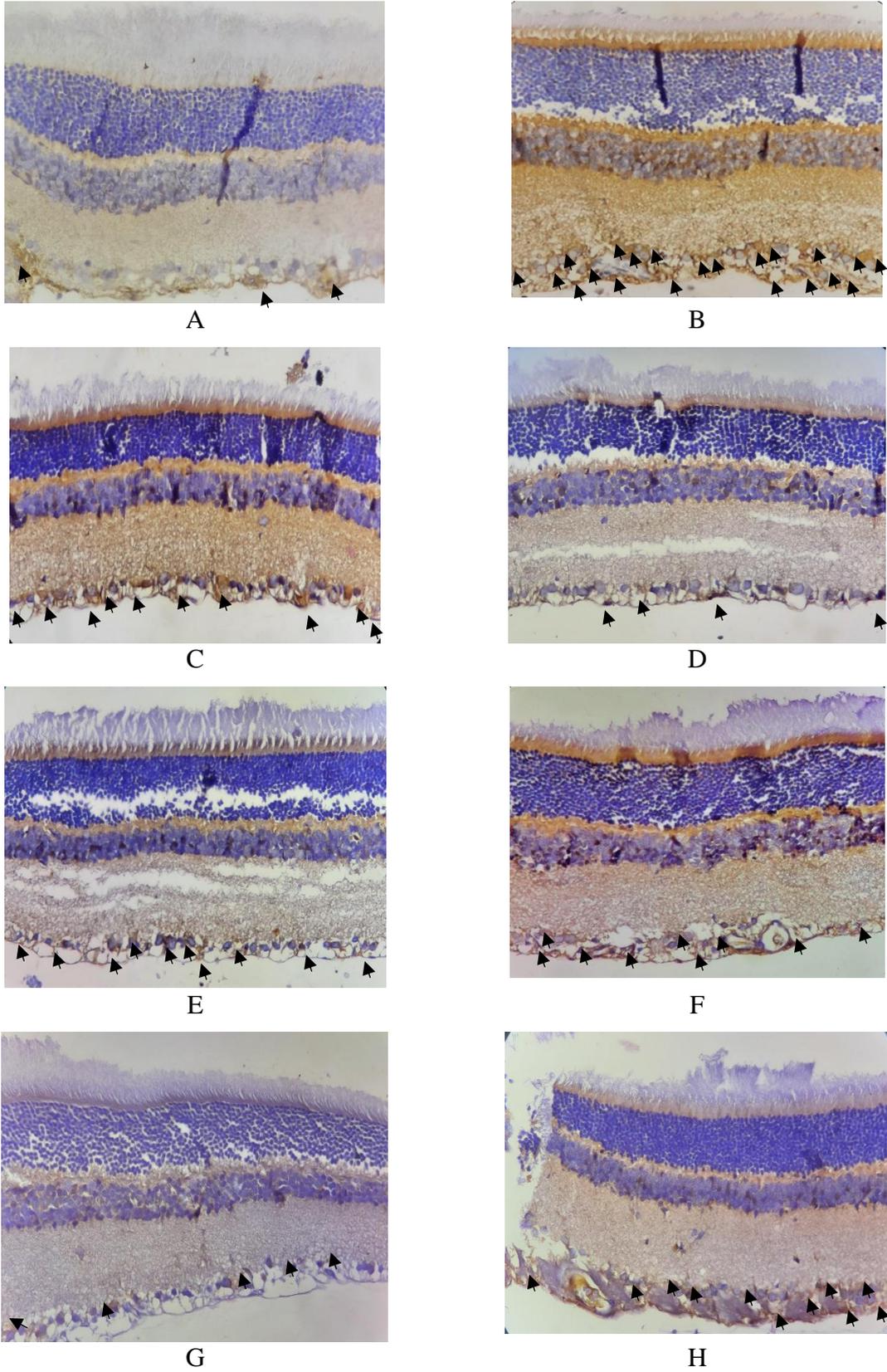
19   **Figure. 5.** Immunohistochemistry staining for Caspase-7 expression. A = group 1, B = group
20   2, C = group 3, D = group 4, E = group 5, F = group 6, G = group 7, H = group 8. The black
21   arrow shows caspase 7
22

RESPONSE TO REVIEWERS

<mark>Reviewer #1:</mark>

Abstract:

1. In the Methods, please replace BW by twice per week. This frequency is not mentioned in the Methods section of the manuscript. Please mention it.
   <mark>Response:</mark>
   We have revised this point into "Alloxan 150mg/kgBW single dose was used to develop animal models". The abbreviation "BW" we meant in the previous version was "body weight". In this study, we only did once injection intraperitoneally without any repetitions, so we did not write 'twice per week' in the latest version but rather 'a single dose injection' (revised manuscript line 27-28).

2. "…which were then separated into eight groups and treated with retinol, α-tocopherol, or a combination of both". It seems that all eight groups were treated (please rewrite this).
   <mark>Response:</mark>
   We have made improvements to this sentence by describing in detail that sample separated into eight groups consisted of one negative control group without any intervention, one positive control group which only induced with alloxan without retinol or tocopherol supplementation and six groups that given retinol, α-tocopherol, or a combination of both (revised manuscript line 29-31).

3. Hematoxylin-eosin and immunohistochemistry do not need to be abbreviated in the Abstract.
   <mark>Response:</mark>
   We have removed the abbreviation (revised manuscript line 32-33).

4. Please state that density of photoreceptor and ganglion cells will be evaluated as well as caspase-3 and caspase-7 expression (in this case, to evaluate apoptosis).
   <mark>Response:</mark>
   We have revised this sentence by writing that "Histopathological examination on retinal layers was performed using Haematoxylin-Eosin staining to evaluate the photoreceptor and retinal ganglion cell densities and Immunohistochemistry staining to evaluate caspase-3 and 7 expressions on photoreceptor and retinal ganglion cell as apoptotic markers" (revised manuscript line 31-34).

Background:

1. Please include a sentence that explains that alloxan can be used to induce diabetic models.
   <mark>Response:</mark>
   We have added a paragraph to explain the role of alloxan to induce hyperglycemia. In the manuscript it has been written that "The most often utilized diabetogenic drugs are alloxan and streptozotocin.(Ighodaro et al., 2017) Alloxan is a highly potent diabetogenic cyclic-urea derivative. Alloxan generates reactive oxygen species (ROS) in a cyclic reaction with its reduction product, dialuric acid, in the presence of intracellular thiols, particularly glutathione. Alloxan's beta cell toxicity is begun by the free radicals produced during this redox reaction. Autoxidation of dialuric acid produces superoxide radicals (O2), hydrogen peroxide (H2O2), and hydroxyl radicals in a final iron-catalyzed reaction step (OH-). These hydroxyl radicals ultimately cause beta cells to die due to their innately limited ability for antioxidative defense and the resulting state of insulin-dependent "alloxan diabetes." As a thiol reagent, alloxan inhibits glucose-induced insulin secretion selectively by oxidizing functionally important thiol

1

groups in the glucokinase protein, disrupting oxidative metabolism and the glucose sensor function of this beta cell signaling enzyme (Lenzen et al., 1996). The dose of alloxan used to cause diabetes in rats varies between 40-200 mg/kgBW via intraperitoneal or intravascular route (Sheriff et al., 2020)" (revised manuscript line 56-67).

2. Please include a sentence that explains why caspase-3 and caspase-7 expression will be evaluated. It is well explained in the Discussion section, but it could briefly mentioned in the Background section.
<mark>Response:</mark>
In our manuscript, we have added information that explains why caspase-3 and caspase-7 expression will be evaluated in our study (revised manuscript line 73-77).

Caspases are a family of endoproteases that provide critical links in cell regulatory networks controlling inflammation and cell death. Caspases are a family of genes important for maintaining homeostasis through regulating cell death and inflammation. Caspases involved in apoptosis have been subclassified by their mechanism of action and are either initiator caspases (caspase- 8 and -9) or executioner caspases (caspase-3, -6, and -7) (McIlwain et al., 2015). The cell death machinery is evolutionarily conserved and composed of caspases and their regulatory components that include activators and repressors. These key components of the death machinery are linked to signalling pathways that are activated by either ligation of death receptors expressed at the cell surface or intracellular death signals. (Nuñez et al., 1998) In this process, caspase-3 and -7 regulate mitochondrial events in the apoptotic pathway (Kuribayashi et al., 2006).

Caspase-3 with caspase-7 was considered to be similar because these related cysteine proteases share an optimal peptide recognition sequence and have several endogenous protein substrates in common. In addition, both caspases are proteolytically activated by the initiator caspase-8 and -9 during death receptor- and DNA-damage-induced apoptosis, respectively. However, a growing body of biochemical and physiological data indicate that caspase-7 also differs in significant ways from caspase-3 (Lamkanfi & Kanneganti, 2010).

Caspase-3 is mostly activated in disorders resulting in photoreceptor degeneration (Zeiss et al., 2004). The caspase-3 inhibitor was transiently effective in delaying retinal degeneration through inhibition of the apoptosis of photoreceptor cells in rd gene-carrying mice. The use of caspase-3 inhibitors may have therapeutic applications in the treatment of human retinal degeneration (Yoshizawa et al., 2002). It is also similar with another study by Choudhury (2015) who was indicate that caspase-7 plays a critical role in optic nerve injury-induced RGC death, and inhibition of caspase-7 activity may be a novel therapeutic strategy sfor some neurodegenerative diseases of the retina (Choudhury et al., 2015).

