

Protective effect of pomegranate peel extract on diabetic ocular structural changes in experimental diabetic rats: A histological, immunohistochemical and clinical study

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ABSTRACT

Ocular complications of diabetes mellitus are rapidly progressing and have become a leading cause of vision impairment. The present study evaluated the potential protective role of pomegranate peel extract, as a powerful antioxidant agent, on the ocular complications of diabetes. One hundred, 8 weeks old, male Wistar rats were subdivided into three groups; the first control group (non-diabetic) was injected intraperitoneally with a single dose (2 ml/animal) of citrate buffer alone. The second group (diabetic untreated) was subjected to induction of diabetes by an intraperitoneal single dose of streptozotocin (60 mg/kg body weight). The third group (diabetic treated) was subjected to induction of diabetes as the second group and given simultaneously, 500 mg/kg body weight of pomegranate peel extract orally once per day with the drinking water. Clinical examination of lens and fundus was made, and the biochemical analysis was done at 8, 12, 16, 20, 24, 28, 32, 36 and 40 weeks after diabetes induction. At the end of the investigation (40 weeks), the rats were anesthetized, and the eyeballs were removed and sampled for histopathological, immunohistochemical, and ultrastructural investigations. The study revealed that the lens of 12 rats from a total of 40 rats of the second group (diabetic untreated) depicted the onset of cataract at 16 week, which increased gradually. The retina of the same group showed gradual inter-retinal microvascular abnormalities and histopathological changes. The third group (diabetic treated) showed marked amelioration as compared to the second group. ANOVA test for the biomedical indicators showed a statistically significant improvement in the third group. In conclusion, the pomegranate peel extract possesses strong potential for its development as a protective agent against ocular complications of diabetes.

Keywords: Pomegranate peel extract, diabetes mellitus, ocular structure, rats.

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INTRODUCTION

Ocular complications of diabetes mellitus are rapidly progressing and have become the most evident cause of morbidity worldwide (Sayin et al., 2015). Diabetic eye problems include diabetic retinopathy (DR), cataract, glaucoma, ocular surface diseases, and papillopathy (Threatt et al., 2013). Diabetic retinopathy is the most widespread microvascular complication of diabetes causing loss of vision (Sayin et al., 2015; Gardner et al.,

1998). The vision-threatening complications of DR are related to progressive retinal ischemia associated with retinal vascular permeability and upgrowth of retinal neovascularization (proliferative DR) (Zheng et al., 2007). Moreover, diabetes caused a deleterious effect on vision through the generation of cataract and glaucoma (Curtis et al. 2009).

Pomegranate (*Punica granatum*), is a sweet fruit

cultivated, primarily, in Asian and Mediterranean countries. A considerable number of therapeutic activities have been reported for different pomegranate extracts (Ashanth et al., 2001; Afaq et al., 2005; Bagri et al., 2009). Pomegranate peel extract (PPE) holds a high content of polyphenolic compounds (such as ellagic tannins, flavanols, anthocyanins, ellagic acid, and gallic acid) (Gozlekçi et al., 2011), that possess stronger biological activity as compared to the juice including anti-cancer (Brusselmans et al., 2005), anti-inflammatory (Lansky and Newman, 2007), and anti-diabetic activities (Althunibat et al., 2010; Galal et al., 2014). Moreover, antioxidant activity correlated with antifibrotic and radioprotective properties of PPE have also been reported (Toklu et al., 2007; Toklu et al., 2009; Belal et al., 2020). Kalaycıoğlu and Erim (2017) reported that the total phenolic contents and total flavonoid contents of PPE were 5.9-fold and 12.4-fold, respectively, higher than that of juice extract. Also, the antioxidant activity of PPE was 23-fold higher than juice extract (Kalaycıoğlu and Erim, 2017).

The purpose of the present study was to investigate the potential protective role of pomegranate peel extract against the diabetic-induced pathological changes of the retina and lens in diabetic rats, as a powerful antioxidant agent that may have a safer preventive and therapeutic impact.

MATERIALS AND METHODS

Experimental animals

A total of one hundred, 8 weeks old, male Wistar rats, weighing 250 to 300 g have been used. All animals were housed in an air-conditioned room ($25 \pm 2^\circ\text{C}$) with a normal light/dark cycle. They were allowed free access to commercial standard rat chow and water. We have treated animals after The Association for Research in Vision and Ophthalmology "ARVO" protocol for the use of animals in vision research. The study protocol was approved by the Research Ethics Committee, College of Medicine, Taif University (protocol number 1-432-1346).

Chemicals and reagents

All the reagents and chemicals were obtained from MilliporeSigma (St. Louis, MO, USA).

Preparation of pomegranate peel extract (PPE)

Extract preparation was performed according to the procedure followed by Fischer et al. (2011). Fresh mature *Punica granatum* L. fruits (Yemini varieties) were obtained from the local market, Taif City, Saudi Arabia, and used after identification of the plant taxonomy by the Biology Department, Taif University. The pomegranate peel was dried, before grounding, for 10 days in the shade. After grounding the material was immersed in absolute methanol (50 mg/500 ml) for 24 h and then filtered through several layers of gauze. Centrifuging of the filtrate was done at 8000 rpm (GL 23m Hunan Xiangyi, Hunan, China) for 15 min. After collection of the clear supernatant, the methanol was evaporated under

reduced pressure using a rotatory evaporator (RE-2010 Rotatory Evaporator, Lanphan industry, China) at 45°C . The crude extract was preserved at 20°C for further usage.

