

Original Article

The protective effect of procyanidins on high glucose-induced oxidative damage in the rabbit lens

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Abstract: Excessive exposure to oxygen has been indicated as a risk factor for cataracts. This study aims to explore the protective role of procyanidins (PCs) on rabbit lens oxidation damage induced by high glucose (HG). The rabbits were randomly divided into four groups: (A) blank control, (B) HG treatment, (C) Vitamin C (VC) + HG treatment (200 mg/L), and (D) PCs + HG treatment (200 mg/L). The lenses were cultivated in vitro. At 48 h, 72 h and 96 h, the opacity of the lenses in each group was detected separately. The contents of superoxide dismutase (SOD), catalase (CAT) and malondialdehyde (MDA) were analyzed. The morphology of the lens epithelial cells was observed by H&E staining. Immunohistochemistry was applied to detect the expression of Bcl-2 and Bax. The turbidity of the lenses from Group B was significantly higher than in Group A. Groups C and D showed lower turbidity than Group B. The SOD and CAT contents were decreased in Group B, while treatment with VC or PCs clearly reversed HG-induced SOD and CAT reduction. Regular morphology could be observed in Group A, while Group B showed broken fibers and many empty air bubbles. In comparison, fewer empty air bubbles and lens flaws could be observed in Group C and Group D. Decreased Bcl-2 and enhanced Bax expression was detected in Group B, while VC or PCs treatment partially reversed these effects. PC protects lenses from HG-induced oxidative injury through enhancing SOD and CAT production, as well as reducing cell apoptosis.

Keywords: Procyanidins, cataract, lens, rabbit, oxidative damage

Introduction

Cataracts mainly result from the reduction or loss of the opacity of the lens. A number of studies show that cataracts remain the world's main cause of blindness [1-3]. The incidence of cataracts is 10%~20% at the age of 40 years and 80%~90% at the age of 80 years. Age-related cataracts are the main reason for the decreased visual acuity in the elderly. Currently, the only effective treatment for cataracts is surgical treatment due to the poor effects of drug treatment. However, many patients with cataracts are the elderly, who often have other systemic diseases or eye disease. Therefore, they cannot undergo cataract surgery, or the post-operative recovery is not satisfactory [4]. Thus, the cause and the mechanism of cataracts should be deeply explored. Currently, their pathogenesis is still unclear.

At present, there are many factors affecting cataracts, such as aging, ultraviolet irradiation, diabetes and others. Recent studies have indi-

cated that oxidative damage and apoptosis of the lens capsule membrane cells are considered the main reasons [5, 6]. Many studies have proven that antioxidants can prevent or delay the occurrence and development of cataracts [7-9].

Procyanidins (PCs) are a polyphenol mixture [10]. In nature, there is a wide distribution of PCs, which is consisted of different monomers [11]. For many years, researchers have extracted PCs from various substances and they are characterized by a strong antioxidant capacity [12]. In this study, rabbit lenses were placed in a high-glucose environment and the effects of PCs on diabetic cataracts were explored which may provide a practical basis for their clinical application.

Materials and methods

Lens culture

A total of 45 white rabbits were selected, and ocular diseases were excluded through slit

PCs protect lens from HG injury

Table 1. The lens turbidity of group D was significantly less than in group B after 48 h

	I	II	III	IV	V
A	9	0	0	0	0
B ^e	0	4	5	0	0
C ^f	5	4	0	0	0
D ^g	6	3	0	0	0

ps: I, II, III, IV and V indicating the 5 levels of lens opacity; e, $P < 0.01$ vs. A; f, $P < 0.01$ vs. B; g, $P < 0.01$ vs. B.

Table 2. The lens turbidity of group D was significantly less than in group B after 72 h

	I	II	III	IV	V
A	7	2	0	0	0
B ^e	0	0	2	5	2
C ^f	4	4	1	0	0
D ^g	5	4	0	0	0

P values: I, II, III, IV and V indicating the 5 levels of lens opacity; e, $P < 0.01$ vs. A; f, $P < 0.01$ vs. B; g, $P < 0.01$ vs. B; $P < 0.01$.

