Pomegranate juice supports therapeutic –treatment of atorvastatin against maternal hypercholesterolemia induced retinopathy of rat offspring

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ABSTRACT
Dietary consumption of processed high fat diet led to the development of metabolic diseases. This study aimed to demonstrate the retinopathy in a rat offspring maternally fed on high cholesterol and the improvement of pomegranate juice (PJ) administration and atorvastatin as potential treatment. Eighty pregnant Wistar albino rats were categorized into eight groups (n = 10); the control, PJ supplementation [0.4 mL (20%)]; atorvastatin-treatment (10 mg/kg); and hypercholesterolemia (fed 3% cholesterol for 6 weeks prior to conception, during pregnancy and lactation period. Offspring of 21-day-old rat offspring were investigated. Offspring maternally fed on a hypercholesterolemic diet exhibited internal retinal hemorrhage at the interphase between the pigment epithelium and photoreceptors leading to retinal detachment. In addition, widespread of degenerated stacked membrane of the photoreceptor outer segment and the neovascularization within the nuclear layers were observed. Offspring retina of a hypercholesterolemic mother exhibited a significant depletion of assayed neurotransmitters coinciding with a significant increase of the vacuolar endothelial growth factor apoptotic markers expressing pressing damaging of retinal cells. Co-administration of pomegranate juice to hypercholesterolemic mother rats treated with atorvastatin significantly improved the retinal structure and the assayed biochemical markers of offspring than that with atorvastatin-treatment. In conclusion, PJ showed a potent antiapoptotic and anti-angiogenesis activity and improved the histocytological pictures of rat offspring maternally subjected to hypercholesterolemia.

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**Introduction**

Consumption of processed high fat food stuffs led to increased blood low-density lipoprotein (LDL), triglycerides, and cholesterol levels contributed to the induction of cardiovascular diseases [1,2], reaching 34 million patients in the United States. The glial cells are the elementary components of the retina involved in cholesterol biosynthesis. However, increased blood levels of cholesteryl esters was associated with retinal accumulation of astrogliosis and drusen-debris in rabbit model of hypercholesterolemia [3], disrupted synaptic structures [4], increased thrombosis of cerebral fine blood vessels [5] and development of age-related macular degeneration [6] particularly in the outer segment associated with deposition of soft and hard drusen [7]. Patients with type 2 diabetes developed atherosclerosis [8] and thickening of the retinal arterial wall [9].

Dietary high cholesterol levels were also contributed to the damage of retinal cells [10]. Rabbit ingested a cholesterol rich diet, exhibited an increased level of 27-hydroxycholesterol (27-OHC) in the hippocampus and pointed out the onset of Alzheimer’s disease (AD) [11]. Also, mutations in the 7-dehydrocholesterol reductase (DHCR7) have resulted in excessive accumulation of 7-dehydrocholesterol and increased retinal degeneration [12].

Pomegranate is the oldest fruit grown in the Middle East [13] and produces phenolic compounds such as tannins hydrolysis (punicalin, pedunculagin, punicalagin, Gallic and ellagic acid) and flavonoids (anthocyanins, catechins and flavonoids) extracted from around about 26%-30% of the total fruit weight [14].

Administration of pomegranate juice (PJ) to diabetic rats was found to reduce cholesterol biosynthesis [15]. It is also associated with increased serum paraoxonase 1 activity
associated with the decreased thickness of tunica intima [16] and alleviated oxidative end products malondialdehyde and 8-oxyhydroxy-deoxyguanosine in diabetic rats [17].

Furthermore, administration of PJ in type 2 diabetic rats has led to a reduction of plasma levels of low-density lipoprotein, cholesterol, triglycerides and inflammatory biomarkers [18]. Statins are also a class of medication involved in reducing blood cholesterol levels by inhibiting the cholesterol synthesizing enzyme, 3-hydroxyl 3 methylglutaryl coenzyme A [19], decreased mevalonate formation involved in synthesis of cholesterol [20], increased endothelial function and improved the activity of antioxidant [21]. Simvastatin also attenuated the cholesterol induced damage in the rabbit hippocampus [22], alleviated cell death of hippocampal cells and improved the synaptic function in AD mice model [23]. Statins can also contribute to improve dry eye disease, corneal ulcer, glaucoma, uveitis, cataracts, proliferative retinopathy, diabetic retinopathy, macular degeneration, and choroidal melanoma [24].