Experimental design and treatment

The animals were subdivided into three groups; the first control group, 20 rats (non-diabetic) were injected intraperitoneally with a single dose (2 ml/animal) of citrate buffer alone. The second group, 40 rats (diabetic untreated) was subjected to induction of diabetes by an intraperitoneal single dose (60 mg/kg body weight) of streptozotocin dissolved in sodium citrate buffer. The third group, 40 rats (diabetic treated) was subjected to induction of diabetes as the second group and given, simultaneously, 500 mg/kg body weight of PPE orally with the drinking water once per day. Clinical evaluation of lens and ophthalmic fundus examination was made, and the biochemical measurements were evaluated at 8, 12, 16, 20, 24, 28, 32, 36 and 40 weeks after diabetic induction. At the end of the investigation period, 40 weeks, rats were anesthetized using an intraperitoneal injection of xylazine hydrochloride (5 mg/kg) and ketamine hydrochloride (100 mg/kg). After completing this procedure, the eyeballs were removed and sampled for histopathological, immunohistochemical, and ultrastructural investigation.

Clinical evaluation

Clinical examination of lens and ophthalmic fundus examination for evaluation of the retina were made at 8, 12, 16, 20, 24, 28, 32, 36 and 40 weeks after induction of diabetes, using a direct ophthalmoscope (Ka We Piccolight E56, Kirchner & Wilhelm, Germany). The procedure was carried out as described by Jekl et al. (2015). Briefly, Sodium pentobarbital 3% was used for anesthesia (doses ranged from 38 mg/kg to 48 mg/kg, ip). Eye drops of sterile saline were used topically to provide a fluid interface between the cornea and telescope lens. During the procedure, the rats were put on a water-heated platform ($37 \pm 0.5^\circ\text{C}$) for maintenance of body temperature in a lateral recumbency position. A mydriatic agent (1% atropine P-O) was applied for detailed visualization of the lens and retina. The tip of the telescope was moved carefully by the operator and always kept 1 to 2 mm from the cornea to avoid its damage. At the end of examination, topical eye gel containing dexpanthenol was applied to ensure that the cornea was not got dry and healing any minor corneal damage.

Biochemical analysis

Blood samples were taken from the tail veins and the biochemical measures were evaluated at 8, 12, 16, 20, 24, 28, 32, 36 and 40 weeks after induction of diabetes. Total cholesterol (TC), triglycerides (TG) alongside the blood glucose were measured using an automatic analyzer (Ax-200 Balio Diagnostics, France). Glycated hemoglobin was determined using the affinity chromatography method (Abraham et al., 1983) (Glyc-Affin_GHb, Seikagaku, Tokyo, Japan).

Histopathological analysis

After removal of the anterior half of the eyeball, the specimens were prepared as described by Kato et al. (2003). The specimens were fixed through a 1% glutaraldehyde fixative with cacodylate buffer (0.1 mol/L). Then, the eyeball was treated with microwave irradiation (LG NeoChef microwave, LG group, Seoul, South Korea) for 6 s, left overnight at 4°C in the same fixative, and embedded in

paraffin. Sections of 4 μm thickness taken from the central part of the eyeball (retina) were cut, dehydrated, and stained with Hematoxylin & Eosin (H&E) for general histological observation by light microscopy.

Immunohistochemistry

Four-micrometer thick sections of the eye retina were cut, dehydrated, and subsequently stained by immunohistochemical methods, as described by Zheng et al. (2007), to detect vascular endothelial growth factor (VEGF) as an indicator of the formation of new blood vessels in the retina. The sections were managed with microwave irradiation for 10 min at low power in sodium citrate buffer (10 mmol/L, pH 6.0) after deparaffinization and dehydration. After cooling the slides to room temperature, inactivation of endogenous peroxidase was made by immersing the sections for 20 min in 3% hydrogen peroxide in methanol. The sections were incubated overnight at 4°C with VEGF (VEGF₁₂₁ and VEGF₁₆₅), as a primary monoclonal mouse antibody. Biotinylated horse anti-mouse immunoglobulin G secondary antibody was applied. After that, sections were incubated in horseradish peroxidase-conjugated streptavidin. The color was developed as a chromogen with 3,3'-diaminobenzidine tetrahydrochloride. The slides were counterstained through hematoxylin. The analysis was done by the same investigator and was recorded on a scale ranging from 0 to 3 where 0: non, 1: weak, 2: average, and 3: strong reaction.

Transmission electron microscope

Ultrathin sections have been prepared from the retina, as detailed in a previous study (Dosso et al., 1999). Investigation through a transmission electron microscope (JEM-1200EX transmission electron microscope, Japan) was made for evaluation of the basement membrane thickness of the retinal blood vessels.