Table 3. The lens turbidity of group D was significantly less than in group B after 96 h

	I	II	III	IV	V
A	7	2	0	0	0
B ^e	0	0	0	2	7
C ^f	3	4	2	0	0
D ^g	4	5	0	0	0

P values: I, II, III, IV and V indicating the 5 levels of lens opacity; e, $P < 0.01$ vs. A; f, $P < 0.01$ vs. B; g, $P < 0.01$ vs. B.

lamp examination. Animals were sacrificed through air embolism, and the eye was rapidly removed. Then, the vitreous was dissected, and the lens was cultured in saline (500 ml saline containing penicillin 800,000 U + gentamycin 80,000 U) under sterile conditions. One minute later, the lenses were placed in twelve-well plates containing low Dulbecco's Modified Eagle's Medium (L-DMEM, Gibco) containing 10% fetal bovine serum (FBS), 100 U/ml streptomycin and 100 U/ml penicillin.

Grouping

Group A (blank control group): Twenty-one transparent lenses were placed in a 12-well culture plate containing L-DMEM medium (final glucose concentration 5.56 mM).

Group B (HG treatment group): Twenty-one transparent lenses were placed in H-DMEM cul-

ture medium (final glucose concentration at 55.6 mmol/L).

Group C (VC treatment group): Twenty-one transparent lenses were placed in a 12-well culture plate containing H-DMEM medium, and the final concentration of VC was 200 mg/L. To ensure adequate nutrition, the culture medium was replaced every 12 h.

Group D (PC treatment group): Twenty-one transparent lenses were placed in a 12-well culture plate containing H-DMEM medium, and the final concentration of PCs was 200 mg/L. To ensure adequate nutrition, the culture medium was replaced every 12 h.

Determination of lens opacity

Nine lenses were selected from each group. According to Azuma grading standards, the degree of lens opacity was divided into 5 levels: I, the lens was transparent and opaque; II, vacuoles were found in the periphery of the lens, which showed mild turbidity; III, the number of vacuoles was increased, and the cavity was expanded into the center. IV: the vacuoles were expanded to the nucleus, and the opacity of the lens was increased; V, the lens was opaque with the formation of cataract.

Enzyme activity assay

Twelve lenses were selected from each group. The lenses were cultured for 48 h, 72 h and 96 h for the enzyme activity assay. The weight of the lens was measured, and then physiological saline was added. Next, a 10% tissue homogenate was prepared. The tissue homogenate was centrifuged at 2000 rpm/min for 10 min. The contents of total protein, SOD, CAT and MDA were determined using the assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

Specimen preparation

The remaining lenses cultured at 48 h, 72 h and 96 h were randomly selected and placed in 4% paraformaldehyde for 48 h. Through gradient alcohol dehydration, transparent xylene and paraffin embedding, 5 μ m thick slices were prepared for H&E staining and immunohistochemical staining.

PCs protect lens from HG injury

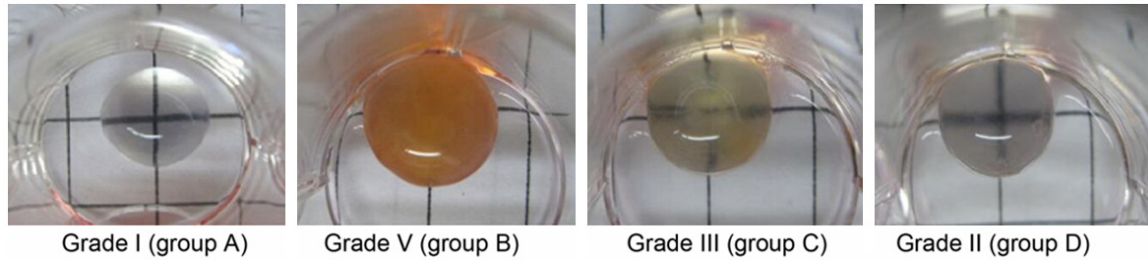


Figure 1. Representative figures of lens in different groups suggested that PCs treatment significantly improved lens turbidity in group D.

Table 4. Compared with group B, SOD contents were much reduced in group C and group D ($\bar{x} \pm s$, n=4, U/mg protein)

	A	B	C	D
48 h	23.883±0.507	11.478±0.535 ^a	17.483±0.448 ^{b,d}	20.605±0.302 ^{c,e,f}
72 h	22.653±0.410	6.213±0.546 ^a	13.516±0.599 ^{b,d}	17.399±0.403 ^{c,e,f}
96 h	18.447±0.609	4.305±0.351 ^a	10.478±0.456 ^{b,d}	16.534±0.479 ^{c,e,f}

$P < 0.01$, a, b, c vs. A; $P < 0.01$, d, e vs. B; $P < 0.01$, f vs. C.