However, there are limited studies on the developmental origin of retinal disorders in offspring maternally fed on a hypercholesterolemic diet. The current study was conducted to demonstrate the therapeutic role of PJ in alleviating the hypercholesterolemia associated with the developmental origin of retinopathy and initiating the therapeutic potential of atorvastatin.

### Materials and methods

This research was conducted according to the National Institute of Health guidelines for the use of laboratory animals (NIH Publication No.8523, updated 1996) and confirmed by the local Experimental Animal ethical committee of Mansoura University, Egypt.

**Preparation of hypercholesterolemic diet**

The high cholesterol diet was prepared according to the study carried out to the study of Enkhmaa et al. [25]. The diet formula is illustrated in Table 1. Rats were fed on a high cholesterol diet for 6 weeks before conception, throughout gestation and lactation period. The control group fed on a standard diet free from cholesterol.

| Ingredient (%) | Control | High Cholesterol diet |
|----------------|---------|------------------------|
| Crude protein  | 20      | 20                     |
| Soybean oil    | 6       | 7                      |
| Cholesterol    | 0       | 3                      |
| Cholic acid    | 0       | 2                      |
| Thiouracil     | 0       | 1                      |
| Cellulose      | 16      | 16                     |
| Casein         | 13      | 13                     |
| L-cystine and choline bitartarate | 1 | 1 |
| Vitamins       | 3       | 3                      |
| Minerals       | 6       | 3                      |
| Sucrose        | 10      | 10                     |
| Carbohydrate   | 11      | 8                      |
| Corn starch    | 14      | 14                     |
| Total          | 100     | 100                    |

**Administration of PJ**

Pomegranate fruits were brought from the market, crushed and squeezed. The PJ was diluted 1:5 (v/v) with a saline solution immediately before administration. Each of the pregnant rats was intragastrically administered a 0.5 mL³ (20%) of fresh juice throughout the pregnancy and lactation period until 21 days postpartum. The applied dose was prepared according to the studies carried out by Aviram et al. [26], Türk et al. [27] and Dulcich and Hartman [28].

**Atorvastatin-treatment**

Atorvastatin is used to reduce the biosynthesis of cholesterol by inhibiting the precursor enzyme 3-hydroxyl-3-methylglutaryl-coenzyme A (HMG-CoA) reductase. The applied dose of 0.5 g atorvastatin calcium trihydrate (Sigma – Aldrich, company) was dissolved in 1 mL 70% ethyl alcohol and diluted with 50 mL saline.
solution. Applied dose of 1 mL\(^3\) containing 10 mg/kg body weight was administered by sterile stomach tube on alternating days from the 6\(^{th}\) day of conception, during gestation and lactation period up to 21 days post-partum [29].

**Experimental work**

A total of 80 virgin females and 16 fertile male albino Wister rat rats weighing approximately 125 g, were obtained from the Ministry of Health, Cairo, Egypt, Breeding farm. They were acclimatized and kept in good ventilation under approximately 12 h light and dark period. They were given free access to a standard diet and water ad-libitum. Mating was carried out and the visualization of sperms in the vaginal smears the next morning predicted the onset of gestation. The pregnant rats were arranged into eight groups (n = 10); control, PJ, atorvastatin, atorvastatin and pomegranate, hypercholesterolemia, hypercholesterolemia and pomegranate and hypercholesterolemia and atorvastatin and pomegranate. Twenty-one-day-old offspring of the studied groups were fastened from food overnight, euthanized by intraperitoneal injection of ketamine (40 mg/kg), sacrificed and their ocular organs were dissected and retinae were separated and processed for procedures detailed in Figure 1

**Assessments of maternal blood glucose levels and serum lipogram**

Serum levels of total cholesterol (TC) were assayed according to the method of Deeg and Ziegenhorn [30] and high-density lipoproteins (HDL) were measured using the study of Grove [31] For low-density lipoproteins (LDL), it was calculated from the total concentrations of triglycerides, cholesterol (TC) and HDL-cholesterol [32]. The glucose was measured by one contact blood glucometers (Life Scan Milipitas, CA, and USA).