Statistical analysis

The data that gave numerical values were expressed as mean \pm standard deviation for each group and subjected to one-way analysis of variance (ANOVA) with multiple comparisons between groups. SPSS13.0 for windows was used. A *P*-value below 0.05 was considered statistically significant.

RESULTS

Clinical evaluation

The clinical evaluation of group I (control non-diabetic) revealed a normal lens and retina. However, in the second group (diabetic untreated), the lens of 12 rats from a total of 40 rats showed an onset of cataract at 16 weeks after diabetes induction, which increased gradually. The retina seemed normal up to the 24th week. After that, it gradually showed inter-retinal microvascular abnormalities, microaneurysms, dilated and tortuous vessels, and small "dot and blot" hemorrhage. As compared to the second group, the third group (diabetic treated) showed marked amelioration. Only 7 rats of this group showed an onset of cataract at 24 weeks, which increased gradually. Moreover, abnormal vascularizations

and hemorrhage spots have been found in the third group (diabetic treated) but less numerous as compared to the second group (diabetic-untreated).

Biochemical analysis

Blood glucose level

The main values of different groups throughout the experiment were plotted in Figure 1. At the end of the experiment, the level of blood glucose in the control group animals was ranged from 72.45 to 82.9 mg/dl with a mean value of 77.83 mg/dl. The second group (diabetic nontreated) showed the highest level ranged from 252.67 to 294.6 mg/dl with a mean of 261.36 mg/dl. The third diabetic treated group showed marked amelioration but the value still higher than that of the control group, with a mean range of 173.07 mg/dl. The differences between groups were statistically significant ($P < 0.001$, *F* value = 2261.1) (Table 1).

Total cholesterol

The main values of different groups throughout the experimental period were plotted in Figure 2. At the end of the experiment, the main value of the total cholesterol in the control group was 97.08 ± 4.57 . The second diabetic nontreated group showed the highest total cholesterol level ranging from 190.46 mg/dl to 214.1 mg/dl, with a mean value of 203.32 mg/dl. Marked improvement of total cholesterol level was observed among the animals of the treated group ranging from 152 to 174.92 mg/dl with a mean of 163.12 mg/dl. The differences between groups were statistically significant ($P < 0.001$, *F* value = 1323.9) (Table 1).

Triglycerides

The main values of different groups throughout the experiment were plotted in Figure 3. At the end of the experiment, the highest level of blood triglycerides was recorded among rats of the diabetic nontreated group ranging from 153 to 168 mg/dl with a mean of 163.42 mg/dl. The treated group showed a marked reduction of the triglycerides level ranging from 99 to 135 mg/dl with a mean of 125.50 mg/dl. There were significant statistical differences between groups ($P < 0.001$, *F* value = 1398.2), as depicted through the ANOVA test (Table 1).

Glycated hemoglobin

The main values of different groups throughout the experiment were plotted in Figure 4. At the end of the

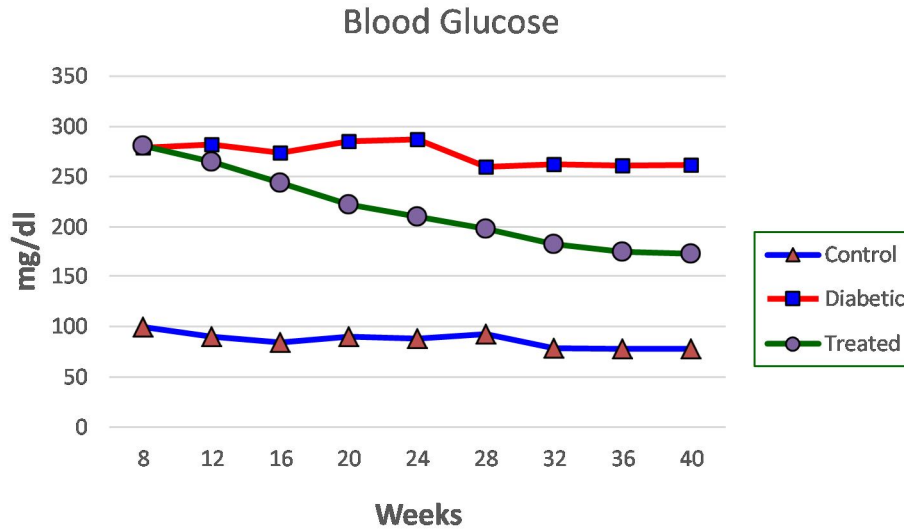


Figure 1. Blood glucose level of different groups throughout the experiment. Blood glucose is represented by the mean values of each determination.

Table 1. Mean values of biomedical parameters in different groups at the end of the experiment.