Table 5. PCs treatment could reduce the MDA contents induced by HG ($\bar{x} \pm s$, n=4, nmol/g protein)

	A	B	C	D
48 h	0.635±0.189	6.569±0.158 ^a	1.31±0.089 ^{b,d}	1.063±0.009 ^{c,e,f}
72 h	1.313±0.512	8.174±0.099 ^a	2.356±0.216 ^{b,d}	1.849±0.019 ^{c,e,f}
96 h	1.758±0.077	11.535±0.247 ^a	3.663±0.232 ^{b,d}	2.844±0.026 ^{c,e,f}

$P < 0.01$, a, b, c vs. A; $P < 0.01$, d, e vs. B; $P < 0.01$, f vs. C.

Table 6. PCs treatment could enhance CAT activity compared with HG pre-incubation ($\bar{x} \pm s$, n=4, nmol/g protein)

	A	B	C	D
48 h	6.253±0.142	2.268±0.111 ^a	4.756±0.126 ^{b,d}	5.305±0.127 ^{c,e,f}
72 h	5.501±0.129	1.535±0.066 ^a	4.532±0.569 ^{b,d}	5.056±0.102 ^{c,e,f}
96 h	5.335±0.417	0.921±0.072 ^a	4.038±0.149 ^{b,d}	4.765±0.089 ^{c,e,f}

$P < 0.01$, a, b, c vs. A; $P < 0.01$, d, e vs. B; $P < 0.01$, f vs. C.

H&E staining

Sections were dewaxed routinely and stained with hematoxylin for 4 min. The sections were then differentiated with 1% hydrochloric acid for 20 s following water flushing for 1 min. After eosin staining for 2 min, the sections were washed with ddH₂O for 1 min. Then, after dehydration and transparency, the sections were mounted with neutral gum.

Immunohistochemical staining

Sections were conventionally dewaxed in water and incubated at room temperature for 10 min

with 3% H₂O₂ to inactivate the endogenous peroxidase. The slides were washed 3 times with ddH₂O (5 min/time). After heat antigen repair (heating 0.01 mM citrate buffer (pH 6.0) to boiling), the slides were cooled at room temperature and washed with PBS (3 times, 5 min/time). The slides were blocked with normal goat serum and incubated at room temperature for 10 min. Then, the primary antibodies for Bcl-2 (1:50, CST) and Bax (1:50, CST) were added and incubated at 4°C overnight. The slides were washed with PBS three times (5 min/time), and SABC solution was added. Then, the slides were incubated at 37°C for 30 min and washed again with PBS 3 times. Finally, the sections were stained with diaminobenzidine (DAB), and the nucleus was counterstained

with Meyer's hematoxylin. A negative control was obtained through replacing the primary antibody with PBS.

The calculation was performed as follows: under a light microscope, 50 lens epithelial cells were selected randomly, and the positive cells were calculated as a percentage of the total number of cells. The percentage was applied to determine the expression levels: 0%-10% indicating negative (-), 11%-20% indicating weak positive (+), 21%-30% indicating positive (++), 31%-40% suggesting strong positive (+++), and 41% indicating very strong positive (++++).

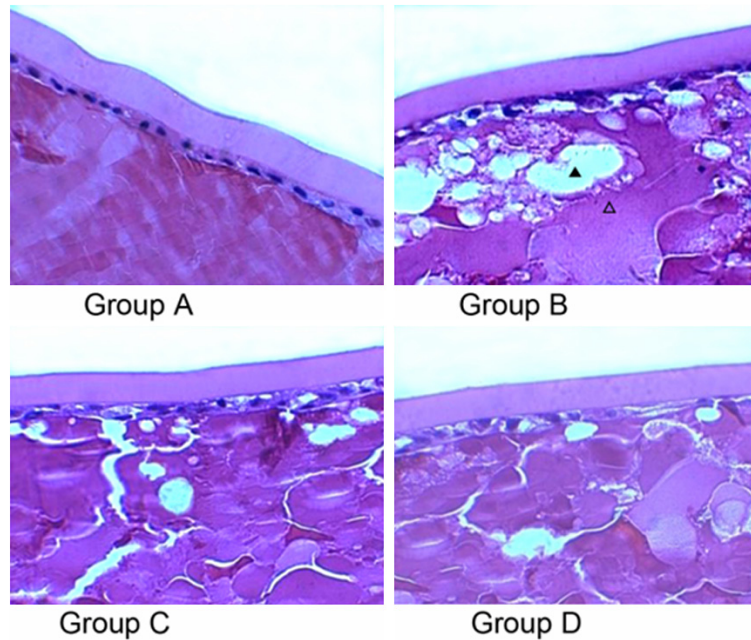


Figure 2. Representative lenses of each group stained by H&E (magnification, $\times 400$). Far fewer vacuoles and nearly normal fiber arrangement were observed by PCs treatment than HG treatment. \blacktriangle represents vacuole; Δ represents fiber.