In this study, we examined caspase-3 and caspase-7 expression in diabetic rats to determine the effect of retinol and α-tocopherol supplementation. The purpose of this study is to evaluate cell death condition that occurs as a result of alloxan-induced diabetes, also the effect of retinol and α-tocopherol supplementation on cell apoptosis inhibition. Because we did not conduct theoretical studies on the mechanism by which alloxan induces apoptosis, we did not employ caspase-8 or -9 as biomarkers in this work. Moreover, we use caspase-3 and caspase-7 due to their similarities in biomechanism of action, so the study results will be more accurate and could be validated.

3. Neurodegeneration is not a symptom of diabetic retinopathy. Please correct this.
   We have corrected the statement and wrote "Neurodegeneration is one of the underlying pathomechanism for DR and is often found to precede the visible vasculopathy" (revised manuscript line 49-50).

Methods:
1. Where was the study conducted?
   This study was conducted at the Animal Laboratory and Pathology Laboratory of Hasanuddin University, Indonesia (revised manuscript line 113-114).

2. What was the study period?
   Experimental study was conducted from June to November 2021.

3. Was there an animal Ethics Committee approval?
   This study was received an ethical approval from The Ethics Committee of Medical Research, Faculty of Medicine, Hasanuddin University with approval number: 725/UN4.6.4.5.31/ PP36/2021 (revised manuscript line 114-116 and 381-383). In this ethics commission there is a division that specifically oversees animal research and has worked in accordance with the protocol of the institutional animal care and use committee (IACUC).

4. "Retinol compounds up to 900 mcg/day were administered to groups 3 and 6 and α-tocopherol compounds up to 15mg/day were provided to groups 4 and 7". This dose is probably dependent on the animal weight. Please clarify this.
   In this study we used the recommended dose according to the recommended dose allowance (RDA) standard for α-tocopherol and retinol activity equivalents (RAE) for retinol. In these studies, there are no difference in the dose given referring to the body weight. Therefore, all experimental animals in this study were given a uniform dose of retinol 900mcg/day and 15mg/day for α-tocopherol (National Institues of Health, 2020; Rasmussen & Johnson, 2013) (revised manuscript line 138-141).

5. "To calculate the density of retinal ganglion and photoreceptor cells, retinal tissue was cut using a microtome with a thickness of 5m". Is this measurement correct?
   Thank you for your accurate correction. We have revised this section by writing "To calculate the density of retinal ganglion and photoreceptor cells, retinal tissue was cut using a microtome with a thickness of 5μm" (revised manuscript line 150).

Results
1. The results were mainly presented in tables and graphics. They could also be better explained in a text.
   Thank you for your kind advice. In our revised manuscript, we have added some explanations about the results of this study, including cell densities and the expression of caspase-3 and caspase-7 in each group (revised manuscript line 169-199).

Table 1 showed that the photoreceptor cell density showed the highest value in group 1 while the lowest value was shown in group 2. Furthermore, in the treatment group, the most effective value for approaching the normal group of mice was shown in group 7 that received α-tocopherol supplementation for 14 days (pre and post alloxan induction). The statistical test results showed that there was a significant difference in the photoreceptor cell density among groups (p=0.002). It also shown in figure 1.a.

This result is in line with the measurement of ganglion cell density where the highest value was also obtained in group 1 and the lowest value in group 2, while for the treatment group, the most effective supplementation was shown in group 7 (Fig 1.b). The results of the statistical test showed that there was a significant difference in the photoreceptor cell density among groups (p=0.010).

In calculating the expression value of caspase-3, apoptosis in photoreceptor cells showed the lowest in group 1, while the highest expression value was found in group 2. In the treatment group, values close to the normal group were shown by groups 4 and 7 (a-tocopherol supplementation group). It also shown in figure 2a. Statistical analysis showed that there was a significant difference in caspase-3 expression among groups (p=0.016).

The above results are in line with the results of expression calculations in ganglion cells where the lowest value was found in group 1, while the highest value was found in group 2. The results of observation in the treatment group were found to be the most effective in the combination group of retinol and α-tocopherol for 14 days (pre and post alloxan induction). It also shown in figure 2b. Statistically, these results also showed a significant caspase-3 expression among groups (p=0.010).

The expression value of caspase-7, apoptosis in photoreceptor cells showed the lowest in group 1, while the highest expression was found in groups 5, 6 and 8. Observations in the treatment group indicated that groups 4 and 7 (supplementation of α-tocopherol for 7 and 14 days) showed the lowest expression. Based on these results, it was obtained statistically that this result did not show a significant difference on caspase-7 expression among groups (p = 0.069). The value of caspase-7 expression in ganglion cells showed the lowest value in group 1, while the highest value was found in group 2. In the treatment group, the expression values that close to the normal group were samples in groups 4 and 7, respectively. This statistically indicated that there was a significant difference between caspase-7 expression among groups with (p=0.010). It also shown in figure 2c and 2d.

2. Figure 3: please show the photoreceptors and ganglion cells.
   Response:
   In the figures file that we sent, we have indicated a "yellow line area" as outer nuclear layer of the retina which contains the nucleus of photoreceptor cells, and a "red line area" that indicating the area of retinal ganglion cells (Figure revision file, page 3).

Discussion
1. This section is excessively long and includes many theoretical concepts. This section repeats some concepts presented in the Background section.
   Response:
   We have restricted and summarized this paper by eliminating some repetitive data or information. In the discussion section, we have removed the theoretical explanation of cataractogenesis and retinal photodeterioration, the pharmacodynamics of tocopherols, hyperglycemia-induced oxidative stress, pathophysiological cause of ectopic

4

202	neovascularization in DR and all other explanations that are not related to the scope of this
203	study or the paragraphs previously explained in the introduction.
204	
205	2. Please, make this section more succinct and try to emphasize the discussion of the study
206	results.
207	<mark>Response:</mark> In our manuscript, detailed informations have been added to explain the
208	comparative analysis of retinal cell density and the expression of caspase-3 and -7 obtained in
209	this study. Those are written in the revised manuscript line 235, 253, 277, 283, 316, 322, 332,
210	and 343. In the revised manuscript, we have emphasized the discussion of our study results.
211	Hope it could fulfil the reviewer's requirement.
212	
213	3. What are the study limitations?
214	<mark>Response:</mark>
215	In our study, the limitations were:
216	1. The unavailability of experimental animal strains that match the type 2 diabetic rat model.
217	In our research center, there is only one Rattus novergicus pure strain rats. Therefore, in
218	this study only a rat model of type 1 diabetes could be obtained.
219	2. In this study, we used a standard dose according to the RDA without giving a graded dose.
220	This is a suggestion for further research in order to evaluate the most effective dose that
221	can be used as a standard for prevention therapy of diabetic retinopathy progression.
222	
223	Conclusions
224	It would be better to state that further studies, including in humans, are needed to establish
225	the definite role of tocopherol therapy in the care of diabetic retinopathy.
226	<mark>Response:</mark> We agree with your suggestion. In this section, we have added a statement about
227	the importance of further studies to get the best results in this study. In the manuscript, we
228	have written that "Retinol and α-tocopherol compounds have a protective effect of
229	maintaining the retinal cells' densities and preventing the cells from apoptotic process.
230	Moreover, the α-tocopherol compound showed better results compared to retinol compound
231	or a combination of both. Future studies including in humans are needed to demonstrate the
232	better understanding of α-tocopherol supplementation in preventing diabetic retinopathy
233	progression" (revised manuscript line 355-359).
234	

1. Congratulations by your extensive review and data about the physiopathology of Diabetic Retinopathy.Although, you should adequate your paper to bring us the information in a lighter and fluider way. It is too much dense and we could even find your results in the text.
   Response:
   We have restricted and summarized this paper by eliminating some repetitive data or information. In the discussion section, we have removed the theoretical explanation of cataractogenesis and retinal photodeterioration, the pharmacodynamics of tocopherols, hyperglycemia-induced oxidative stress, pathophysiological cause of ectopic neovascularization in DR and all others explanations that are not related to the results of this study or the paragraphs previously explained in the introduction. Moreover, in our manuscript, detailed informations have been added to explain the comparative analysis of retinal cell density and the expression of caspase-3 and -7 obtained in this study. Those are written in the revised manuscript line 235, 253, 277, 283, 316, 322, 332, and 343. In the revised manuscript, we have emphasized the discussion of our study results. Hope it could fulfil the reviewer's requirement.

2. You should correct your title, the word "Photoreceptor" is missing a letter;
   Response:
   We have corrected this word in the revised manuscript line 2.

3. You should input, necessarily, your results at the "Results" area;
   Response:
   In our manuscript, we have added some explanations about the results of this study, including the blood sugar levels, cell densities and the expression of caspase-3 and caspase-7 in each group (revised manuscript line 169-199).

4. Your figures are not mentioned on the text, so it is not possible to understand it.
   Response:
   We have written a description of the image in the results section and the discussion section. Figure 1.a (line 176), Figure 1.b (line 179), Figure 2.a (line 183), Figure 2.b (line 189), Figure 2.c (line 198) and 2.d (line 198), Figure 3 (line 276), Figure 4 and 5 (line 300-301).

Reviewer #3:

1. Please provide details as to how severe and fatal mortality associated with alloxan induced animal models was prevented? Did any of the animals succumb during the induction of diabetes?
   Response:
    At the beginning of this study, we used 10 rats as a pre-eliminary study sample. We used the upper limit dose for alloxan induction which was 200mg/kg body weight (BW) referring to the study by Ighodaro (2017). After 14 days observation, 4 out of 10 rats were died. After that, we decreased the dose of alloxan induction to 150mg/kgBW and observed there was no animal subject died after 14 days observation. Therefore, in the main study, we used a single dose of 150mg/kgBW intraperitoneally. In our experimental study, there are 4 out of 40 rats died during data collection (1 rat in each group 3, 4, 5 and 8). Moreover, blood glucose follow-up was carried out on the second, fifth and seventh days after induction and showed consistent values above 200 mg/kgBW (revised manuscript line 131-135).