Parameter	Control G	Diabetic untreated G	Diabetic treated G	P value
Blood glucose (mg/dl)	77.83 ± 3.41 ^a	261.36 ± 12.94 ^c	173.07 ± 9.73 ^b	.013*
Total cholesterol (mg/dl)	97.08 ± 4.57 ^a	203.32 ± 8.14 ^c	163.12 ± 6.52 ^b	.021*
Triglycerides (mg/dl)	68.05 ± 3.12 ^a	163.42 ± 4.72 ^c	125.50 ± 7.66 ^b	.011*
Glycated hemoglobin (%)	3.8 ± 1.7 ^a	12.7 ± 1.4 ^c	8.2 ± 1.7 ^b	.027*

*significant at $p < 0.05$.

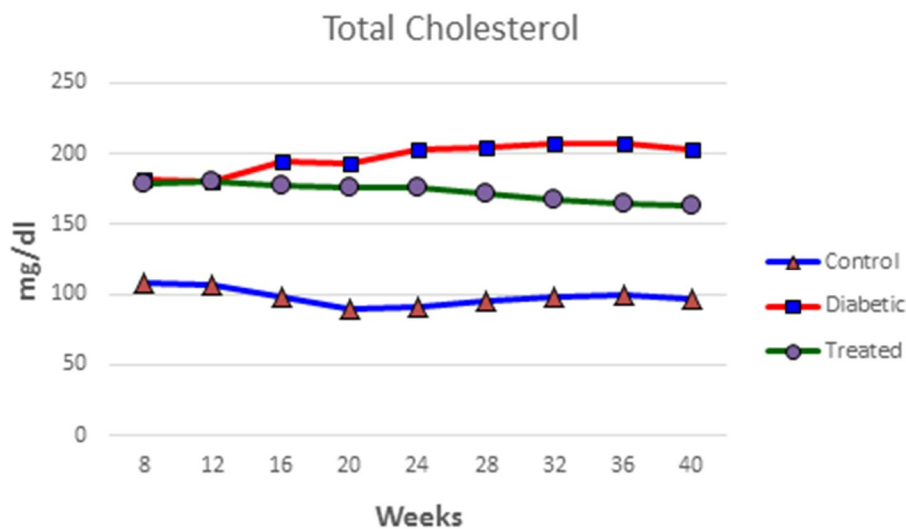


Figure 2. Total cholesterol blood level of different groups throughout the experiment. Total cholesterol is represented by the mean values of each determination.

investigation, the levels of glycated hemoglobin were ranged from 2.7 to 6.5% with a mean of $3.8 \pm 1.7\%$ in the

control group. The diabetic group showed levels ranged from 10.1 to 14.1% with a mean value of $12.7 \pm 1.4\%$.

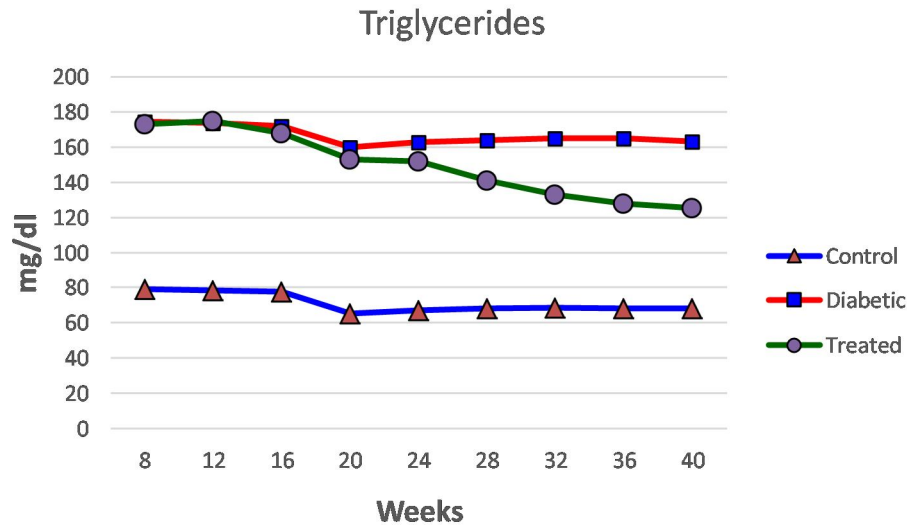


Figure 3. Triglycerides level of different groups throughout the experiment. Triglycerides level is represented by the mean values of each determination.

