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Puerarin antagonizes peroxyntrite-induced injury in retinal pigment epithelial cells☆

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Abstract

A rat model of diabetes mellitus was established by intraperitoneal injection of streptozotocin. Three days later, the rats were intraperitoneally administered 140 mg puerarin/kg daily, for a total of 60 successive days. DNA ladder results showed increased apoptosis over time in retinal pigment epithelial cells from rats with streptozotocin-induced diabetes mellitus. Western blot analysis, Reverse transcription-PCR, immunohistochemistry, and flow cytometry results showed increased expression of 3-nitrotyrosine, a peroxyntrite marker, as well as inducible nitric synthase and Fas/FasL, in retinal pigment epithelial cells. Puerarin reversed these changes, and results demonstrated that puerarin inhibited Fas/FasL expression and alleviated peroxyntrite injury to retinal pigment epithelial cells. These results suggested that puerarin inhibited production of inducible nitric oxide synthase and directly antagonized peroxyntrite injury in retinal pigment epithelial cells.

Key Words: apoptosis; Fas/FasL; inducible nitric oxide synthase; oxidative stress; puerarin; retinal pigment epithelial cells

Abbreviations: RPE, retinal pigment epithelium; AMD, age-related macular degeneration; STZ, streptozotocin; NT, nitrotyrosine

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INTRODUCTION

Retinal pigment epithelium (RPE) plays many important roles essential to the visual process. Age-related macular degeneration (AMD) is an idiopathic retinal degenerative disease and is the leading cause of irreversible vision loss[1]. RPE cell apoptosis is an important feature of advanced forms of AMD. Although vision loss in AMD is due to photoreceptor damage in the central retina, RPE atrophy is a prominent disease component[1].

The traditional oxygen-free radical stress mechanism pays more attention to the role of hydrogen peroxide (H_2O_2), nitric oxide (NO), and superoxide anion ($O_2^{\cdot-}$), while the new theory includes $ONOO^-$, a product from rapid reaction of NO and $O_2^{\cdot-}$, which may be an important mediator of cytotoxicity in oxidation[2-9]. $ONOO^-$ plays a key role in the development and progression of diabetic retinopathy[10-11].

Puerarin is one of the major phytoestrogens isolated from the root of a wild leguminous creeper, Puerarialobata [Willd.]. Puerarin is widely available in common foods and is used in alternative medicine owing to its wide spectrum of biological activity, particularly its estrogenic and antioxidant properties. It is widely employed for the treatment and prevention of cardiovascular diseases, diabetes mellitus, as well as diabetic complications, cancer, and osteoporosis[12-16]. The present study hypothesized that puerarin could antagonize oxidative stress by decreasing RPE cell apoptosis. The role of puerarin on decreased inducible nitric oxide synthase (iNOS) expression, which is partly induced by $ONOO^-$ *via* Fas/FasL signal activation, in RPE cells was analyzed to determine whether puerarin is involved in RPE cell apoptosis.

RESULTS

Quantitative analysis of experimental animals

A total of 110 rats were initially included in the study and randomly assigned to three groups: control (n = 36), $ONOO^-$ (n = 38), and puerarin (n = 36). Sprague-Dawley rats from the $ONOO^-$ and puerarin groups were intraperitoneally injected with streptozotocin (STZ) to establish an animal model of diabetes. In addition, rats in the puerarin group were intragastrically administered puerarin. At the end of experimentation, two rats from the $ONOO^-$ group were excluded from further analysis due to diabetic crisis.

In total, there were 108 rats included in the final analysis.

Puerarin improved diabetic symptoms in rats

Typical diabetic symptoms, including increased drinking, urination, and food intake, as well as low weight, were observed in the puerarin and ONOO$^-$ groups. Puerarin significantly increased body weight and reduced glucose concentration in tail vein blood of diabetic rats at 20, 40, and 60 days after streptozotocin administration ($P < 0.01$; Table 1).