2. Alloxan induced diabetes is multiphasic and the chances of achieving stable hyperglycaemia after 1 month is considered limited? How then were all animals found to be consistently hyperglycemic at the study interval of 4- 6 weeks?
    Response:
    In this study, we treated the animal models only for 14 days whereas the hyperglycemia condition basically only lasted for 7 days. The induction process is illustrated as follows:
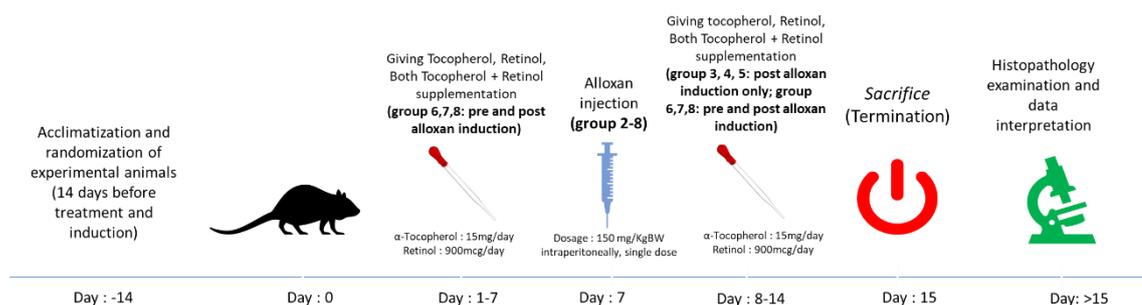
    In our study, all experimental animals were induced on the same day (day-7). Groups 3, 4 and 5 were supplemented for 7 days after induction, while groups 6, 7 and 8 were supplemented for 7 days before and 7 days after alloxan induction. Therefore, maintenance of hyperglycaemic conditions was only carried out for 7 days for the entire treatment group (revised manuscript line 120-128).

3. Alloxan itself induces damage to pancreatic beta cells by ROS damage? Why is it not possible that a similar damage is being induced in the retina, and hence unrelated to the presumed hyperglycaemia induced damage?
    Response:
    Based on Rohila and Ali (2012), they reported that alloxan's cytotoxic effect is mostly due to the production of reactive oxygen species (ROS). Alloxan and its reduction product, dialuric acid, initiate a redox cycle by producing superoxide radicals. These radicals are dismutated to generate hydrogen peroxide (H_2O_2), followed by the creation of more reactive hydroxyl radicals. Then, the concentration of cytosolic calcium rises dramatically, resulting in the fast death of beta cells in pancreatic islets.(Rohilla & Ali, 2012) Moreover, based on El-Esawy (2016), alloxan-induced hyperglycaemia has been described as a useful experimental model to study the activities of hypoglycemic agents because it selectively destroys the pancreatic ß-cells of rats (El-esawy et al., 2016).

Besides, according to Bouterse and Kowluru (2008), in the retina, oxidative stress is considered as one of the crucial contributors in the pathogenesis of diabetic retinopathy, but oxidative stress appears to be highly interrelated with other biochemical imbalances that lead to structural and functional changes and accelerated loss of capillary cells in the retinal microvasculature and, ultimately, pathological evidence of the disease. Therefore, tissue damage to the pancreas tends to be more severe than damage in the retinal tissue.(Bouterse & Kowluru, 2008)

4. Alloxan induces features of type 1 diabetes and not type 2. How then would the results be applicable to type 2 diabetes, which constitutes more than 90-95% of all diabetics?
   Response:
   Induction of type 2 diabetes is generally carried out using rodent strains Lepob/ob mouse, Leprdb/db mouse, Zucker Diabetic Fatty (ZDF) rat, C57BL/6J mouse, TALLYHO/Jng mice, and the KK-Ay mouse (Fang et al., 2019). In this study we had limitations on the availability of experimental animals in our research center. In our center, we only have a pure strain of rattus novergicus, so it is quite difficult to find a type 2 DM model. Moreover, study in type 2 DM need a very long time to reach the DR model when using this strain. According to study by Kern and Engerman (1994), it is known that diabetic retinopathy was present in rats who having insulin-deficient diabetes for 18-22 months. Lesions included pericyte ghosts, acellular capillaries, and thickened retinal capillary basement membrane (Kern & Engerman, 1994). However, we believe that the results of this study can still be used to represent patological condition with type 2 diabetes because both diseases are based on a similar pathway (hyperglycemia).

5. The results are exceedingly brief but the introduction and discussion are exceedingly redundant. Please keep these sections more concise and to the point.
   Response:
   We have restricted and summarized this paper by eliminating some repetitive data or information. In the discussion section, we have removed the theoretical explanation of cataractogenesis and retinal photodeterioration, the pharmacodynamics of tocopherols, hyperglycemia-induced oxidative stress, pathophysiological cause of ectopic neovascularization in DR and all others explanations that are not related to the results of this study or the paragraphs previously explained in the introduction. Moreover, in our manuscript, detailed informations have been added to explain the comparative analysis of retinal cell density and the expression of caspase-3 and -7 obtained in this study. Those are written in the revised manuscript line 235, 253, 277, 283, 316, 322, 332, and 343. In the revised manuscript, we have emphasized the discussion of our study results. Hope it could fulfil the reviewer's requirement.

6. What is the sensitivity of the methods used to measure thickness of relevant cellular layers? Were the results blinded and repeated? How was the possibility of bias eliminated?
   Response:
   - Quantitative approach was used to interpretate cell density using an Olympus CX23 binocular microscope with 40-fold objective magnification, and the results were expressed as a mean with standard deviation. Besides, the intensity of caspases expression in photoreceptor cells was categorized qualitatively using the Immunoreactive Scoring System (IRS) modification method. To get an objective standard, counting measurement are carried out by three examiners by discussed and objectified the calculation results based on published references.
   - In this study, the research team came from multidisciplinary fields where only the ophthalmology department team knew the type of treatment given to each group. The

pathologists who perform tissue examination did not know the treatment group being examined, so this maintains the objectivity of the examination.
- All bias factors were eliminated.
  - Selection/sampling bias in this study was restricted by homogenizing the sample. All of the selected experimental animals were similar in terms of age, weight, and location of origin. Experimental animals were also acclimatized for 2 weeks and then randomized before the treatment was carried out.
  - We have eliminated design bias by conducting a structured research method before starting the research. It is also based on related references to achieve audit objectivity.
  - Measurement bias is eliminated by using the same inspection methods and tools. In addition, we conclude the results of the examination through a discussion process involving three examiners. So that subjectivity and interobserver bias can also be minimized.
  - Performance bias is eliminated by calibrating each tool being inspected. We carried out calculations and data analysis using a computerized system where it was also concluded based on the results of the discussions of the entire research team.

7. Was the study sponsored by any company related to the manufacture/ marketing of tocopherol?
   Response:
   In this study, we did not collaborate with manufacturers or distributors of tocopherol products. This research uses synthetic retinol and α-tocopherol compounds which are ordered independently without cooperating with each other.

   For additional information, based on the result of this study, we will continue further research by analyzing the effect of α-tocopherol from local Indonesian plant sources to be used as candidate for supplementation in the prevention of retinal apoptosis in diabetic rat.

   The final goal of this series experiment is the creation of herbal product for the prevention of diabetic retinopathy progression that could be applied in diabetic patients.

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COVER LETTER

To
The Editor,
International Journal of Retina and Vitreous

I am enclosing herewith a **<u>revised manuscript</u>** 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;
4. The contents of this manuscript are not now under consideration for publication elsewhere;
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,

Andi Muhammad Ichsan
Department of Ophthalmology, Medical Faculty, Hasanuddin University
Makassar, Indonesia

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

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ABSTRACT

Background: Diabetic retinopathy (DR) is the most common microvascular complication of diabetes. Retinol and α-tocopherol of diabetic models prevent the damage of photoreceptor and retinal ganglion cells (RGC) caused by hyperglycemia. **Objective:** This study aims to examine the effect of retinol and α-tocopherol on photoreceptor and RGC densities with the expression of caspase-3 and -7 on the layers of the diabetic rat model. **Methods:** Alloxan 150 mg/kgBW single dose was used to develop animal models, which were separated into eight groups. These consist of one group without intervention (1), one positive control only induced with alloxan (2), and others receiving retinol (3 and 6), α-tocopherol (4 and 7), or their combination (5 and 8). Furthermore, histopathological examination was performed using Hematoxylin-Eosin staining to evaluate the photoreceptor and RGC densities, while immunohistochemistry staining evaluated the caspase-3 and -7 expressions. **Results:** In the treatment group, the highest and lowest densities were identified in diabetic rats given α-tocopherol (group 7) and retinol (group 3). The caspase-3 and -7 expression showed that the group given α-tocopherol (group 7) had the lowest value. **Conclusion:** In diabetic rats, retinol and α-tocopherol compounds maintained densities and prevented photoreceptor as well às RGC death. However, α-tocopherol was more promising than retinol or cell densities and caspase expression combinations.

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 worldwide (Song & Wong, 2014). Based on the abnormalities of the retinal microvasculature, it is a microvascular complication of diabetes. However, a recent pathophysiological model has highlighted that neurodegeneration is a crucial and early component of this complication. Neural apoptosis, response gliosis, glutamate excitotoxicity, the decline in neuroprotective components, and debilitation of the neurovascular coupling are depicted as causes of retinal neurodegeneration. One of the underlying pathomechanisms for DR found to precede visible vasculopathy is neurodegeneration (Jonsson *et al*., 2016). Previous study showed that the neuronal unit of the retina and DR are strongly related because retinal neurons and glial cells demonstrate biochemical defects and functional abnormalities. This involves rapid neuronal death, microglial cell activation, and enhanced oxidative stress generation by photoreceptors (Kowluru & Mishra, 2015).