Table 7. VC treatment and PC treatment enhanced Bcl-2 expression in the lens epithelial cells at 48 h

	-	+	++	+++	++++
A	0	0	0	1	8
B ^a	5	3	1	0	0
C ^b	0	0	0	2	7
D ^c	0	0	0	1	8

a, $P < 0.01$ vs. A; b, $P < 0.01$, vs. B; c, $P < 0.01$, vs. B.

Table 8. VC treatment and PC treatment enhanced Bcl-2 expression in the lens epithelial cells at 72 h

	-	+	++	+++	++++
A	0	0	0	1	8
B ^a	6	2	1	0	0
C ^b	0	0	0	3	6
D ^c	0	0	0	2	7

a, $P < 0.01$ vs. A; b, $P < 0.01$, vs. B; c, $P < 0.01$, vs. B.

Statistical analysis

The two-tailed unpaired student's t-tests were used for comparisons of two groups. The ANOVA multiple comparison test (SPSS 13.0) followed by Turkey *post hoc* test were used for

comparisons of two more groups. $P < 0.05$ was considered to be statistically significant. All the experimental data are represented as the mean \pm SD. The statistical analyses were performed using the SPSS software (SPSS Standard version 19.0, SPSS Inc. Chicago, IL, USA).

Results

Comparison of lens opacity in different groups

Along with the culture time, the lenses of each group were different. After 48 h, no change was found in group A. For group B, 4 (44.44%) lenses demonstrated grade II turbidity, and 5 (55.56%) showed grade III turbidity. In group C, 4 (44.44%) demonstrated grade II turbidity, and 3 (33.33%) showed grade II opacity in group D. After 72 h, 2 (22.22%) lenses demonstrated grade II turbidity, and 2 (22.22%) showed grade V opacity in group B. In comparison, 1 lens (11.11%) demonstrated grade III turbidity in group C, and after 96 h, lens opacity had expanded from the equator to the cortex. In group B, 7 (77.78%) lenses showed grade V opacity. In comparison, 2 (22.22%) lenses showed grade III turbidity in group C, and only 5 (55.56%) lenses showed grade II turbidity in group D, while the rest of the lenses were completely transparent. Clearly, the lens turbidity of group D was significantly less than in group B and group C (Tables 1-3; Figure 1).

Determination of SOD, MDA and CAT contents

No significant changes were detected in group A with increasing culture time, while SOD contents were significantly increased in group B. In comparison with group B, SOD contents were much reduced in group C and group D. Group D also demonstrated better results than group C, suggesting that PCs could obviously improve SOD activity (Table 4).

After 48 h, the MDA activity of group B was much higher than in group A. Meanwhile, the

Table 9. VC treatment and PC treatment enhanced Bcl-2 expression in the lens epithelial cells at 96 h

	-	+	++	+++	++++
A	0	0	0	2	7
B ^a	9	0	0	0	0
C ^b	0	0	0	4	5
D ^c	0	0	0	3	6

a, $P<0.01$ vs. A; b, $P<0.01$, vs. B; c, $P<0.01$, vs. B.

MDA contents of group C and group D were higher than in group A, but much lower than in group B. Group D was lower than group C, nearly reduced to the normal level. These results indicate that PCs could reduce the MDA contents induced by high glucose (**Table 5**).

CAT can protect the intracellular environment and maintain cellular morphology. CAT activity was not reduced in group A with increasing culture time but was significantly decreased in group B. Compared with group B, CAT activity in groups C and D was increased, with higher levels in group D. These data indicate that PCs could increase the activity of CAT (**Table 6**).

Comparison of lens morphology

HE staining showed that the lenses of group A were transparent after 96 h, showing compact, neat, round and oval linear arrangement (**Figure 2**). Group B was found to have a large number of vacuoles (**Figure 2**), and the structure of epithelial cells was not complete. In group C, fiber breakage and vacuoles could also be observed, but were much improved over group B (**Figure 2**). In group D, there were far fewer vacuoles than in group B, and the fiber arrangement was nearly normal (**Figure 2**).