Table 1 Effects of puerarin on blood glucose (mM) and body weight (g) in diabetic rats

Group	Time after streptozotocin injection (day)		
	20	40	60
Control			
Blood glucose	3.77±0.15	3.69±0.13	3.59±0.24
Body weight	322.24±2.66	387.22±2.33	466.73±2.38
ONOO$^-$			
Blood glucose	21.78±0.22^{bc}	22.09±0.25^{bc}	23.87±0.23^{bc}
Body weight	223.11±1.65^{bc}	187.07±2.13^{bc}	149.87±2.17^{bc}
Puerarin			
Blood glucose	11.01±0.33^{a}	9.88±0.27^{a}	7.09±0.46^{a}
Body weight	321.11±2.32^{a}	343.16±2.44^{a}	454.44±2.52^{a}

$^{a}P < 0.05$, $^{b}P < 0.01$, *vs.* control group; $^{c}P < 0.01$, *vs.* puerarin group. Data are expressed as mean ± SD ($n = 12$ rats for each time point/group) and were analyzed using one-way analysis of variance followed by Fisher *post hoc* test for multiple comparisons.

Puerarin decreased nitrotyrosine (NT) expression in rat RPE cells

Western blot analysis showed that NT was slightly expressed in RPE cells in the control group, but NT expression gradually increased in the ONOO$^-$ group at 20, 40, and 60 days after STZ administration. NT expression increased during the period from 20 to 40 days, but decreased again by 60 days (Figure 1).

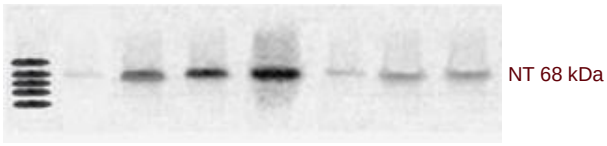


Figure 1 Nitrotyrosine (NT) protein expression in retinal pigment epithelium (RPE) cells of a diabetic rat model (western blot).

The experiment was repeated at least three times. Lane M: Marker; lane 1: control group; lanes 2–4: ONOO$^-$ group at 20, 40, and 60 days after streptozotocin (STZ) administration, respectively; lanes 5–7: puerarin group at 20, 40, and 60 days after STZ administration, respectively.

Weak NT expression is observed in the control group. Weak to strong NT expression is observed at different time points in the ONOO$^-$ group. However, NT expression in the puerarin group increases during the period from 20 to 40 days after STZ administration, but decreases again by 60 days.

In the puerarin group, NT expression in rat RPE cells decreased compared with the ONOO$^-$ group at 20, 40, and 60 days after STZ administration ($P < 0.05$ or 0.01, Table 2).

Table 2 Quantification of nitrotyrosine (NT) protein expression (absorbance) in retinal pigment epithelium cells (western blot)

Days	Control group	ONOO$^-$ group	Puerarin group
20	32.33±2.35	74.44±3.00^{bc}	72.78±2.64^{a}
40	34.33±1.73	145.00±3.94^{bc}	77.22±2.44^{b}
60	33.44±1.94	235.78±5.97^{bd}	74.04±2.98^{a}

$^{a}P < 0.05$, $^{b}P < 0.01$, *vs.* control group; $^{c}P < 0.05$, $^{d}P < 0.01$, *vs.* puerarin group.

Data are expressed as mean ± SD ($n = 3$ eyes/group) and were analyzed using one-way analysis of variance followed by Fisher *post hoc* test for multiple comparisons.

RPE cell apoptosis

There was no appearance of a DNA ladder band in the RPE layer of the control group, but there was a distinct and typical DNA ladder band in the ONOO$^-$ with time. In the puerarin group, expression of a DNA ladder band gradually grew stronger during the period from 20 to 40 days after STZ administration, but significantly decreased again by 60 days (Figure 2).

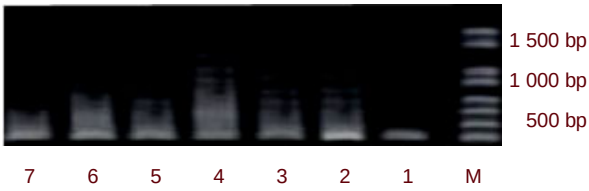


Figure 2 DNA ladder for apoptosis of retinal pigment epithelium (RPE) cells in diabetic rats.

The experiment was repeated at least three times. Lane M: Marker; lane 1: control group; lanes 2–4: ONOO$^-$ group at 20, 40, and 60 days after streptozotocin (STZ) administration, respectively; lanes 5–7: puerarin group at 20, 40, and 60 days after STZ administration, respectively.

There is no appearance of DNA ladder band in the control group, but there is a distinct and typical DNA ladder band in the ONOO$^-$ group over time.