The most often utilized diabetogenic drugs are alloxan and streptozotocin (Ighodaro et al., 2017). Alloxan is a highly potent diabetogenic cyclic-urea derivative that can generate reactive oxygen species (ROS) in a cyclic reaction with its reduction product, dialuric acid, in the presence of intracellular thiols, particularly glutathione. The beta cell toxicity is begins by the free radicals produced during the redox reaction. Autoxidation of dialuric acid produces superoxide radicals (O_2-), hydrogen peroxide (H_2O_2), and hydroxyl radicals in a final iron-catalyzed reaction step ($OH-$). These hydroxyl radicals ultimately cause beta cells to die due to their innately limited ability for antioxidative defense and the resulting state of insulin-dependent alloxan diabetes. As a thiol reagent, alloxan inhibits glucose-induced insulin secretion selectively by oxidizing important thiol groups in the glucokinase protein, disrupting oxidative metabolism and this beta-cell signaling enzyme (Lenzen et al., 1996). The dose used

66   to cause diabetes varies between 40-200 mg/kgBW through the intraperitoneal or intravascular

67   route (Sheriff et al., 2020).

68   Caspases involved in apoptosis have been subclassified by their mechanism of action and

69   are either initiator (caspase- 8 and -9) or executioner (caspase-3, -6, and -7) (McIlwain et al.,

70   2015). Caspase-3 with -7 are similar because the cysteine proteases share an optimal peptide

71   recognition sequence and have several endogenous protein substrates in common. In addition,

72   they are proteolytically activated by the initiator caspase-8 and -9 during death receptor- and

73   DNA-damage-induced apoptosis (Lamkanfi & Kanneganti, 2010). Caspase-3 and -7

74   expression as apoptotic markers might be used to investigate the alterations in the retina

75   following the diabetes condition. These are two of the essential caspase effectors in apoptotic

76   pathways, and the indicators can assess the level of tissue damage caused by the induction

77   agent (Kowluru & Koppolu, 2002; Lamkanfi & Kanneganti, 2010).

78   The amounts of damaged DNA and nitrosylated proteins are higher in the diabetic retina

79   due to increased oxidative stress (OS) and compromised antioxidant defense enzymes. Diabetic

80   experimental animals and humans have a lower level of antioxidant enzymes and vitamins

81   2022/4/13. Antioxidants can be used to alleviate metabolic and functional abnormalities as a

82   result of the close relationship between OS and dysmetabolism associated with the

83   pathogenesis of DR. They can work on various levels, such as inhibiting the generation of

84   reactive oxygen species (ROS), lowering free radicals, or enhancing enzyme capacities. The

85   finding demonstrated that medicinal and aromatic plants' dietary or local bio factors could help

86   manage diabetes. OS triggers other unfavorable pathways to DR development and causes a

87   vicious circle of injury to macromolecules by magnifying additional ROS. Therefore, OS and

88   ROS are considered to have a role in DR by increasing glucose and significant metabolic

89   abnormalities (Madsen-Bouterse & Kowluru, 2008; Silva et al., 2010).

90   The extensive investigation of vitamins A, C, E, and carotenoids are well-known

91   antioxidants produced from food. Antioxidants can limit the generation of reactive oxygen

92   species (ROS), scavenge free radicals, or boost the enzyme capabilities to reduce oxidative

93   stress-induced damage to the retina (Silva *et al*., 2010).

94   Retinol, retinal, retinoic acid, and provitamin A carotenoids are unsaturated nutritional

95   chemical molecules that make up vitamin A (Zhong *et al*., 2012). Painstaking biochemical

96   reconstitution experiments have enabled recent improvements in the molecular knowledge of

97   the retinoid cycle in the mammalian retina. Furthermore, natural or synthetic animal models

98   with known genetic lesions backed this claim with human studies of target genetic blinding

99   diseases. Critical retinal enzymes and proteins as well as their substrates and ligands have been

100   identified using structural and membrane biology in a cellular context (Kiser & Palczewski,

101   2016). In a reversible reaction catalyzed by the reduced nicotinamide adenine dinucleotide

102   phosphate (NADPH) -dependent all-trans-retinol dehydrogenase, all-trans-retinal in the

103   cytoplasm were degraded to all-trans-retinol. This product diffuses into the retinal pigment

104   epithelium, which is esterified by lecithin retinol acyltransferase (LRAT) (Palczewski, 2010).

105   Meanwhile, in vitro and in vivo studies showed a protective impact of α-tocopherol, a vitamin

106   E derivative, on eye tissues. For up to 24 hours of exposure, a biomolecular compound of α-

107   tocopherol can protect the retina against light damage (Ritch, 2007). Therefore, this study

108   aimed to investigate the protective effect of retinol and α-tocopherol on the photoreceptor and

109   retinal ganglion cells apoptosis in a diabetic rats model.

110

111   **METHODS**

112   **Design**

113   This study was conducted with a post-test group of forty animal subjects at the Animal and

114   Pathology Laboratory of Hasanuddin University, Indonesia. Alloxan monohydrate (SIGMA

USA, Cat. No. A7413) 150 mg/kgBW single dose intraperitoneally was used to induce the diabetic model. Supplementation was performed with retinol (SIGMA USA, Product No. R7632, CAS Number: 68-26-8) and α-tocopherol (SIGMA USA, Cat. No.258024) compounds. Furthermore, eight groups of animals were created, where 1 is the negative control (wild type), 2 is the positive control (alloxan induction without treatment), 3 describes diabetic rats on retinol for 1 week (after alloxan induction), 4 represents the diabetic rats on α-tocopherol for 1 week (after alloxan induction), 5 is the sample given a combination of retinol and α-tocopherol for 1 week (after alloxan induction), 6 represents diabetic rats on retinol for 14 days (1 week each, before and after alloxan induction), 7 describes the samples given α-tocopherol for 14 days (1 week each, before and after alloxan induction), and 8 is the combination of retinol and α-tocopherol for 14 days (1 week each, before and after alloxan induction).

Established animal experiment

Male Wistar rats (*Rattus norvegicus*) of 8-12 weeks old, weighing 160-200 g, were used for this study. All animals were given standard feed and access to *ad libitum* drinking water in a room with a 12-hour light-dark cycle. Each experimental animal in groups 2-8 received 150 mg/kg body weight an intraperitoneal injection of Alloxan monohydrate. Induction was considered successful where blood glucose levels were >200 mg/dl. Furthermore, blood sugar measurements were performed three times before alloxan injection, 3 days later, and a day before sacrifice. All samples in group 1 had a blood glucose level <200 mg/dl, while those in groups 2 to 8 had a blood glucose level >200 mg/d after being induced. Retinol compounds up to 900 mcg/day were administered to groups 3 and 6 (National Institutes of Health, 2020). α-tocopherol compounds up to 15 mg/day were also provided to groups 4 and 7 (Rasmussen & Johnson, 2013), while 5 and 8 received both.

Sample collection and processing

The rats were sacrificed before enucleation by placing in a closed container filled with cotton and ether for approximately ten minutes until there was no motoric reaction, neurological reflexes, or heartbeat. Subsequently, 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. Finally, all eyes were fixed with 10% formalin and transported to the pathology laboratory.

Retinal tissue was cut using a microtome with a thickness of 5 µm and stained with hematoxylin and eosin (HE) to calculate the density of ganglion and photoreceptor cells. Caspase-3 (Cat No. C9598, Sigma USA) and -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. Immunohistochemistry staining was conducted using a primary and secondary antibody (Cat. No. UCS015-IFU, ScyTek USA) to identify caspase-3 and -7. The intensity of expression in photoreceptor cells was categorized qualitatively using the Immunoreactive Scoring System (IRS) modification method. The three categories are negative (caspase expression shows <5%), low (5-20% expression), and high (>20% expression). Meanwhile, the intensity of caspase expression in retinal ganglion cells was calculated quantitatively by counting the number of cells and apoptotic bodies.

Data Analysis

Statistical analysis used an Independent T-test and Kruskal Wallis for the quantitative and qualitative data (sig. $p < 0.05$).

RESULTS

According to Table 1, the blood sugar level of the negative control was 82±2 mg/dl compared to the diabetic groups (276±15 to 426±45 mg/dl). This showed that the experimental animal could be used as a model for type 1 diabetes rats because they have hyperglycemic conditions.

The photoreceptor cell density showed the highest and lowest value in groups 1 and 2, respectively. In the treatment group, the most effective value for approaching the normal group of mice was in 7, which received α-tocopherol supplementation for 14 days (pre and post-alloxan induction). The statistical test results showed a significant difference in the photoreceptor cell density among groups (p=0.002), as shown in Figure 1. a. This result is in line with the measurement of retinal ganglion cell density, where the highest and lowest value was also obtained in groups 1 and 2, respectively. For the treatment group, the most effective supplementation was shown in group 7 (Figure 1.b). In addition, the statistical test result showed that there was a significant difference in the photoreceptor cell density among groups (p=0.010).

In calculating the expression of caspase-3, apoptosis in photoreceptor cells showed the lowest and highest expression in groups 1 and 2, respectively. Values close to the normal were shown by groups 4 and 7 (α-tocopherol supplementation group), as presented in Figure 2.a. Statistical analysis showed a significant difference in the difference caspase-3 expression among groups (p=0.016).

The above results are in line with the those of expression in retinal ganglion cells, where the lowest and highest value was found in groups 1 and 2, respectively. The observation results in the treatment group were found to be the most effective in the group given α-tocopherol supplementation (groups 4 and 7), as shown in Figure 2. b. Statistically, these results showed a significant caspase-3 expression among groups (p=0.010).