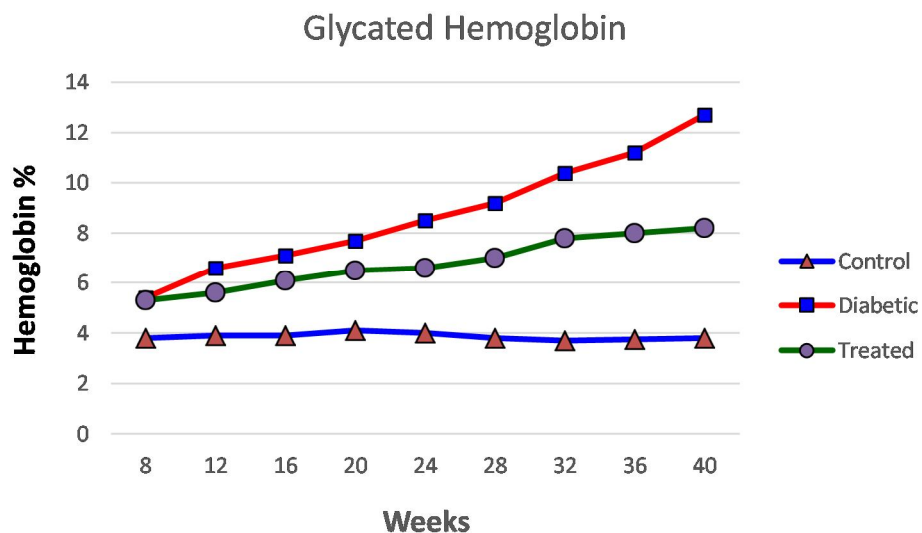


Figure 4. Glycated hemoglobin of different groups throughout the experiment. Glycated hemoglobin is represented by percentage for each determination.

Marked reduction of the glycated hemoglobin percentage was observed among animals of the treated group ranging from 6.5 to 11.0% with a mean of $8.2 \pm 1.7\%$ (Table 1).

Histopathological analysis

The retina of the control group showed normal highly organized layers and normal capillaries [Figure 5(A)]. Among the rats of the second group (diabetic non-treated), the retina was disorganized with impaired layers and the retinal cells number was markedly reduced. The

outer plexiform layer (OPL) and inner nuclear layer showed marked vacuolizations as demonstrated in Figure 5(B). Moreover, the retina of the third group (diabetic treated) has shown marked amelioration of retinal cell layers [Figure 5(C)].

Immunohistochemistry

Immunohistochemical staining for VEGF and counterstained with hematoxylin in paraffin sections of the retina of the studied groups showed a negative reaction in the control group. Otherwise, the diabetic non-