**Determination of retinal neurotransmitters**

Homogenization of fresh retinal specimens was carried out in 10% ice-cold 2.5 mM-tris buffer (pH 7.5), containing 1.0 mM EDTA, centrifuged at 14,000 x g for 60 min at 4°C and supernatants were separated and kept in the refrigerator. Serotonin (5-HT) and dopamine (DA) were determined fluorometrically following Schlumpf et al. [33]. In addition, gamma-aminobutyric acid (GABA) was determined by a high performance liquid chromatography using the precolumn PTC derivatization [34].

**Assessments of caspase 3 and 7, vascular endothelial growth factor and 8-hydroxydeoxy-guanosine**

Caspase-3 was determined calorimetrically using a Stressgen (catalog No. 907–013). The supernatant of homogenized retinal tissues were placed with a caspase-specific peptide which conjugated to the p-nitroaniline (pNA). The cleaved peptide was quantitated spectrophotometrically at a wavelength of 405 nm.

Caspase-7 was also determined using an enzyme-linked immunosorbent assay (ELISA)
kit from Usn Life Science Inc., Wuhan, China, Cat. No.: E0449Ra. The microtiter plate was pre-coated with the antibody of caspase-7 compared to the standard. The addition of avidin facilitated the conjugation with horseradish peroxidase (HRP) and subsequently with a TMB substrate solution. The reaction ended and the color reaction is measured spectrophotometrically at a wavelength of 450 nm.

Vascular endothelial growth factor (VEGF) is demonstrated using an ELISA kit obtained from KOMA BIOTECH INC. The level of 8-OHdG was measured through quantitative assessments of DNA with 8-OHdG adducts using an in vitro ELISA from the Japan Institute for the control of aging (Catalog. no. KOG-2005/E.)

**Histological investigations**

Retinal tissues were fixed in a 10% phosphate-buffered formalin (pH 7.4) and processed for histological investigations. Histological 5 µm thick sections were cut, stained with hematoxylin and eosin and examined under a bright field light microscopy.

**Transmission electron microscopy**

The specimens were fixed in phosphate buffered 2% glutaraldehyde (pH 7.4), followed by post fixation in 1% osmium tetroxide at 4°C, dehydrated in ascending concentrations of ethyl alcohol, and mounted in epoxy resin. Ultrathin sections were cut with an LKB Ultratome IV (LKB Instruments, Bromma, Sweden) stained with uranyl acetate and lead citrate, and examined in a Joel 100CXI transmission electron microscope at the Mansoura University lab (Musashino 3- chome; Akishima, Tokyo, Japan).

**Single cell gel electrophoresis (comet assay)**

The retinal tissues were lysed with (2.5 M NaCl, 0.1 M Na_2_ ethylenediaminetetraacetic acid (EDTA), 0.01 M Tris, and 10 g/L sodium N-lauroylsarcosine at pH 10. To allow the unwind of DNA, the specimens were mounted in a buffer solution composed of 300 mM NaOH and 1 mM EDTA (pH 13). Following done electrophoresis at 300 mA and 0.7 V/cm and neutralization with 0.4 M Tris–HCl buffer (pH 7), the slides were stained with a 100 µL of a 2 µg/mL ethidium bromide. About 50 cells per each slide were investigated using the comet assay II automatic digital analysis system method at a Leitz Orthoplan (Wetzlar, Germany) fluorescence microscope. It is carried out by staining with acridine orange and measured with the fluorescence microscope photometer with a × 40 objective using a Phloem Pak Filter block H2 giving excitation at 390–490 nm. The tail length (DNA migration) and DNA concentration (density) were measured automatically by image analysis software to quantify the DNA damage [35].

**Statistical analysis**

Data are presented as means ± standard error (SE). The statistical studies were carried out using post-hoc analysis of a one-way-variant analysis of the SPSS (version 13) software program. At p < 0.05, the F-test was estimated and become statistically significant.

**Results**

**Biochemical observations**

The serum of hypercholesterolemic mother administered pomegranate and/or atorvastatin revealed improvement of blood glucose levels and the serum levels of LDL, triglycerides, total cholesterol and HDL relative to mothers who consumed a high cholesterol diet and control groups (Figure 2).

The retina of offspring maternally fed on a hypercholesterolemic diet showed a significant decrease of dopamine and serotonin coinciding with increased retinal levels of VEGF, 8-OHdG and caspase 3 and 7. However, co-administration
of PJ to hypercholesterolemic mother rats treated with or without atorvastatin showed a significant decrease of the sharp rise of VEGF, 8-OHdG, and caspase 3 and 7 and nearly normalized the retinal dopamine and serotonin levels in the retina of the offspring (Tables 2 & 3).