187   The expression value of caspase-7, apoptosis in photoreceptor cells showed the lowest in

188   group 1, while the highest was found in 5, 6, and 8. Observations in the treatment group

189   indicated that groups 4 and 7 (supplementation of α-tocopherol for 7 and 14 days) showed the

190   lowest expression. Based on these results, it was obtained that this result did not significantly

191   affect caspase-7 expression among groups ($p = 0.069$). The value of the expression in retinal

192   ganglion cells showed the lowest and highest value in groups 1 and 2, respectively. In the

193   treatment group, the expression values close to the standard were samples in 4 and 7,

194   respectively. Figures 2. c and d indicated a significant difference between caspase-7 expression

195   among groups ($p=0.010$).

196

197   **DISCUSSION**

198   The retina is a weak, thin layer of tissue that originates from the neuroectoderm, comprising of

199   nine layers of sensory neurons in the visual pathway (Gupta *et al*., 2015). Photoreceptors are

200   visual system sensors that transform photon capture into a nerve signal through a process

201   known as phototransduction. Photoreceptor terminals interrelate with surrounding

202   photoreceptors and interneurons of horizontal and bipolar cells. They are required for

203   transmitting visual information and early processing in the retina (Fielder & Alistair, 2011).

204   Photoreceptors in the healthy retina are among the most active oxygen consumers in the

205   body, and the choroidal circulation supplies the majority of the oxygen to photoreceptors. As

206   a result, oxygen tension drops quickly from the Bruch's membrane to the retina's outer nuclear

207   layer, where it reaches the lowest values. This reduces oxygen reserve in photoreceptors, and

208   even a minor disruption of oxygen flow in diabetes can result in severe hypoxia. The creation

209   of acellular capillaries, capillary blockage, and capillary dropout can contribute to retinal

210   hypoxia, hence, the vascular pathology of DR (Becker *et al*., 2020).

On the other layer, retinal ganglion cells process and convey information from the retina to visual centers in the brain. These output neurons comprise subpopulations with distinct structures and functions (Sernagor et al., 2001). As a result, there is a remarkable diversity of RGCs. The various subtypes have unique morphological features and pathways linking the inner retina to the relevant brain areas (Kim et al., 2021). Retinal ganglion cells carry visual signals from the eye to the brain but do not make chemical synapses with other neurons. However, they form gap junctions with other RGCs and amacrine cells, allowing RGC signals to feedback into the inner retina (Vlasiuk & Asari, 2021).

A pathogenic disease, such as diabetic retinopathy causes a decrease in the electrical activity of neurotransmitters from photoreceptors and RGC cells to the nerve fiber layer (Antonetti, 2012). DR is a duration-dependent disease infrequently discovered during the early years of diabetes. However, it substantially develops with time, nearly 90% of patients showing 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). Therefore, to protect 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).

This study showed a substantial difference in cell density between diabetic and non-diabetic rats after alloxan induction as well as supplementation with retinol and α-tocopherol substances. Retinol supplementation appeared to affect maintaining the retinal cell densities positively. However, it was not better than the α-tocopherol and combination supplementation groups. The higher density values proved this in groups 3 and 6 compared with 2. The study

by Zhong et al. (2012) reported that retinoids might create cation radicals due to interactions with different radicals or photoexcitation with light. Furthermore, 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).

In the retinoid cycle, retinol is an excellent substrate for LRAT and quickly converted into fatty acid esters. Their propensity to form oil droplets excludes fatty acid esters from circulation (Kiser & Palczewski, 2016). The mechanism of vitamin A transport is mediated by the plasma retinol-binding protein (RBP), a specific and sole carrier in the blood. The specific membrane receptor stimulated by retinoic acid 6 (STRA6) mediates cellular vitamin A uptake. (Zhong et al., 2012) Structural and membrane biology have been used to detect critical retinal enzymes and proteins as well as their substrates and ligands, placing them in a cellular context. The most presently accepted modulators of the retinoid cycle have demonstrated promising results in animal models of retinal degeneration (Kiser & Palczewski, 2016).

The α-tocopherol supplementation group was closest to the normal values for photoreceptor and retinal ganglion cell densities. A similar result was found in Ritch (2007), which stated that the α-tocopherol had been suggested to protect against retinal phototoxicity and central nervous system ischemia (Ritch, 2007). 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. By inhibiting the peroxidation of membrane lipids and scavenging lipid peroxyl radicals, it 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).

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

261 the eight forms (Gagné *et al.*, 2009). Vitamin E is crucial for erythrocytes' stability as well as

262 central and peripheral nerves conductivity. Therefore, several countries have established

263 dietary vitamin E recommendations (Péter *et al.*, 2015).

264   The loss of photoreceptors in the diabetic retina is still debatable, and various optical

265 coherence tomography (OCT) studies in diabetic patients show that the thickness of the inner

266 retina, including the nerve fiber, retinal ganglion cell, and inner plexiform layers, decreases

267 with the duration of diabetes (Becker *et al.*, 2020). In a diabetic animal model study, the outer

268 nuclear layer thickness is frequently reduced, specifically in models of type 1 disease with

269 early-onset. Furthermore, various studies supported the notion that photoreceptor loss increases

270 with disease duration (Kern & Berkowitz, 2015). A similar condition was found, where cell

271 densities significantly decreased in group 2 compared to others in photoreceptor and retinal

272 ganglion cells (Figure 3).

273   A high dose of α-tocopherol following positive results in a diabetic rat model to prevent

274 diabetes-related vascular damage was examined in the clinic for administration. This was

275 performed to restore retinal blood flow in diabetic type I patients, which was discovered to

276 control levels. Furthermore, the α-tocopherol is useful in DR by the nonenzymatic free radical

277 scavenging action outside the cell. Antioxidant therapy with α-tocopherol has been shown in

278 humans to improve retinal vascular hemodynamics (Silva *et al.*, 2010).

279   In this study, the administration of the combination of retinol and α-tocopherol did not

280 show any more effective results to maintain the retinal cell densities than the single

281 supplementation of α-tocopherol. A previous study reported that supplementation with

282 substantial doses of retinol was demonstrated to reduce the bioavailability in growing pigs and

283 calves (Hymoller *et al.*, 2016). Due to its abundance in human and animal tissues, α-tocopherol

284 is a significant contributor to dietary lipid peroxidation in vivo. As a result, there have been

285 various investigations about its effects on lipid peroxidation and combinations with other

286 antioxidants (Wang & Quinn, 1999).

287   A resonance-stabilized phenoxyl radical is created during the α-tocopherol donation of

288 electrons. This has a reduced reaction compared to lipid-derived peroxyl radicals and does not

289 quickly reproduce the radical chain in lipid peroxidation. Subsequently, certain biological

290 reductants, such as ascorbate (vitamin C), ubiquinol, or dihydrolipoic acid, convert the

291 tocopherol radical back to tocopherol. Retinoids' interaction with hydroxyl radicals, peroxyl

292 radicals, such as trichloromethyl peroxyl radical, or the photoionization of retinoids by

293 exposure to ultraviolet light is responsible for the cation radicals production (El-Agamey *et al*.,

294 2017).

295   The apoptosis can be characterized by the expression of biochemical markers called

296 caspases (Lavrik et al., 2005; Nuñez et al., 1998). The expression of caspase-3 and -7 (Figures

297 4 and 5) was also conducted in this study. Apoptosis, which called programmed cell death, is

298 a morphologically unique process that includes cell shrinkage, cytoplasm condensation, plasma

299 membrane blebbing, and fragmentation of chromatin and DNA into oligonucleosomes (Park *et*

300 *al*., 2020).

301   The caspases are a family of genes essential for maintaining homeostasis through

302 regulating cell death and inflammation. This biomarker produces active signaling molecules

303 that aid in apoptosis and are divided into two types based on their modes of action, including

304 initiator (-8 and -9) and executioner caspases (-3, -6, and -7) (McIlwain et al., 2015).

305   Caspase-3, a key effector caspase in apoptotic pathways, is 32-kDa proenzyme that is not

306 active. This is broken at the aspartate residue to form the p12 and p17 subunits necessary for

307 producing the active caspase-3 enzyme. Furthermore, this is in charge of morphological and

308 biochemical alterations during apoptosis and can be used in computing the apoptotic index

309 (Huang *et al*., 2017). Caspase-7 is also an executioner caspase that plays a critical role in optic

310   nerve injury and retinal ganglion cell death. The inhibition might be a novel therapeutic strategy

311   for some neurodegenerative diseases of the retina (Choudhury et al., 2015).

312   This study showed that the percentage of cell staining at each intensity level was used to

313   grade the interpretation of caspase-3 and -7 expressions in photoreceptor cells. The degree of

314   positivity using Immunoreactive Scoring System (IRS) modification classified Huang, et al.

315   (2017) method into negative = <5%, low = 5-20%, and high = >20% (Huang *et al*., 2017).

316   Moreover, quantitative measurement was obtained for the expression of the caspase in the

317   retinal ganglion cells.

318   The statistical analysis found a significant difference in caspase-3 and -7 expressions

319   among groups, and α-tocopherol groups showed a better effect on retinal cell apoptosis

320   prevention than others. Antioxidants prevented the progression of retinopathy in diabetic rats'

321   retinas, which showed elevated oxidative stress. According to previous studies, apoptosis of

322   retinal neuronal cells is increased in experimental diabetes in rats and humans. The apoptosis-

323   induced cell death leads to ongoing neurodegeneration, where neurons are destroyed before

324   another histopathology occurs (Abu El-Asrar *et al*., 2007). The significant results of the α-

325   tocopherol groups could be due to its biochemistry compound (2,7,8-trimethyl-2- (2'-

326   carboxyethyl)-6-hydroxychroman (Ɣ-CEHC)) that suppresses cyclo-oxygenase activity with

327   an anti-inflammatory effect (Gagné *et al*., 2009).