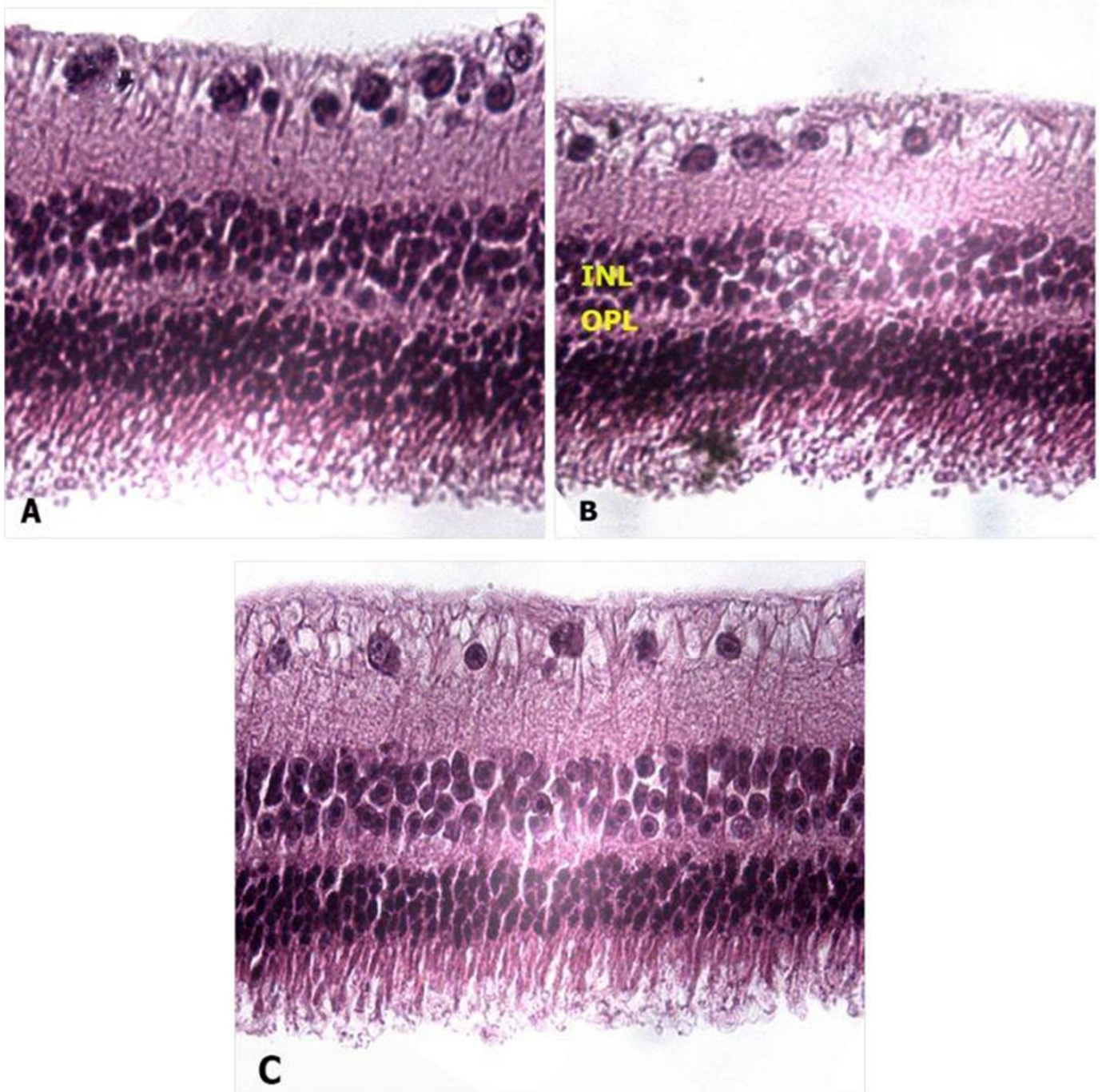


Figure 5. (A) The retina of the control group showed normal highly organized layers and normal capillaries. (B) The retina of the diabetic group was disorganized with impaired layers and the retinal cells number was markedly reduced. The outer plexiform layer (OPL) and inner nuclear layer showed marked vacuolization. (C) The retina of the diabetic treated group showed a marked amelioration of retinal cell layers. (H&E X 400).

treated group revealed a brown coloration of cytoplasm in the cells of inner plexiform, ganglion cells, and nerve fiber layers of the retina (positive immune reaction); whereas there was a weak positive reaction (light brown coloration of cytoplasm of these cells) among the diabetic treated group (Figure 6).

Transmission electron microscope

The transmission electron micrograph of capillaries of the retina showed a normal capillary wall among the rats of the control group. However, the diabetic group that received no treatment showed a markedly thickened

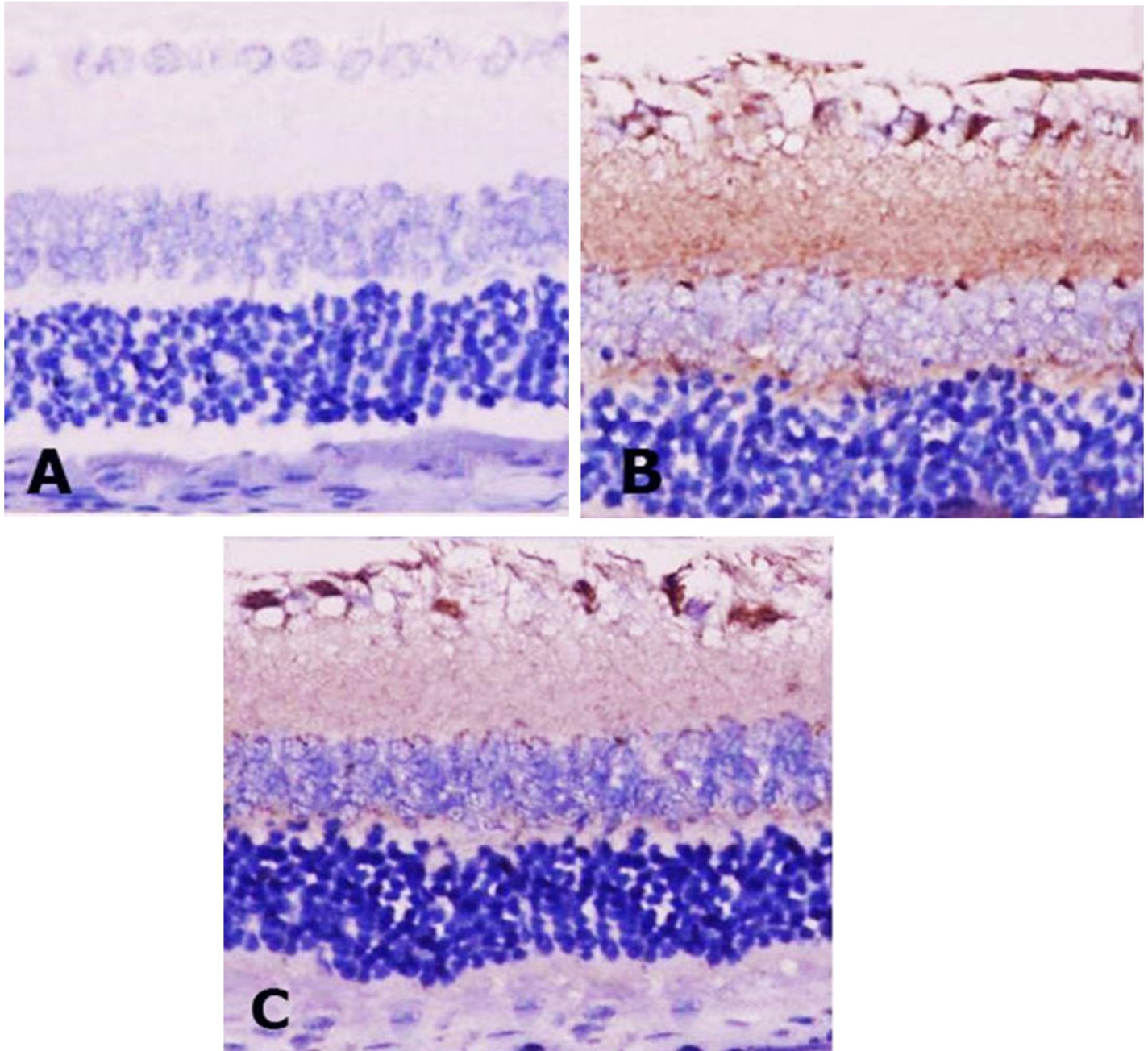


Figure 6. The retina stained immunohistochemically for VEGF and counterstained with hematoxylin X 400. (A) The retina of the control group: showed a negative immune reaction. (B) The retina of the diabetic group: showed brown coloration of cytoplasm in the cells of inner plexiform, ganglion cells, and nerve fiber layers of the retina (positive immune reaction). (C) The retina of the treated group showed a weak positive immune reaction (light brown coloration of these cells) among the diabetes treated group.