Analysis of Bcl-2 and Bax expression in different groups

Immunohistochemical staining showed that both the Bax and Bcl-2 proteins were expressed in the cytoplasm of epithelial cells, and the specific brown granules in the cytoplasm of the epithelial cells were positive.

At 48 h, a small amount of Bcl-2 was expressed in group B, and no Bcl-2 was detected at 96 h. Compared with group A, the expression of Bcl-2 was significantly reduced in group B. In comparison with group B, VC treatment and PC

treatment enhanced Bcl-2 expression (**Tables 7-9; Figure 3A**).

Compared with group A, the expression of Bax was significantly enhanced in group B, and the expression gradually increased with culture time. However, treatment with VC or PC could significantly reverse this HG-induced Bax expression enhancement (**Tables 10-12; Figure 3B**).

Discussion

Blood glucose in diabetic patients cannot be maintained at normal levels, resulting in the activation of the polyol pathway of aldose reductase (ALR) [13, 14]. Therefore, a large amount of glucose can be dispersed into the lens. The activation of ALR breaks the balance of glucose metabolism, leading to a loss of sorbitol accumulation and NADPH [15]. The intracellular aggregation of sorbitol leads to a hyperosmotic state. Because of its non-fat-soluble characteristics, sorbitol cannot easily penetrate the cell membrane. Because of the increase in intracellular moisture, the crystal fiber of the lens epithelial cells is expanded, and the permeability of the lens capsule is increased. Therefore, the material leakage in the lens causes oxidative stress, which damages the lens capsule epithelial cells and lens fiber cells and ultimately results in lens opacity and cataract formation [16-18]. The results of this study showed that the opacity of the lenses in the B group was higher than in the control group, suggesting that high glucose induced cataracts.

PCs have a protective effect on lens in vivo because they can eliminate free radicals and improve the activity of antioxidant enzymes [19]. Furthermore, they can reduce the damage caused by free radicals and decrease the oxidative stress of cells [20]. PCs not only enhance the activity of antioxidant enzymes, but also enhance their gene expression [21]. For instance, the activity of H_2O_2 and CAT decomposition are increased, while the formation of hydroxyl radicals is decreased. SOD is a superoxide dismutase and its main role is to remove superoxide anion free radicals in the cells [22]. The activities of CAT are to decompose hydrogen and oxygen free radicals, thereby maintaining a stable cell environment. In this study, the SOD value for group A was 23.883 ± 0.507 U/

PCs protect lens from HG injury

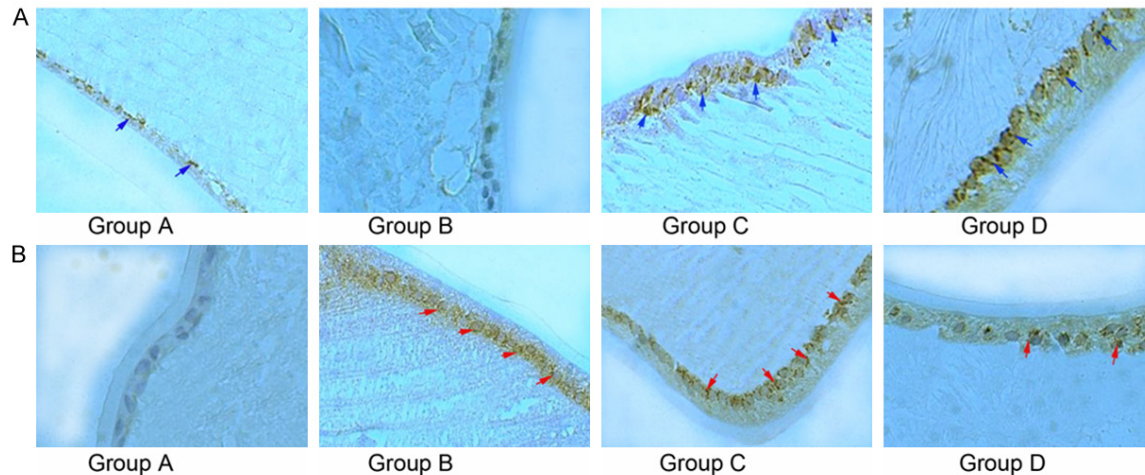


Figure 3. The expression of Bcl-2 (A) and Bax (B) in different groups at 96 h as shown by immunohistochemical staining (magnification, $\times 400$).