328   This study did not show a significant effect in combination of retinol and α-tocopherol

329   supplementation to prevent retinal cell apoptosis. It could occur because the apparent

330   synergism between α-tocopherol and other antioxidants is based on recycling. Furthermore, α-

331   tocopherol decreases and recycles other semi-oxidized forms such as cation radicals of vitamin

332   A (El-Agamey & Fukuzumi, 2011; Li *et al*., 2013).

333   The findings are comparable to the study conducted by Salerno (2007) on the effects of

334   α-tocopherol consumption on apoptosis. It was stated that α-tocopherol (10, 20, 50, or 100 μM

335   in 0.25 M MetOH) was the only agent capable of inducing a slight statistically significant

336   reduction in intracellular caspase-3 activity (P<0.05). The combinations of α-tocopherol and

337   carotenoid cleavage products (13 μg/ml) showed a high up-regulation of intracellular caspase-

338   3 activity, and the treatment had more significant effect than carotenoid derivatives (Salerno *et*

339   *al*., 2007). The different results were shown because they used a combination of α-tocopherol

340   and carotenoid cleavage products. In contrast, a combination of α-tocopherol and retinol, which

341   is pure forms of vitamin A was used. The administration of vitamins C and E reduced

342   superoxide generation in the retina, and diabetic mice given this combination experienced a

343   partial reduction in retinal neovascularization. The benefits of retinal cell survival become

344   increasingly well-known once antioxidants such as ascorbic acid, acetate, α-tocopherol, Trolox

345   cysteine, β-carotene, and selenium are consumed. The same components can also minimize

346   lipid peroxides and prevent superoxide dismutation with catalase reduction. Therefore, it is

347   suggested to increase the application or consumption of a broader range of antioxidants as an

348   effective strategy to prevent retinopathy (Silva *et al*., 2010).

349

350   **CONCLUSIONS**

351   Retinol and α-tocopherol compounds have a protective effect of maintaining the retinal cells'

352   densities and preventing the cells from apoptotic process. Moreover, the α-tocopherol

353   compound showed more promising results in cell density and caspases expression compared

354   to retinol compounds or a combination of both. Future studies are needed to demonstrate the

355   definitive function of α-tocopherol supplementation in preventing diabetic retinopathy.

356

357   **List of abbreviations**

358   DR: Diabetic retinopathy, OS: oxidative stress, ROS: reactive oxygen species, LRAT: retinol

359   acyltransferase, IRS: Immunoreactive Scoring System, RDA: Recommended Daily

360 Allowance, PKC: Protein kinase C, NADPH: Nicotinamide adenine dinucleotide phosphate

361 (NADPH) oxidase, VEGF: vascular endothelial growth factor., IHC: immunohistochemistry,

362 RBP: retinol-binding protein, STRA6: stimulated by retinoic acid 6 (STRA6), OCT: optical

363 coherence tomography.

364

365 **DECLARATION**

366 **Ethics approval and consent to participate**

367 This study received approval from The Ethics Committee of Medical Research, Faculty of

368 Medicine, Hasanuddin University, with number: 725/UN4.6.4.5.31/ PP36/2021.

369

370 **Consent for publication**

371 Not applicable

372

373 **Availability of data and materials**

374 The data supporting these findings are available from the corresponding author upon

375 reasonable request.

376

377 **Competing interests**

378 The authors declare that they have no competing interests.

379

380 **Funding**

381 This study is funded by Hasanuddin University in term of "Dana Penelitian Dasar Universitas

382 Hasanuddin 2021" program (Contract No.: 915/UN4.22/PT.01.03/2021).

383

Author Contribution

AMI: design of the work, medical procedure execution (alloxan injection, feeding retinol and α-tocopherol, sacrifice animal model), data analysis and interpretation, drafting the work for publication. **AB:** work conception, animal care, statistical data analysis, and interpretation. **SL:** work conception, treatment material selection, work drafting, and publication revision. **UAM:** performing the medical procedure (tissue preparation and interpretation) and 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. **HSM:** work conception and data analysis.

Authors' information

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

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528

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

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ABSTRACT

Background: Diabetic retinopathy is the most common microvascular complication of diabetes. Retinol and α-tocopherol are thought to save photoreceptor and retinal ganglion cells (RGC) damage caused by hyperglycemia. **Objective:** This study aims to examine the effect of retinol and α-tocopherol on photoreceptor and RGC densities and the expression of caspase-3 and -7 on those layers of the diabetic rat model. **Methods:** Alloxan 150mg/kgBW single dose was used to develop animal models, which were then separated into eight groups consisted of one group without intervention (1), one positive control which only induced with alloxan (2), and other groups that given retinol (3 and 6), α-tocopherol (4 and 7), or combination of both (5 and 8). Histopathological examination was performed using Hematoxylin-Eosin staining to evaluate the photoreceptor and RGC densities and immunohistochemistry staining to evaluate the caspase-3 and -7 expression on photoreceptor and RGC as apoptotic markers. **Results:** In the treatment group, the highest density of photoreceptor cells and RGC was identified in diabetic rats given α-tocopherol (group 7) while the lowest value was discovered in the group given retinol (group 3). Identification on the caspase-3 and -7 expression revealed that the group given α-tocopherol (group 7) was had the lowest value. **Conclusion:** In diabetic rats, retinol and α-tocopherol compounds help to maintain densities and prevent the photoreceptor and RGC death. However, α-tocopherol was more promising than retinol or combinations in terms of cell densities 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 one of the underlying patomechanism for DR and is often found to precede the 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 most often utilized diabetogenic drugs are alloxan and streptozotocin.(Ighodaro et al., 2017) Alloxan is a highly potent diabetogenic cyclic-urea derivative. Alloxan generates reactive oxygen species (ROS) in a cyclic reaction with its reduction product, dialuric acid, in the presence of intracellular thiols, particularly glutathione. Alloxan's beta cell toxicity is begun by the free radicals produced during this redox reaction. Autoxidation of dialuric acid produces superoxide radicals (O_2-), hydrogen peroxide (H_2O_2), and hydroxyl radicals in a final iron-catalyzed reaction step ($OH-$). These hydroxyl radicals ultimately cause beta cells to die due to their innately limited ability for antioxidative defense and the resulting state of insulin-dependent alloxan diabetes. As a thiol reagent, alloxan inhibits glucose-induced insulin secretion selectively by oxidizing functionally important thiol groups in the glucokinase protein, disrupting oxidative metabolism and the glucose sensor function of this beta cell

67 signaling enzyme (Lenzen et al., 1996). The dose of alloxan used to cause diabetes in rats varies

68 between 40-200 mg/kgBW via intraperitoneal or intravascular route (Sheriff et al., 2020).

69 Caspases involved in apoptosis have been subclassified by their mechanism of action and

70 are either initiator caspases (caspase- 8 and -9) or executioner caspases (caspase-3, -6, and -7)

71 (McIlwain et al., 2015). Caspase-3 with caspase-7 was considered to be similar because these

72 related cysteine proteases share an optimal peptide recognition sequence and have several

73 endogenous protein substrates in common. In addition, both caspases are proteolytically

74 activated by the initiator caspase-8 and -9 during death receptor- and DNA-damage-induced

75 apoptosis, respectively (Lamkanfi & Kanneganti, 2010). Caspase-3 and -7 expression as

76 apoptotic marker might be used to investigate the alterations in the retina following diabetes

77 condition. These are two of the most essential caspase effectors in apoptotic pathways. Both

78 indicators have the potential to assess the level of tissue damage caused by the induction agent

79 (Kowluru & Koppolu, 2002; Lamkanfi & Kanneganti, 2010).

80 The amounts of oxidatively damaged DNA and nitrosylated proteins are higher in the

81 diabetic retina due to increased oxidative stress (OS), and antioxidant defense enzymes are

82 compromised. Diabetic experimental animals and humans have a lower level of antioxidant

83 enzymes and potential antioxidant vitamins (Nita & Grzybowski, 2016). As a result of the close

84 relationship between OS and dysmetabolism associated with the pathogenesis of DR, suitable

85 antioxidants can be used to alleviate metabolic and functional abnormalities. Antioxidants can

86 work on various levels, such as inhibiting the generation of reactive oxygen species (ROS),

87 lowering free radicals, or enhancing antioxidants enzyme capacities. The finding demonstrated

88 that using dietary or local bio factors of medicinal and aromatic plants could help manage

89 diabetes. OS triggers other pathways that are unfavorable to DR development and causes a

90 vicious circle of injury to macromolecules by magnifying additional ROS. Therefore, OS and

91 ROS are considered to have a role in DR, by increasing glucose and significant metabolic

92 abnormalities (Madsen-Bouterse & Kowluru, 2008; Silva et al., 2010).

93   Vitamins A, C, E, and carotenoids, which have been extensively investigated, are well-

94 known antioxidants produced from food. Additionally, antioxidants can limit the generation of

95 reactive oxygen species (ROS), scavenge free radicals, or boost the antioxidant enzyme

96 capabilities. This supplementation has proven to reduce oxidative stress-induced damage to the

97 retina (Silva *et al.*, 2010).