**Light and transmission electron microscopy**

Compared with the control (Figure 3(A & B) and Figure 4(A)), the retina of offspring of mothers fed on a high cholesterol diet indicated damaging of the ganglion cells, inner and outer nuclear layer and vacuolated photoreceptor outer segment (Figure 3(C)). Toluidine blue staining retinal semithin sections of offspring maternally fed on a high cholesterol diet revealed numerous neovascularization of blood vessels within the ganglion and the interphase between the inner and outer nuclear layers. In addition dense hemorrhagic spots were identified between the photoreceptor

![Figure 2](image.png)

**Figure 2.** Blood glucose level (Gl) and serum levels of triglyceride (TG), total cholesterol (TC), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) of hypercholesterolemic mother treated with atorvastatin and/or pomegranate juice supplementation. Stars means significant at P < 0.05.

| Table 2. Biochemical markers of retinal function of 2-week-old offspring maternally fed on a high cholesterol diet and and/or atorvastatin and pomegranate juice. |
|-----------------------------------------------|
|                  | DA (ng/mg) | 5-HT (ng/mg) | GABA (ng/mg) | VEGF (pg/100 mg) | 8-OHdG (ng/100 mg) | Casp3 (ng/100 mg) | Casp7 (ng/100 mg) |
|------------------|------------|--------------|--------------|-----------------|-------------------|----------------|-----------------|
| Control          | 18.22 ± 2.34 | 3.84 ± 0.21 | 60.25 ± 2.56 | 341 ± 4.58      | 0.29 ± 0.01       | 0.39 ± 0.01     | 3.18 ± 0.34     |
| A (10 mg/kg)     | 14.05 ± 2.13* | 3.53 ± 0.17 | 48.05 ± 4.15 | 339 ± 3.76*     | 0.26 ± 0.02       | 0.47 ± 0.17     | 3.78 ± 0.65     |
| A + P            | 18.09 ± 2.76 | 3.75 ± 0.26 | 56.64 ± 5.10 | 340 ± 3.53      | 0.31 ± 0.02       | 0.41 ± 0.03     | 3.53 ± 0.38     |
| H                | 13.59 ± 1.46* | 2.75 ± 0.21 | 45.64 ± 2.73*| 364 ± 3.54*     | 0.56 ± 0.03*      | 0.52 ± 0.04*    | 4.15 ± 0.63*    |
| H + A            | 14.53 ± 1.59* | 2.91 ± 0.21 | 48.20 ± 2.75*| 348 ± 3.54*     | 0.45 ± 0.02       | 0.46 ± 0.02*    | 4.04 ± 0.52*    |
| H + P            | 17.63 ± 2.25 | 3.41 ± 0.24 | 56.50 ± 4.85 | 356 ± 4.64      | 0.34 ± 0.01       | 0.42 ± 0.02     | 3.87 ± 0.21     |
| H + A + P        | 17.43 ± 2.98 | 3.56 ± 0.31 | 56.67 ± 3.87 | 343 ± 4.65      | 0.39 ± 0.02       | 0.58 ± 0.02*    | 3.98 ± 0.31     |

Each result represents the mean ± SE (n = 5), * Significant at P < 0.05. Abbreviations; A, atorvastatin; H, hypercholesterolemia; Casp3, caspase3; casp7, caspase 7; 5-HT, serotonin; GABA, gamma amino butyric acid; 8-OHdG, 8-hydroxydoxy-guanosine; P, pomegranate juice; VEGF, vascular endothelial growth factor.
Table 3. Biochemical markers of retinal function of 3 week-old offspring maternally fed on a high cholesterol diet and/or atorvastatin and pomegranate juice.