Table 10. Treatment with VC or PC could significantly reverse this HG-induced Bax expression enhancement at 48 h

	-	+	++	+++	++++
A	5	3	1	0	0
B ^a	0	0	1	3	5
C ^b	4	3	2	0	0
D ^c	4	4	1	0	0

a, $P < 0.01$ vs. A; b, $P < 0.01$, vs. B; c, $P < 0.01$, vs. B.

Table 11. Treatment with VC or PC could significantly reverse this HG-induced Bax expression enhancement at 72 h

	-	+	++	+++	++++
A	6	2	1	0	0
B ^a	0	0	1	2	6
C ^b	5	3	1	0	0
D ^c	5	4	0	0	0

a, $P < 0.01$ vs. A; b, $P < 0.01$, vs. B; c, $P < 0.01$, vs. B.

Table 12. Treatment with VC or PC could significantly reverse this HG-induced Bax expression enhancement at 96 h

	-	+	++	+++	++++
A	8	1	0	0	0
B ^a	0	0	0	1	8
C ^b	6	2	1	0	0
D ^c	6	3	0	0	0

a, $P < 0.01$ vs. A; b, $P < 0.01$, vs. B; c, $P < 0.01$, vs. B.

mg protein, and the SOD value for group B was 1.478 ± 0.535 , while the values for groups C and D were 17.483 ± 0.448 and 20.605 ± 0.302 , respectively. At 48 h, the CAT value for group A was 6.253 ± 0.142 and for group B was 2.268 ± 0.111 . In comparison, the CAT values for groups C and D were 4.756 ± 0.126 and 5.305 ± 0.127 , respectively. These data indicated that PCs could enhance the antioxidant capacity.

MDA is an end product of polyunsaturated fatty acid lipid peroxidation, which causes peroxidation when the free radical attacks the biofilm [22]. MDA content reflects the degree of oxidative damage in the body. In this study, the MDA value of the PC treatment group was significantly lower than in group B. For example, at 48 h, the MDA values were 0.635 ± 0.189 , 6.569 ± 0.158 , 1.31 ± 0.089 and 1.063 ± 0.009 for groups A, B, C and D, respectively. These data showed that PC has an inhibitory effect on lipid peroxidation. The results of this study showed that PCs can reduce the oxidation of isolated lens epithelial cells. Anwar, Yildirim and Yogici found that the activity of SOD in the epithelial cells was clearly enhanced and the MDA content was decreased by PC treatment, which was consistent with our experimental results [22, 23].

PCs were found to maintain the stability of the cell membrane. The cell membrane and organelles are the main components involved in lipid

peroxidation. Meanwhile, lens epithelial cells are the centers of oxidative damage. Due to lipid peroxidation and increased Ca^{2+} flux, the cell membrane permeability was increased, resulting in the substantial loss of functional substances and changes in cell metabolism [24]. The results of this study showed obvious lens fiber breakage and arrangement disorder in the HG group. Furthermore, HG treatment group demonstrated a large number of vacuoles and destroyed lens capsule membrane structure. Compared with group B, the arrangement of the lens fibers in the PC group is relatively neat with the similar morphology to the control group. This result indicated that the effects of PCs could maintain a stable cell membrane, which rendered the cells more resistant to free radicals and allowed the maintenance of lens transparency.

Furthermore, the apoptosis of lens epithelial cells plays a key role in the formation of diabetic cataract. In epithelial cells, oxidative damage and protein glycosylation can induce apoptosis in the HG environment. Tkanuray et al. report that HG promotes apoptosis in the lens epithelial cells of diabetic cataracts in rats [25]. The anti-apoptotic protein, Bcl-2 and the pro-apoptotic protein, Bax are the two important regulators that exert different functions related to cell apoptosis. It was reported that the expression of Bcl-2 could be up-regulated by PC, while the expression of Bax was downregulated by PC [26]. This study showed that the expression of Bcl-2 protein was decreased and the expression of Bax protein increased in the cytoplasm of the HG-treated group compared with the control group. Furthermore, the expression of Bcl-2 and Bax was correlated with the length of time. PC treatment could enhance the expression of Bcl-2 and decrease the expression of Bax in comparison with HG treatment.

Conclusions

The study showed that high glucose could significantly induce the apoptosis of lens epithelial cells, which can be used as a model of diabetic cataracts. After PC treatment, the apoptosis of lens epithelial cells was decreased, and the lens turbidity was reduced. These findings suggest that PC could be used as an antioxidant reagent for the prevention and treatment of cataracts.

Disclosure of conflict of interest

None.

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