98   Retinol, retinal, retinoic acid, and provitamin A carotenoids are all unsaturated nutritional

99 chemical molecules that makeup vitamin A (Zhong *et al.*, 2012). Painstaking biochemical

100 reconstitution experiments have enabled recent improvements in the molecular knowledge of

101 the retinoid cycle in the mammalian retina. Natural or synthetic animal models with known

102 genetic lesions backed up this claim, as well as human studies of target genetic blinding

103 diseases. Critical retinal enzymes and proteins and their substrates and ligands have been

104 identified using structural and membrane biology and placed in a cellular context (Kiser &

105 Palczewski, 2016). In a reversible reaction catalyzed by the reduced nicotinamide adenine

106 dinucleotide phosphate (NADPH) -dependent all-trans-retinol dehydrogenase, all-trans-retinal

107 in the cytoplasm are degraded to all-trans-retinol. After that, al-trans-retinol diffuses into the

108 retinal pigmen epithelium where it is esterified by lecithin retinol acyl transferase (LRAT)

109 (Palczewski, 2010). Meanwhile, in vitro, and in vivo studies showed a protective impact of α-

110 tocopherol, a derivative of vitamin E on practically all eye tissues. For up to 24 hours of

111 exposure, a biomolecular compound of α-tocopherol can protect the retina against light damage

112 (Ritch, 2007).

113   The aims of this study was to investigate the protective effect of retinol and α-tocopherol

114 on the photoreceptor and retinal ganglion cells apoptosis in diabetic rats model.

115

METHODS

Design

This study was conducted at the Animal laboratory and Pathology Laboratory of Hasanuddin University, Indonesia. This study was a true experimental with only a post-test group which made use of forty animal subjects. Alloxan monohydrate (SIGMA USA, Cat. No. A7413) 150mg/kgBW single dose intraperitoneally was 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 α-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 α-tocopherol for 14 days (1 week each, before and after alloxan induction), and 8 was diabetic rats given combination of retinol and α-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

6

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 Instiues of Health, 2020) then 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 sacrificed before enucleation, by placing them in a closed container filled with cotton and ether. Subsequently, the animals were placed for approximately ten minutes until there was no motoric 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 laboratory.

To calculate the density of retinal ganglion and photoreceptor cells, retinal tissue was cut using a microtome with a thickness of 5 μm 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 (caspase expression shows <5%), low (5-20% expression), and high (>20% expression). Meanwhile,

164   the intensity of caspase expression in retinal ganglion cells was calculated quantitatively by

165   counting the number of cells and apoptotic bodies that express binding colour.

166

167   **Data Analysis**

168   Statistical analysis of the data were using an Independent T-test for the quantitative data

169   and Kruskall Wallis test for qualitative data (sig. $p<0.05$).

170   **RESULTS**

171   According to the table 1, the blood sugar level of the negative control group was 82 ± 2

172   mg/dl compared to the diabetic groups (276 ± 15 mg/dl to 426 ± 45 mg/dl). This revealed that the

173   experimental animal can be used as a model for type 1 diabetes rats because they have been in

174   a hyperglycaemic condition.

175   The photoreceptor cell density showed the highest value in group 1 while the lowest

176   value was shown in group 2. Furthermore, in the treatment group, the most effective value for

177   approaching the normal group of mice was shown in group 7 that received α-tocopherol

178   supplementation for 14 days (pre and post alloxan induction). The statistical test results showed

179   that there was a significant difference in the photoreceptor cell density among groups

180   ($p=0.002$), that can be seen in figure 1.a. This result is in line with the measurement of retinal

181   ganglion cell density where the highest value was also obtained in group 1 and the lowest value

182   in group 2, while for the treatment group, the most effective supplementation was shown in

183   group 7 (Fig. 1.b). The result of the statistical test showed that there was a significant difference

184   in the photoreceptor cell density among groups ($p=0.010$).

185   In calculating the expression of caspase-3, apoptosis in photoreceptor cells showed the

186   lowest expression in group 1, while the highest expression was found in group 2. In the

187   treatment group, values close to the normal group were shown by groups 4 and 7 (α-tocopherol

8

188 supplementation group), as shown in figure 2.a. Statistical analysis showed that there was a

189 significant difference in caspase-3 expression among groups (p=0.016).

190   The above results are in line with the results of expression calculations in retinal ganglion

191 cells where the lowest value was found in group 1, while the highest value was found in group

192 2. The results of observation in the treatment group were found to be the most effective in the

193 group that given α-tocopherol supplementation (group 4 and 7), as can be seen in figure 2.b.

194 Statistically, these results also showed a significant caspase-3 expression among groups

195 (p=0.010).

196   The expression value of caspase-7, apoptosis in photoreceptor cells showed the lowest in

197 group 1, while the highest expression was found in groups 5, 6 and 8. Observations in the

198 treatment group indicated that groups 4 and 7 (supplementation of α-tocopherol for 7 and 14

199 days) showed the lowest expression. Based on these results, it was obtained statistically that

200 this result did not show a significant difference on caspase-7 expression among groups (p =

201 0.069). The value of caspase-7 expression in retinal ganglion cells showed the lowest value in

202 group 1, while the highest value was found in group 2. In the treatment group, the expression

203 values that close to the normal group were samples in groups 4 and 7, respectively. Figure 2.c

204 and 2.d indicated that there was a significant difference between caspase-7 expression among

205 groups (p=0.010).

206

207 **DISCUSSION**

208   The retina is a fragile thin layer of tissue that originates from the neuroectoderm. It is

209 made up of nine layers of sensory neurons that begin the visual pathway (Gupta *et al*., 2015).

210 Photoreceptors are visual system sensors that transform photon capture into a nerve signal

211 through a process known as phototransduction. Photoreceptor terminal ends interrelate with

212 surrounding photoreceptors and interneurons of horizontal and bipolar cells, and they are

213 required for visual information transmitting and early processing in the retina (Fielder &

214 Alistair, 2011).

215   Photoreceptors in the healthy retina are among the most active oxygen consumers in the

216 body. The choroidal circulation, not the retinal blood vessels, supplies the majority of the

217 oxygen to photoreceptors. As a result, oxygen tension drops quickly from the Bruch's

218 membrane to the retina's outer nuclear layer, where it reaches its lowest values. This reduces

219 oxygen reserve in photoreceptors and even a minor disruption of oxygen flow in diabetes can

220 result in severe hypoxia. The creation of acellular capillaries, capillary blockage, and capillary

221 dropout are all thought to contribute to retinal hypoxia and hence, the retinal vascular pathology

222 of DR (Becker *et al*., 2020).

223   On the other layer, retinal ganglion cells process and convey information from the retina

224 to visual centers in the brain. These output neurons comprise subpopulations with distinct

225 structure and function (Sernagor et al., 2001). There is a remarkable diversity of RGCs and

226 the various subtypes have unique morphological features and characteristic pathways linking

227 the inner retina to the relevant brain areas (Kim et al., 2021). Retinal ganglion cells carry visual

228 signals from the eye to the brain, but do not make chemical synapses onto other retinal neurons.

229 Nevertheless, they form gap junctions with other RGCs and amacrine cells, providing

230 possibilities for RGC signals to feed back into the inner retina (Vlasiuk & Asari, 2021).

231   A pathogenic disease, such as diabetic retinopathy, caused a decrease in electrical

232 activity of neurotransmitter from photoreceptor and RGC cell to the nerve fiber layer

233 (Antonetti, 2012). Diabetic retinopathy is a duration-dependent disease, that is infrequently

234 discovered during the early years of diabetes but substantially develops with time, with nearly

235 90% of patients showing indications of retinopathy after 20–25 years of diabetes (Kowluru &

236 Mishra, 2015). After cellular membranes are damaged and intracellular components are

237 released, oxygen-derived free radicals mediate tissue injury (Nur Azlina & Nafeeza, 2008).

238   Antioxidants have the potential of preventing retinopathy development in diabetic rats

239   and the implicated retinal metabolic abnormalities (Silva *et al*., 2010). For the protection of the

240   retina and choroid, optimal combinations of vitamins B1, B2, B6, L-methylfolate,

241   methylcobalamin (B12), C, D, natural α-tocopherol complex, lutein, zeaxanthin, α-lipoic acid,

242   and n-acetylcysteine are necessary (Rasmussen & Johnson, 2013)**.**

243   In this study, there was a substantial difference in cell density between diabetic and non-

244   diabetic rats after alloxan induction and supplementation with retinol and α-tocopherol

245   substances. Retinol supplementation appeared to have a positive effect on maintain the retinal

246   cell densities, although it was not better than the α-tocopherol and combination

247   supplementation groups. This was proven by the higher density values in groups 3 and 6

248   compared with group 2. A study by Zhong et al. (2012) reported that retinoids may create

249   retinoid cation radicals as a result of interactions with different radicals or photoexcitation with

250   light. Also, there is an indication that semi-oxidized retinoids can oxidize certain amino acids

251   and proteins, and that α-tocopherol can scavenge retinol and retinoic acid cation radicals

252   (Zhong *et al*., 2012).

253   In the retinoid cycle, retinol is an excellent substrate for LRAT and is quickly converted

254   into fatty acid esters. Their propensity to form oil droplets excludes fatty acid esters of retinol

255   from the circulation (Kiser & Palczewski, 2016). The mechanism of vitamin A transport is

256   mediated by the plasma retinol binding protein (RBP), a specific and sole carrier of vitamin A

257   in the blood, and its specific membrane receptor stimulated by retinoic acid 6 (STRA6), which

258   mediates cellular vitamin A uptake. (Zhong et al., 2012) Structural and membrane biology have

259   been used to detect critical retinal enzymes and proteins and their substrates and ligands,

260   placing them in a cellular context. The most presently accepted modulators of the retinoid cycle

261   already have demonstrated promising results in animal models of retinal degeneration.(Kiser

262   & Palczewski, 2016)

263   In this study, the α-tocopherol supplementation group was the group closest to the normal

264   values for both photoreceptor and retinal ganglion cell densities. Similar result was found in

265   the study by Ritch (2007) which stated that the α-tocopherol has been suggested to protect

266   against retinal phototoxicity and central nervous system ischemia (Ritch, 2007). Once the fat

267   is oxidized and free radical reactions propagate, α-tocopherol is a powerful chain-breaking

268   antioxidant that counteracts reactive oxygen species molecules creation. By inhibiting the

269   peroxidation of membrane lipids and scavenging lipid peroxyl radicals, α-tocopherol protects

270   essential cellular structures from damage produced by oxygen free radicals and reactive

271   products of lipid peroxidation (Kanter *et al*., 2009). This also protects the polyunsaturated fatty

272   acids found in membrane phospholipids and plasma lipoproteins, because of its peroxyl radical

273   scavenging activity (Rizvi *et al*., 2014).