capillary basement membrane with a focal accumulation of fibers in some parts. The diabetes treated group showed a basement membrane of capillaries with less thickness (nearly normal capillary wall) as compared to the untreated diabetic group. However, some focal thickness in the capillary wall with focal accumulation of fibers in some parts were observed in this group (Figure 7).

DISCUSSION

Oxidative stress plays an important role in the underlying mechanism of microvascular damage in diabetic retinopathy (Kowluru et al., 1996). Diabetes causes modification of the tight junctions of the retinal blood vessels leading to leakage of fluids and/or blood into the tissue causing retinal edema (Joussen et al., 2004). In

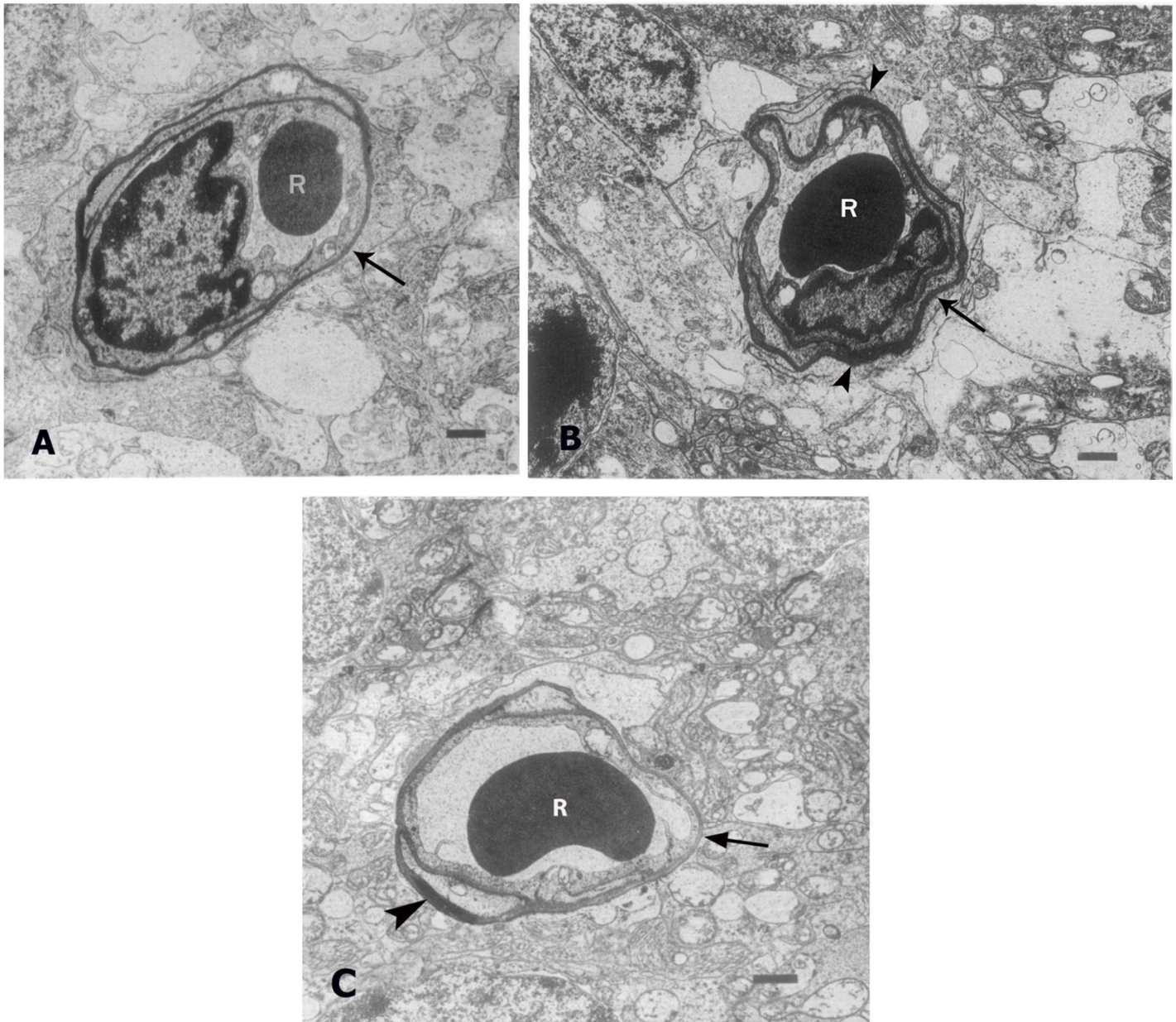


Figure 7. (A) Transmission electron micrograph of a capillary of the retina of the control group showed not thickened capillary wall (arrow). The par represented 1 μ . (B) Transmission electron micrograph of a capillary of the retina of the diabetic group showed markedly thickened capillary wall (arrow) with focal accumulations of fibers in some parts (arrowheads). The par represented 1 μ . (C) Transmission electron micrograph of a capillary of the retina of the treated group showed nearly normal capillary wall (arrow) with focal accumulations of fibers in some parts (arrowheads). The par represented 1 μ .