Expression of the DNA ladder band in the puerarin group increases during the period from 20 to 40 days after STZ administration, but decreases again by 60 days.

iNOS mRNA expression in the rat RPE layer

Expression of iNOS mRNA was not detected in the control group, but iNOS mRNA significantly increased in the ONOO$^-$ group with time. In the puerarin group, iNOS mRNA expression increased during the period from 20 to 40 days after STZ administration, but decreased again by 60 days (Figure 3). Puerarin significantly decreased iNOS mRNA expression in PRE cells of diabetic rats ($P < 0.05$ or 0.01, Table 3).

Figure 3 Reverse transcription-PCR for expression of inducible nitric oxide synthase (iNOS) mRNA in retinal pigment epithelium (RPE) cells.

Lane M: Marker; lane 1: control group; lanes 2–4: $ONOO^-$ group at 20, 40, and 60 days, respectively; lanes 5–7: puerarin group at 20, 40, and 60 days, respectively.

Expression of iNOS mRNA is not detected in the control group, but iNOS mRNA expression increases in the $ONOO^-$ group with time.

In the puerarin group, iNOS mRNA expression increases during the period from 20 to 40 days, but decreases again by 60 days.

Fas/FasL expression in the RPE layer of rats

Immunohistochemistry staining revealed Fas/FasL-positive cells, with staining in the cell nucleus and cytoplasm of RPE cells. In the control group, faint expression was observed. From 20 to 60 days after STZ administration, expression increased in the cell nucleus and cytoplasm of the $ONOO^-$ group. From 20 to 60 days after STZ administration, Fas/FasL expression gradually decreased in the $ONOO^-$ and puerarin groups (Figure 4).

Table 3 Quantification of inducible nitric oxide synthase mRNA expression (absorbance) in rat retinal pigment endothelium cells (reverse transcription-PCR)

Days	Control group	$ONOO^-$ group	Puerarin group
20	2.20±2.21	$4.39±3.58^{bc}$	$4.61±2.44^{a}$
40	2.33±1.86	$7.82±2.77^{bc}$	$6.31±3.24^{b}$
60	2.24±1.72	$14.79±3.66^{bd}$	$5.01±3.25^{a}$

$^{a}P < 0.05$, $^{b}P < 0.01$, *vs.* control group; $^{c}P < 0.05$, $^{d}P < 0.01$, *vs.* puerarin group.

Data are expressed as mean ± SD (n = 3 eyes/group) and were analyzed using one-way analysis of variance followed by Fisher *post hoc* test for multiple comparisons.

Flow cytometry detection revealed very few Fas- and FasL-positive cells in the control group. With increasing time, the number of positive cells increased in the $ONOO^-$ group. On day 20, there were few cells in the puerarin group, but the number of positive cells significantly increased by day 40, and subsequently decreased again by day 60. Puerarin significantly decreased the number of Fas- and FasL-positive cells in PRE cells of diabetic rats ($P < 0.05$ or 0.01; Table 4).