|           | DA (ng/mg) | 5-HT (ng/mg) | GABA (ng/mg) | VEGF (Pg/100 mg) | 8-Ohdg (ng/100 mg) | Casp3 (ng/100 mg) | Casp7 (ng/100 mg) |
|-----------|------------|--------------|--------------|-----------------|-------------------|-------------------|-------------------|
| Control   | 18.12 ± 1.98 | 3.87 ± 0.18  | 58.09 ± 3.78 | 334 ± 5.87      | 0.34 ± 0.01       | 0.51 ± 0.04*     | 3.08 ± 0.19       |
| A (10 mg/kg) | 16.25 ± 1.65* | 2.76 ± 0.13  | 47.15 ± 3.85* | 342 ± 5.12*     | 0.48 ± 0.02       | 0.44 ± 0.03       | 3.98 ± 0.23       |
| A + P     | 13.78 ± 2.37 | 3.56 ± 0.32  | 53.98 ± 3.53 | 338 ± 4.25      | 0.32 ± 0.02       | 0.40 ± 0.02       | 3.63 ± 0.28       |
| H         | 13.86 ± 1.84* | 2.69 ± 0.18  | 46.81 ± 2.86* | 364 ± 2.76*     | 0.59 ± 0.04*      | 0.76 ± 0.02*      | 4.24 ± 0.27*      |
| H + A     | 14.65 ± 1.85* | 3.04 ± 0.18  | 49.59 ± 3.12* | 352 ± 3.43*     | 0.47 ± 0.03       | 0.51 ± 0.04*      | 3.62 ± 0.26*      |
| H + P     | 16.75 ± 2.31 | 3.33 ± 2.37  | 53.68 ± 4.19 | 353 ± 5.12      | 0.39 ± 0.01       | 0.44 ± 0.03       | 3.65 ± 0.28       |
| H + A + P | 16.55 ± 3.12 | 3.49 ± 0.27  | 53.84 ± 2.96 | 348 ± 5.14      | 0.43 ± 0.03       | 0.40 ± 0.03       | 3.75 ± 0.26       |

Each result represents the mean ± SE (n = 5). * Significant at P < 0.05. Abbreviations: A, atorvastatin; H, hypercholesterolemia; Casp3, caspase3; casp7, caspase 7; 5-HT, serotonin; GABA, gamma amino butyric acid; 8-Ohdg, 8-hydroxydoxy-guanosine; P., pomegranate juice; VEGF, vascular endothelial growth factor.

Figure 3. Photomicrographs of cross-histological sections of retina of offspring 21-day old. A. Control showing normal retinal structure. (B). Maternally treated with atorvastatin showing a slight intact of retinal layer with mild damaging cells. (C). Maternally hypercholesterolemic showing thinning of ganglion layer, hyaline degeneration of inner plexiform, reduction of nuclear cells and vacuolated photoreceptor outer segment. (D). Maternally hypercholesterolemic and pomegranate-supplementation showing improvement. (E). Maternally hypercholesterolemic and treated with atorvastatin showing moderate improvement. (F). Maternally hypercholesterolemic and treated with atorvastatin and pomegranate showing a considerable amelioration. Abbreviations: BV, blood vessel; GL, ganglion layer; ILM, inner limiting membrane, INL, inner nuclear layer; IPL, inner plexiform layer; NFL, nerve fiber layer; ONL, outer nuclear layer; OPL, outer plexiform layer; PE, pigment epithelium; PS, photoreceptor.
outer segment and pigmented epithelium (Figure 4(B)).

On the other hand, the administration of PJ to a hypercholesterolemic mother presented an improved retinal structure of their offspring (Figures 3(D) and 4(C)). However, the atorvastatin-treatment to mother rats fed on high cholesterol developed signs of retinal cytotoxicity characterized by decreased densities of the outer nuclear cells (Figure 3(E)). In addition, co-administration of PJ and/or atorvastatin-treatment improved the retinal histological picture of offspring compared
Figure 5. TEM micrograph of retinal pigmented epithelium and photoreceptor outer segment of 21d-old pup. (A-B). Control showing normal pigmented epithelium with underlying choriocapillaris and photoreceptor outer segment with normal traversed stacked membranes. (C & D). Maternally fed on a high cholesterol diet showing internal hemorrhage at the interphase between pigmented epithelium and photoreceptor outer segment. Also, there is a detected degeneration of the stacked membrane of the photoreceptor outer segment. (E & D). Maternally hypercholesterolemia and treated with atorvastatin showing pigmented epithelium with karyolitic of chromatin material (E) and degenerated stacked membranes (F). (G-H). Maternally hypercholesterolemic and treated with atorvastatin and pomegranate-treatment showing improved pigment epithelium and photoreceptor outer segment. Lead citrate and Uranyl acetate. Abbreviations; BL, basal lamina; BM, Bruch membrane; BV, blood vessel; DOS, degenerated outer segment; E, endothelium; IH, internal hemorrhage; M, mitochondria; MV, microvilli; N, nuclei; OS, outer segment.
with that of mother fed on a high cholesterol diet (Figure 3(E & F)).