the later stages, apoptosis of endothelial cells and pericytes are accelerated leading to acellular capillaries and pericytes ghosts (Pfister et al., 2008). In the advanced stage, retinal vascular proliferation (neovascularization) occurs (Crawford et al., 2009). Polyphenols, as powerful antioxidants, have great potential to be a novel promising therapeutic strategy for diabetic retinopathy (Nabavi et al., 2016). Pomegranate peel methanolic extract exhibited high polyphenolic

content and antioxidant capacity compared with other medicinal plants used in the treatment of diabetes and its complications (Althunibat et al., 2010; Al-Rawahi et al., 2014). According to our results, the pomegranate peel extract (PPE) ameliorates the pathological effects of diabetes on the structure of the retina and lens. PPE administration mitigated the microvascular abnormalities, inhibited VEGF expression, and reduced the incidence and severity of cataract. In addition, treatment with PPE

improves, significantly, the biomedical indicators of diabetes. To our knowledge, using PPE, through this study, for the amelioration of diabetic ocular complications was made for the first time. Pomegranate fruit juice (PJ) was used by Tugcu et al. (2017) for this purpose. The authors reported that intake of PJ decreases the oxidative end products and increases the activity of antioxidant enzymes in the retina of diabetic rats.

In agreement with our results, several considerable recent studies using experimental rat models of diabetes suggested that treatment with a number of natural products with a high content of polyphenols can ameliorate diabetic retinopathy. Curcumin (Mrudula et al., 2007; Kowluru and Kanwar, 2007; Gupta et al., 2011; Zuo et al., 2013), green tea (Silva et al., 2013), caffeic acid (Kim et al., 2009), genistein (Kim et al., 2012; Ibrahim et al., 2010), and resveratrol (Ghadiri Soufi et al., 2015; Liu et al., 2013; Yar et al., 2012) have been tried, successfully, for treatment of diabetic retinopathy. These products alleviated diabetic-induced oxidative stress, suppressed retinal neovascularization through inhibition of VEGF expression, reduced retinal vascular hyperpermeability through inhibition of tyrosine kinase, reduced apoptosis of endothelial cells and pericytes. Furthermore, in some clinical human studies, treatment with curcumin improved retinal edema, veno-arteriolar response, and visual acuity (Steigerwalt et al., 2012). In addition, green tea suppressed ocular angiogenesis and vascular permeability through downregulation of VEGF and MMP-9 in diabetic patients (Lee et al., 2014; Skopinski et al. 2004). On the other hand, human studies concerning the anti-diabetic effect of pomegranate supplementation have reported inconsistent results (Jandari et al., 2020; Kim et al., 2016; Das et al., 2001; Parmar and Kar, 2007; Parmar and Kar, 2008). The discrepancy between results of clinical studies might be somewhat attributable to using different types of pomegranate fractions with different doses and duration.

The anti-diabetic effect of pomegranate peel extract is attributable to its phenolic content (Kam et al., 2013; Gil et al., 2000). One key mechanism of ameliorative action of PPE on the diabetic ocular complications is through reducing lipid peroxidation and oxidative stress. This effect may take place by neutralization of generated reactive oxygen species (Banihani et al., 2013). Mitigation of microvascular abnormalities is attributable to inhibition of VEGF and tyrosine kinase (Yar et al., 2012; Ibrahim et al. 2010). In addition, the extract improves insulin sensitivity and inhibits alpha-glucosidase decreasing the conversion of sucrose to glucose (Huang et al., 2005). The lipid-lowering effects of pomegranate peel extract are attributable to enhancing mRNA expression, genes-ABCA1, and HL7702 levels (Zhao et al., 2014).

Limitations of this study include that we used an oral dose of 500 mg/kg body weight in accordance with our

previous study on the rat brain (Belal et al., 2020). Determination of the optimal dose for treatment of diabetic ocular complications needs further well-designed studies with graded doses in experimental animals and clinical trials. Also, the trial was done on the rat model of streptozotocin-induced type 1 diabetes. Further clinical investigations with a large scale and longer duration on type 2 diabetic patients are needed for evaluation of potential beneficial effects of pomegranate peel extract.

CONCLUSION

The findings of the present study have concluded that the pomegranate peel extract possessed a considerable potential for its development as a protective agent against ocular complications of diabetes. Further well-designed investigations with a large scale and longer duration are needed for the evaluation of beneficial effects and tolerability of pomegranate peel extract on diabetic patients.

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Competing interest

The authors declare no competing interest.

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