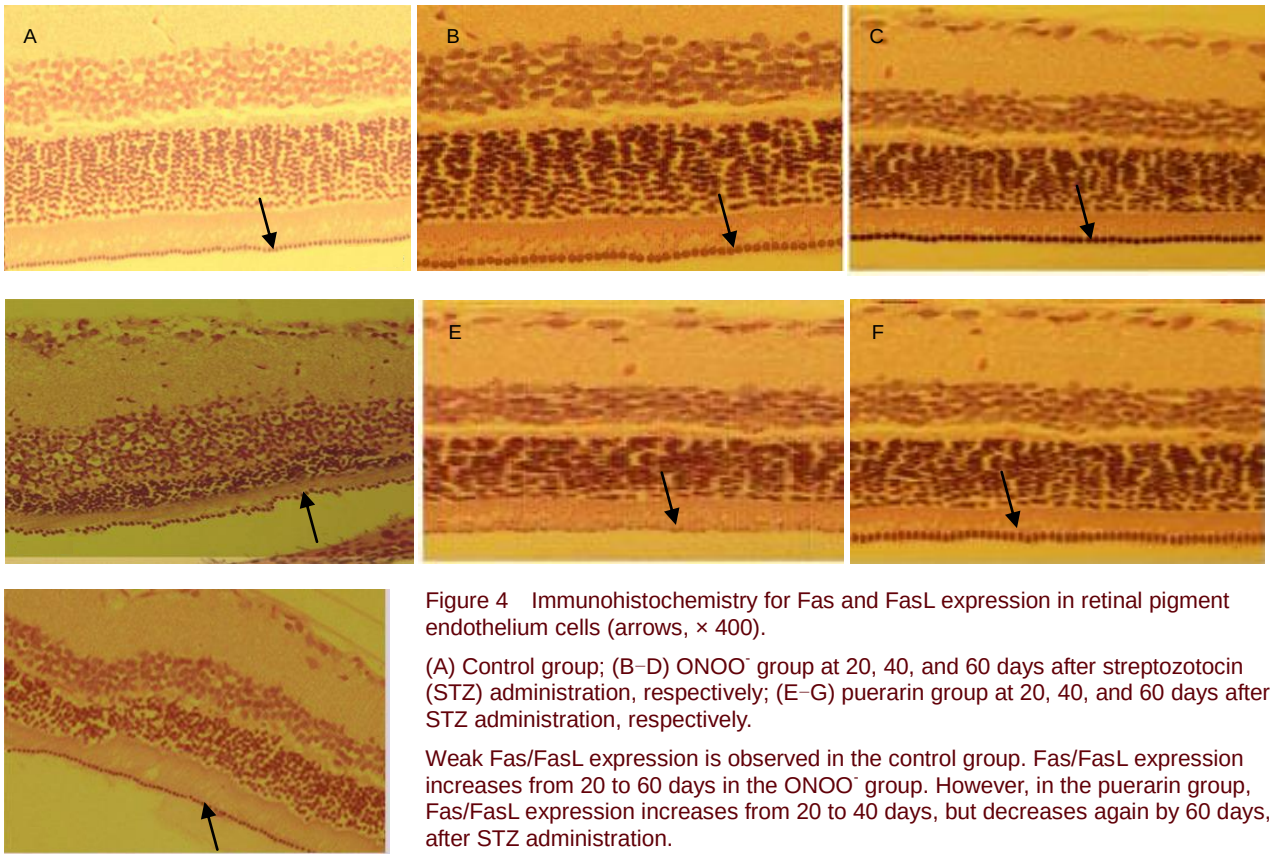


Figure 4 Immunohistochemistry for Fas and FasL expression in retinal pigment endothelium cells (arrows, × 400).

(A) Control group; (B–D) $ONOO^-$ group at 20, 40, and 60 days after streptozotocin (STZ) administration, respectively; (E–G) puerarin group at 20, 40, and 60 days after STZ administration, respectively.

Weak Fas/FasL expression is observed in the control group. Fas/FasL expression increases from 20 to 60 days in the $ONOO^-$ group. However, in the puerarin group, Fas/FasL expression increases from 20 to 40 days, but decreases again by 60 days, after STZ administration.

Table 4 Amount and percentage of Fas- and FasL-positive cells at different time points after streptozotocin administration

Days	Control group		$ONOO^-$ group		Puerarin group	
	Amount	Percentage (%)	Amount	Percentage (%)	Amount	Percentage (%)
20	523±165	2.34±0.88	1161±159[bc]	13.66±0.47[bc]	601±109[a]	3.22±0.55[a]
40	516±139	2.54±0.79	1927±161[bc]	18.56±0.36[bc]	989±115[b]	7.28±0.67[b]
60	524±144	2.38±0.82	2791±142[bd]	21.01±0.64[bd]	604±177[a]	4.75±0.37[a]

[a]$P < 0.05$, [b]$P < 0.01$, *vs*. control group; [c]$P < 0.05$, [d]$P < 0.01$, *vs*. puerarin group. Data are expressed as mean ± SD ($n = 6$ eyes/group) and were analyzed using one-way analysis of variance followed by Fisher *post hoc* test for multiple comparisons.

DISCUSSION

Puerarin alleviated oxidation of RPE cells induced by $ONOO^-$ in diabetic rats, which should decrease free radical production. Results suggested that iNOS contributed to oxidative stress by producing more powerful oxidative agents, such as $ONOO^-$[17-20]. Zhou *et al* [21] reported the influence of iNOS isoforms and $ONOO^-$ generation following experimental stroke. Puerarin inhibits iNOS expression, thereby also decreasing the formation of $ONOO^-$. Results from the present study demonstrated that NT, an $ONOO^-$-mediated protein nitration product, was located in RPE cells of diabetic rats, and NT expression decreased following intervention with puerarin.

It is believed that genes in the inner cell layer directly regulate apoptosis production and development, while related elements in the outer cell layer affects gene expression *via* signal transduction pathways[22-24]. Interaction of the death receptor and death ligand is one of the main ways to induce apoptosis, and the Fas/FasL system is considered a major signal transduction pathway for mediating apoptosis[25-27]. Results from the present study demonstrated that expression of NT, the DNA ladder band, iNOS mRNA, and the number of positive Fas/FasL cells, increased during the period of 20–60 days after STZ administration in the $ONOO^-$ group. In the puerarin group, RPE cell apoptosis increased from 20 to 40 days, but decreased from 40 to 60 days, which suggested a protective role for puerarin on RPE cells.