At the ultrastructural level, compared with normal (Figure 5, 6(A &B) and 7(AA)), the retina of offspring maternally fed on a hypercholesterolemic diet sowed abnormal retinal structure characterized by neovascularization of blood vessels with internal hemorrhage at the internal phase between the pigment epithelium and photoreceptor outer segments. The pigment epithelium exhibited either pyknotic or karyolysed nuclei and distorted cytoplasmic microvillus structures. Atrophied mitochondria were clearly identified. Necrotic patches appeared widespread within the stacked membrane of the photoreceptor outer segment (Figure 5(C) & (D)). In addition, the horizontal and bipolar cells of the inner nuclear cells appeared with an electron-dense accumulation of heterochromatin in their nuclei reflecting a sign of pyknotic cell death. Also, neovascularization was observed at the interphase between the inner and outer nuclear cells (Figure 6(E) & (F)). Many of the ganglion cells were damaged (Figure 7(E)). However, administration of atorvastatin to hypercholesterolemic mother resulted in mild distorted villous cytoplasmic structure of the retinal pigment epithelium of
Figure 7. TEM micrograph of retinal ganglion of 21d-old offspring. A. Control showing normal ganglion cells. B. Maternally supplemented pomegranate-treatment showing normal ganglion cells. C. Maternally treated with atorvastatin showing degenerated ganglion cells. D. Maternally treated with atorvastatin and pomegranate showing intact ganglion cells. E. Maternally fed on a high cholesterol showing damaged ganglion cells. F. Maternally fed on a high cholesterol diet and supplemented pomegranate juice showing amelioration. G. Maternally fed on a high cholesterol diet and treated with atorvastatin showing less damaged ganglion cells. H. Maternally fed on a high cholesterol diet and treated with atorvastatin and pomegranate-treatment showing numerous improved ganglion cells. Lead citrate and Uranyl acetate. Abbreviations; BV, blood vessel; DGC, degenerated ganglion cells; E, endothelium; GC, ganglion cells; ILM, inner limiting membrane; NF, nerve fiber.
offspring (Figure 5(E) & (F)). Minor damage of the nuclear cells was observed (Figure 6(I) & (j)). The ganglion cells were improved (Figure 7(H)). However, co-administration of PJ to hypercholesterolemic mother treated with atorvastatin restored the normal ordinary structure of the
retinal cells especially the pigment epithelium, photoreceptor, nuclear and ganglion cells. There was no sign of neovascularization (Figure 5(G) & (H), 6(G–I), and 7(G) & (H)).

**Comet assay**

After application of comet assay, the single-strand nucleotide of the 21-day-old offspring of controlled retinal cells and those of maternally supplemented PJ tended to possess the ‘dark/red’ round spot without migration. However, those maternally ingested a hypercholesterolemic diet showed a numerical increase of detached tail and DNA damaged retinal cells. Atorvastatin-treatment to mother rats consumed a high cholesterol diet showed few damaged DNA in their offspring’s retinal cells. On the other hand, co-administration of PJ to hypercholesterolemic mothers treated with atorvastatin decreased the frequency of apoptotic retinal cells of offspring (Figures 8 & 9).

**Discussion**

This study revealed the damaging of the retinal cells of offspring maternally fed on a diet with a high cholesterol diet. The pigment epithelium exhibited pyknotic or karyolysed nuclei and distorted cytoplasmic villous structure and mitochondrial atrophy. This reflected the degeneration of the stacked membrane of the photoreceptor outer segment and decreased renewal capacity of the photoreceptor outer segment and consequently altered the visual acuity. Atorvastatin-treatment did not provide an optimal capacity or improvement. The pigmented epithelium and the stacked membrane of the photoreceptors were altered. The changes in RPE reflected the degeneration of the photoreceptors [36], which depend completely on normal RPE function for nutrients transport and phagocytosis at the terminal end of the outer segment [37].