274   Subsequently, the vitamin E refers to eight naturally occurring compounds (α-, β-, γ-, δ-

275   tocopherol, and α-, β-, γ-, δ-tocotrienol). α-tocopherol is the most common form retained in

276   human plasma, out of the eight forms (Gagné *et al*., 2009). Vitamin E is crucial for erythrocytes'

277   stability and central and peripheral nerves conductivity. Several countries have established

278   vitamin E dietary consumption recommendations (Péter *et al*., 2015).

279   The loss of photoreceptors in the diabetic retina is still being topic of debate. Various

280   optical coherence tomography (OCT) studies in diabetic patients show that the thickness of the

281   inner retina, including the nerve fiber, retinal ganglion cell, and inner plexiform layers,

282   decreases with the duration of diabetes (Becker *et al*., 2020). In diabetic animal model study,

283   same result was found in our study that the outer nuclear layer thickness is frequently reduced,

284   especially models of type 1 disease with early onset. Furthermore, various studies support the

285   notion that photoreceptor loss increases with disease duration (Kern & Berkowitz, 2015),

286   similar condition was found in our study that the cell densities significantly decreased in group

287   2 compared to others group both in photoreceptor and retinal ganglion cells (Fig.3).

288   Following positive results in a diabetic rat model for the prevention of diabetes-related

289   vascular damage, high dose of α-tocopherol, the predominant antioxidant in the lipid phase,

290   were examined in the clinic for administration. This was performed to restore retinal blood

291   flow in diabetic type I patients as this was discovered to control levels. Furthermore, the α-

292   tocopherol is useful in DR by the nonenzymatic free radical scavenging action outside the cell.

293   Antioxidant therapy with α-tocopherol has been shown in humans to improve vascular retinal

294   hemodynamics (Silva *et al*., 2010).

295   In this study, administration of the combination of retinol and α-tocopherol did not show

296   any more effective results to maintain the retinal cell densities than the single supplementation

297   of α-tocopherol. Previous study reported that supplementation with substantial doses of retinol

298   was demonstrated to reduce the bioavailability of α-tocopherol in growing pigs and calves

299   (Hymoller *et al*., 2016). Due to its abundance in human and animal tissues, α-tocopherol is the

300   considerable significant inhibitor of dietary lipid peroxidation in vivo. As a result, there have

301   been various investigations about its effects on lipid peroxidation and combinations with other

302   antioxidants than other tocopherols (Wang & Quinn, 1999).

303   A resonance-stabilized phenoxyl radical is created during the α-tocopherol donation of

304   electrons. This has a reduced reaction compared to lipid-derived peroxyl radicals and does not

305   reproduce the radical chain in lipid peroxidation easily. Subsequently, certain biological

306   reductants, such as ascorbate (vitamin C), ubiquinol, or dihydrolipoic acid, then convert the

307   tocopheroxyl radical back to tocopherol. Retinoids interaction with hydroxyl radicals, peroxyl

308   radicals, such as trichloromethylperoxyl radical, or the photoionization of retinoids by exposure

309   to ultraviolet light is responsible for the cation radicals production (El-Agamey *et al*., 2017).

310   The apoptosis can be characterized by the expression of biochemical markers called

311   caspases (Lavrik et al., 2005; Nuñez et al., 1998). In this study, we also observed the expression

312   of caspase-3 and caspase-7 (Fig. 4 and 5). 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).

The caspases are a family of genes important for maintaining homeostasis through regulating cell death and inflammation. This biomarker 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) (McIlwain et al., 2015).

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 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). Besides, the caspase-7 is also an executioner caspase who plays a critical role in optic nerve injury and retinal ganglion cell death. It also reported that inhibition of caspase-7 activity may be a novel therapeutic strategy for some neurodegenerative diseases of the retina (Choudhury et al., 2015).

The result of this study 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, classified it into negative = <5% expression, low = 5-20% expression, and high = >20% expression (Huang *et al*., 2017). Moreover, quantitative measurement was obtained for the caspases expression in the retinal ganglion cells.

Based on the statistical analysis, we found that there was a significant difference in caspase-3 and -7 expressions among groups where in α-tocopherol groups, it showed a better effect on retinal cell apoptosis prevention than other groups. Antioxidants prevented the progression of retinopathy in diabetic rats' retinas, which revealed elevated oxidative stress.

338 According to previous studies, apoptosis of neuronal retinal cells is increased in experimental

339 diabetes in rats and humans. The apoptosis-induced cell death leads to persistent

340 neurodegeneration in diabetic retinas, where neurons are destroyed before another

341 histopathology is seen (Abu El-Asrar *et al*., 2007). The significant results that showed by α-

342 tocopherol groups could be due to it biochemistry compound (2,7,8-trimethyl-2- (2'-

343 carboxyethyl)-6-hydroxychroman (Ɣ-CEHC)) that suppress cyclo-oxygenase activity, and

344 have an anti-inflammatory effect (Gagné *et al*., 2009).

345 Our study also did not show a significant effect in combination of retinol and α-

346 tocopherol supplementation to prevent retinal cell apoptosis. It could be happened because the

347 apparent of antioxidant synergism between α-tocopherol and other antioxidants is based on

348 recycling. Furthermore, α-tocopherol decreases and recycles other semi-oxidized forms such

349 as cation radicals of the two forms of vitamin A such as retinol and retinoic acid (El-Agamey

350 & Fukuzumi, 2011; Li *et al*., 2013).

351 The findings of this study are comparable to those of a study by Salerno (2007) on the

352 effects of α-tocopherol consumption on apoptosis. In that study, α-tocopherol (10, 20, 50, or

353 $100\mu M$ in 0.25 M MetOH) was the only agent that induced a slight statistically significant

354 reduction in intracellular caspase-3 activity ($P<0.05$). Meanwhile, combinations in different

355 amounts of α-tocopherol and carotenoid cleavage products (13 μg/ml), showed an elevated up-

356 regulation of intracellular caspase-3 activity. The combination treatment had a far greater effect

357 than carotenoid derivatives alone (Salerno *et al*., 2007). Their study showed a different results

358 with this study because they used a combination of α-tocopherol and carotenoid cleavage

359 products, while in our study we used a combination of α-tocopherol and retinol, which is pure

360 forms of vitamin A.

361 A provision of vitamins C and E decreased superoxide generation in the retina, and

362 diabetic mice given this vitamin combination showed partial reductions in retinal

363 neovascularization. Once antioxidants such as ascorbic acid, acetate, α-tocopherol, Trolox

364 cysteine, β-carotene, and selenium are consumed, the benefits related to retinal cell survival

365 become increasingly well-known. The same components can also minimize lipid peroxides and

366 prevent superoxide dismutation and catalase reduction. Therefore, it is suggested to increase

367 the application or consumption of a wider range of antioxidants as an effective strategy to

368 prevent retinopathy (Silva *et al*., 2010)

369

370 **CONCLUSIONS**

371 Retinol and α-tocopherol compounds have protective effect to maintain the retinal cells

372 densities, and prevent the cells from apoptotic process. Moreover, α-tocopherol compound was

373 showed more promising results in terms of cell density and caspases expression when

374 compared to retinol compounds or combination of both. Additionally, future studies, including

375 in humans, is needed to demonstrate the definitive function of α-tocopherol supplementation

376 in the prevention of diabetic retinopathy.

377

378 **List of abbreviations**

379 DR: Diabetic retinopathy, OS: oxidative stress, ROS: reactive oxygen species, LRAT: retinol

380 acyl transferase, IRS: Immunoreactive Scoring System, RDA: Recommended Daily

381 Allowance, PKC: Protein kinase C, NADPH: Nicotinamide adenine dinucleotide phosphate

382 (NADPH) oxidase, VEGF: vascular endothelial growth factor., IHC: immunohistochemistry,

383 RBP: retinol binding protein, STRA6: stimulated by retinoic acid 6 (STRA6), OCT: optical

384 coherence tomography.

385

386 **DECLARATION**

387 **Ethics approval and consent to participate**

388 This study was received approval from The Ethics Committee of Medical Research, Faculty of

389 Medicine, Hasanuddin University with Approval number: 725/UN4.6.4.5.31/ PP36/2021.

390 **Consent for publication**

391 Not applicable

392

393 **Availability of data and materials**

394 The data that support the findings of this study are available from the corresponding author

395 upon reasonable request.

396

397 **Competing interests**

398 The authors declare that they have no competing interests.

399

400 **Funding**

403

404 **Author Contribution**

405 **AMI:** design of the work, medical procedure execution (alloxan injection, feeding retinol and

406 α-tocopherol, sacrifice animal model), data analysis and interpretation, drafting the work for

407 publication. **AB:** work conception, animal care, data statistical analysis, and interpretation. **SL:**

408 work conception, treatment material selection, work drafting, and publication revision. **UAM:**

409 performing the medical procedure (tissue preparation and interpretation) drafting the work for

410 publication. **AAD, ICI:** caring for the animal model, performing post-injection follow-up,

411 sacrifice animal model, tissue processing and analysis, composing and critically revising the

412 work for key intellectual content. **HSM:** work conception and data analysis.

Acknowledgment

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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