To summarize, results from the present study suggested that RPE cell apoptosis was partly induced by $ONOO^-$, which could be a novel pathway for oxidative damage in RPE cells. Puerarin decreased RPE cell apoptosis, which was partly induced by $ONOO^-$, suggesting that puerarin could serve as a potential drug for diabetic retinopathy therapy. The mechanism of action on RPE cells could be related to direct inhibition of iNOS formation and subsequent $ONOO^-$ production, as well as reduced $ONOO^-$ damage to RPE cells.

MATERIALS AND METHODS

Design

A randomized, controlled, animal experiment.

Time and setting

This study was performed at the Scientific Research Center in Hebei Province People's Hospital and Hebei Medical University, China from June 2006 to June 2008.

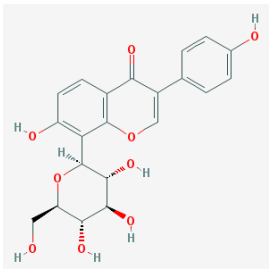
Materials

Animals

Specific pathogen-free, male, Sprague-Dawley rats, aged 5–6 weeks, were provided by Experimental Animal Center of Hebei Medical University (liscience No. SYXK (Ji) 2008-0026). The rats were housed at 22 ± 1°C, 50–70% humidity, and 150–200 lx illumination.

Drugs

Puerarin injection (8-(beta-D-glucopyranosyl-7-hydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one (approval No. H20033577, Kangenbei Pharmaceutical, China) was a product of Kangenbei Pharmaceutical, China. Molecular formula: $C_{21}H_{20}O_9$; molecular weight: 416.38 and chemical structure is shown as follows:



Methods

Animal model establishment and drug administration

Rats were intraperitoneally injected with STZ once daily (45 mg/kg; Sigma, St. Louis, MO, USA) to establish animal models of diabetes. The right eye of each rat was utilized for RPE cell harvest. Rats from the control group were intraperitoneally injected with saline. Three days after start of experiment, rats from the puerarin group were intragastrically injected with 140 mg puerarin/kg daily for 60 days. All rats were anesthetized with ketamine 12 mg/100 g for the following experiments.

Harvesting of RPE sheets

RPE sheets were harvested according to technique modifications described by previous studies[28-30]. Briefly, extraocular tissue from freshly enucleated rat eyes was cleaned. The gelatin film containing the RPE sheet was then incubated in a humidified atmosphere of 5% CO_2/95% air at 37°C for 5 minutes to allow the gelatin to melt and encase the RPE sheet. The specimen was maintained at 4°C for 5 minutes to solidify the liquid gelatin and then stored in CO_2-free medium at 4°C.

Western blot detection of NT expression

Supernatant protein content from the RPE sheet medium was determined using the Bradford method[31]. RPE sheets were homogenized and solubilized in ice-cold phosphate buffered saline (PBS) containing protease inhibitors, phenylmethylsulfonyl fluoride (1 μg/mL), aprotinin (1 μg/mL), leupeptin (1 μg/mL), pepstatin A (1 μg/mL), and ethylenediamine tetraacetic acid (1 mM). The homogenate was centrifuged at 1 505.016 × *g* at 4°C for 10 minutes. The proteins were then separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Invitrogen, Carlsbad, CA, USA) on a 12% linear slab gel under reducing conditions, and the separated proteins were transferred to a polyvinylidene fluoride membrane using a semidry electrophoretic transfer cell (Trans-blot; Bio-Rad, Richmond, CA). Blots were stained at room temperature at a 1:600 dilution of monoclonal mouse anti-NT antibody overnight at 4°C. After washing and incubation with horseradish peroxidase-conjugated secondary antibody (1:1 000 dilution), blots were developed using the enhanced chemiluminescence western blot analysis detection system (ECL Plus; Amersham Pharmacia Biotech, Arlington Heights, IL, USA). Absorbance values of proteins were analyzed using computer photo analysis (Image-Pro Plus Analysis System, USA).

DNA ladder to detect apoptosis

Cell apoptosis in the RPE layer was determined using the DNA ladder technique, as previously described[32].