However, the increased cholesterol level induced neovascularization and internal hemorrhage at the interphase between the pigment epithelium and photoreceptor outer segments. These appeared to be responsible for increasing retinal ischemia associated with neovascularization between the inner and outer nuclear cells and consequently damaging the retinal cells in the offspring of the hypercholesterolemic group. The increase of retinal VEGF supported the genesis of blood capillaries explaining retinal ischemia. Retinal neovascularization (RNV) was also resulted from diabetes mellitus [38,39] and reflected the reduction of visual acuity [40].

Furthermore, maternal hypercholesterolemia damaged the ganglion cells and the inner and outer nuclear cells in their offspring. Varying degrees of necrotic photoreceptor outer segment were observed. These findings reflected retinopathy and visual impairment [41].

Hypercholesterolemia was found to increase LDL and blood sugar levels [42] and enhanced the formation of the advanced glycation end product [43] that produce oxidative stress and increase the production of oxidized LDL. This increased atherosclerotic lesions in the endothelial cell, thereby impaired oxygen and nutrients transport contributing to vision impairment [44,45].

In addition, the observed retinal ischemia reflected the damage of retinal cells confirmed by the significant increase of apoptotic markers caspase 3 and 7 and 8-hydroxydeoxyguanosine and DNA damage by comet assay. This result was consistent with El-Sayyad et al. [42] who reported increased apoptotic retinal cells in pups maternally fed on a hypercholesterolemic diet.

Also, the damaging of the photoreceptors and nuclear cells was confirmed by the significant decrease of the retinal neurotransmitters dopamine, serotonin and γ-aminobutyric acid in offspring of a hypercholesterolemic mother. The current results are consistent with Kim et al. [46], who mentioned that diabetes was mediated by rod-pathway dysfunction and
dopamine impairment. Also, γ-aminobutyric acid was highly expressed in the Müller and photoreceptor cells and altered in diabetic retinopathy [47]. In addition, serotonin impairment in patients with proliferative diabetic retinopathy was associated with a decreased level of serotonin [48]. Dopamine depletion can also lead to loss of pigment epithelium [49]; ganglion cell [50], and development of the stiffness of retinal arteries and consequently retinal ischemia associated with visual impairment [51]. The levels of retinal neurotransmitters reflected the functional activity of neurons, and preserve the visual acuity especially during postnatal life. Dopamine receptors are detected in the mammalian photoreceptors [52] involved in managing the circadian light-adaptation and visual acuity [37]. The reduction in the assayed retinal neurotransmitters confirmed diabetic retinopathy.

Furthermore, co-administration of PJ to a hypercholesterolemic mother treated with atorvastatin synergistically improved the offspring and the retinal cells, coinciding with restoring almost normal retinal levels of γ-aminobutyric acid, serotonin and dopamine. At the same time, caspases, 8-OHdG, and VEGF decreased compared with atorvastatin.

PJ is rich in bioactive compounds such as ellagic acid, ellagitannin, gallic acid, punicalagin and urolithin [53]. Punicalagin is also responsible for >50% of the antioxidant activity of PJ [54], reduced oxidative stress and anti-apoptotic activity of an in vitro culture of retinal pigment epithelium subjected to ultraviolet radiation [55]. Pomegranate fractions punicalagin, ellagic, gallic, oleanolic, ursolic, and gallic acids attenuated type 2 diabetes through oxidative stress reduction and lipid peroxidation [56]. Dietary administration of ellagic acid was found to improve the retinal structure and attenuated the expression of VEGF, and cell death [57].

Taking into consideration, the retinal pigment epithelium cells expressed the retinol-binding protein (RBP) receptor and cell surface receptor for RBP STRA6, which facilitates the uptake of retinol, via conversion to 11-cis retinaldehyde and transported to the photoreceptors to form rhodopsin [58].

Also, Goula et al. [59] extracted carotenoids from a pomegranate. Retinol, rhodopin, canthaxanthin, xanthophylls, lutein (LUT), and zeaxanthin (ZEA) are carotenoids with potent antioxidant activities, capable of crossing the retinal pigment epithelium and accumulated in the macula and protecting against age-related macular degeneration [58]. Carotenoids were found to improve the neovascularization and damage of both retinal cells and photoreceptor outer segment [60].

Finally, the author concluded that PJ contains phytonutrients that activate atorvastatin in improving the retinal structure and function of offspring maternally fed on a high cholesterol diet.

Disclosure statement

The authors declare that there are no conflict of interest

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