Reverse transcription (RT)-PCR to detect iNOS mRNA expression

Equal amounts of total right-eye RNA were used to detect iNOS mRNA levels using RT-PCR (GeneAmp RNA-PCR kit; Applied Biosystems, USA). Total RNA was extracted from the rat retina in three groups, according to the kit manufacture specifications. Sense and anti-sense oligonucleotide primers for rat iNOS mRNA were synthesized by Applied Biosystems, USA. Primer sequences are as follows: iNOS (262 bp) forward, 5'-CGC CCT TCC GCA GTT CT-3', reverse, 5'-TCC AGG AGG ACA TGC AGC AC-3'; β-actin (420 bp) forward, 5'-GAG ACC TTC AAC ACC CAG CC-3', reverse, 5'-GCG GGG CAT CGG AAC CGC TCA-3'. In addition, 4 μg RNA in a total volume of 20 μL (pH 8.3) was used for cDNA synthesis. RT-PCR was initially performed at 24°C for 10 minutes, followed by 42°C for 15 minutes. The reaction mixture was heated to 99°C for 5 minutes, and then the RT product was mixed with DNA polymerase (AmpliTaq; Applied Biosystems, USA) and the sense primer in a buffer containing 20 mM Tris-HCL, 50 mM KCl, 2.0 mM $MgCl_2$ (pH 8.3), and 50 mM of each dNTP in a 100-μL volume. The mixture was then amplified by 29 PCR cycles. The thermal cycle profile used in this study was as follows: initial denaturing at 94°C for 2 minutes and then 45 seconds in each cycle; annealing primer with DNA at 55°C for 45 seconds; and primer extension at 72°C for 10 minutes. All reactions were normalized for iNOS expression. The negative controls consisted of omission of RNA template or reverse transcriptase from the reaction mixture. PCR products were analyzed on a 2% agarose gel. iNOS mRNA expression was analyzed by computer photo-analysis (Image-Pro Plus Analysis System, USA), and results were expressed as absorbance values. Gene Pix Pro 4.0 photo software (Axon Instruments) was used for cluster analysis. Two-fold greater divergence was regarded as a divergence expression of genes. All trials were repeated at least three times.

Immunohistochemistry and flow cytometry for Fas/FasL expression

For immunohistochemistry, the eyeballs were fixed in 10% buffer formalin, embedded with paraffin, and cut into 5-μm thick sections. After washing in PBS, the slides were incubated with H_2O_2 (peroxidase blocking reagent; Daco, Carpinteria, CA, USA) to block endogenous peroxidase activity, and then incubated in 10% goat serum for 30 minutes at room temperature to block non-specific antigen. After rinsing and washing in PBS, the slides were incubated in a 1:200 dilution of monoclonal mouse anti-Fas/FasL antibody (Sigma, St. Louis, MO, USA) at 4°C overnight, followed by incubation with horseradish peroxidase-conjugated secondary antibody (1:600; Daco) at room temperature for 30 minutes. Staining was visualized with streptavidin and biotin chromogen (Liquid DAB+Substrate-Chromogen System; Daco).

For flow cytometry, the eyeballs were dissected *via* a posterior incision under a dissecting microscope; the retina with RPE cells was resected and fixed in 70% ethanol for 24 hours. Fluorescence intensity of Fas/FasL, as well as the number of Fas/FasL-positive cells, in the RPE was analyzed. Cells were washed three times in PBS and then re-suspended in PBS at 2×10^7 cells/mL. After incubating in primary and secondary antibodies (same as immunohistochemistry), the cells were collected and analyzed using a BD FACS Calibur flow cytometer (BD Biosciences, San Jose, CA). Data analysis was performed using BD CellQuest Pro software (BD Biosciences). The percentage of Fas/FasL was analyzed. The DNA distribution pattern and dual parameter dimension pattern were obtained in this way. Cell quantification was calculated using a cell circle analyzing sequence.

Statistical analysis

All data were statistically analyzed using SPSS 15.0 software and were expressed as mean ± SD. Statistical significance was determined using one-way analysis of variance, followed by the Fisher *post hoc* test for multiple comparisons. $P < 0.05$ was considered statistically significant.

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Author contributions: Lina Hao designed the study, conducted the experiment, wrote the manuscript, and was responsible for the entire article. The remaining authors contributed to data analysis.

Conflicts of interest: None declared.

Ethical approval: All animals were treated in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Resolution Statement for the use of Animals in Ophthalmic and Vision Research in USA and China.

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