Curcumin suppresses oxidative stress via regulation of ROS/NF-κB signaling pathway to protect retinal vascular endothelial cell in diabetic retinopathy

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Abstract
Background The retinal vascular endothelial cells can be damaged by oxidative stress even in the early stage of diabetic retinopathy (DR). This study aimed to investigate the protective effect of curcumin on the rat retinal vascular endothelial cells (RRVECs) in high glucose circumstance.

Objective The cultured RRVECs were identified and characterized by both of vWF and CD31 immunofluorescence expression. The activation of ROS/NF-κB signal pathway was examined by electrophoretic Mobility Shift Assay (EMSA), immunohistochemistry and Western blot; the apoptosis of RRVECs was tested by flow cytometry.

Results We found that curcumin reduced the reactive oxygen species (ROS) and relieved the apoptosis in RRVECs exposed to the high glucose by flow cytometry. It was revealed that the increased activity of NF-κB and phosphorylated NF-κB in RRVECs induced by high glucose concentration was significantly suppressed by curcumin.

Conclusion We concluded that curcumin could suppress the oxidative stress via regulation of NF-κB signal to protect the RRVECs in DR.

Keywords Curcumin · Oxidative stress · ROS/NF-κB · Diabetic retinopathy · Retinal vascular endothelial cells

Introduction
Diabetic retinopathy (DR) is one of the leading causes of blindness in people within working-age in mainland China (Xie et al. 2008; Wang et al. 2009). Pathophysiological studies have demonstrated that once diabetic retinopathy appears, it will bring about progressive changes of retinal microvasculature (De Abreu et al. 1994; Alder et al. 1997).

The characteristic early changes in diabetic retinopathy conclude that the pericyte loss, basement membrane thickening, the proliferation of retinal vascular endothelial cell (RVEC) and breakdown of blood-retina barrier (Cunhavaz et al. 1993; Sone et al. 2000).

Several studies (Fan et al. 2016; Stitt et al. 2016; Roy et al. 2017; Tonade et al. 2017) have illustrated that oxidative stress plays an dominant role in the pathogenic mechanism of diabetic retinopathy. Oxidative stress activates multiple pathways in the cell, including cellular signaling cascades, cell apoptosis, and transcription factors, which could in sequence cause the pathophysiological changes of the retinal vasculature in diabetic retinopathy. The RVECs are major participants in diabetic retinopathy. They not only supply oxygen and nutrients for the normal metabolism of the retina, but also contribute to the blood-retinal barrier which is crucial to protect the retina (Ahsan 2015; Kady et al. 2017). NF-κB is a vital transcription factor that commences the transcription of genes participated in the regulation of physiological processes of the cellular and its reaction to oxidative stress. An activating NF-κB can cause cell apoptosis. Some
studies (Ahmed et al. 2016; Kowluru et al. 2016; Rojas et al. 2017) have revealed that NF-κB controls the expression of angiogenic factors in vascular endothelial cells and induces neovascularization in diabetic retinopathy.

Curcumin is a yellow powder abstracted from the tubers of the turmeric plant which is well known for anti-oxidative stress, anti-inflammatory, and anti-tumor effects. Studies (Heger et al. 2013; Aggarwal et al. 2014; Prasad et al. 2014; Ghosh et al. 2015) found that curcumin could reduce glucose intolerance and insulin resistance by its antioxidant effects and anti-inflammatory function. Curcumin is recommended for treatment as a therapeutic drug in diabetes, obesity, and their associated complications (Chuengsamarn et al. 2012; Hassan and Elgharib 2015). Previous studies (Vasquez et al. 2014; Zhu et al. 2015) have demonstrated that curcumin has a distinctly antioxidant effect on retinal pigment epithelial (RPE) cells, and it can inhibit RPE cell proliferation by mediating p53 signaling pathway. However, it is vague whether curcumin has its anti-oxidation effect on retinal vascular endothelial cells (RVECs), and its possible mechanism is still unknown. Therefore, we investigated the effects of curcumin on RVECs exposed to high glucose concentration and analyzed the mechanism of the curcumin’s action on RVECs.

Materials and methods

Experimental animals

Sixty cleaning grade healthy adult male Sprague–Dawley (SD) rats weighing 180 ± 20 g were acquired from the Experimental Animal Center of Soochow University. Animals were housed under the standard conditions based on the guidelines established by the ARRIVE (the Animal Research: Reporting of In Vivo Experiments). The normal room temperature was maintained at 20–23 °C with a relative humidity of 50–70% and the noise was kept < 50 dB. For the use of experimental animals, guidelines were followed from the "Regulations on the Management of Laboratory Animals" promulgated by the National Science and Technology Commission and the Declaration of Helsinki.

Experimental materials

The following materials were obtained for this study: Curcumin [Taishi (Shanghai) Chemical Industry Development Co., Ltd., China], trypsin (Invitrogen, USA), type II collagenase, thiazole blue (MTT), 0.04% trypan blue (Sigma, USA), rabbit monoclonal antibody to von Willebrand factor (Abcam, Cambridge, UK), rabbit anti-CD31 antibody (Abcam, Cambridge, UK), rabbit anti-NF-κB p65 antibody (Abcam, Cambridge, UK), rabbit phosphor-NF-κB p65 antibody (Cell signaling Technology, USA), rabbit anti-IKBα antibody (Abcam, Cambridge, UK), rabbit anti-Bax antibody (Wuhan Sanying Biotechnology Co., Ltd., Hubei, China), rabbit anti-Bcl2 antibody (Wuhan Sanying Biotechnology Co., Ltd., Hubei, China), rabbit anti-IL-1β antibody (Cell signaling Technology, USA), endothelial cell culture medium, fluorescent (Cy3) tagged goat anti-rabbit IgG (Wuhan Biosciences Co., Ltd., Hubei, China), fluorescent (FITC) tagged goat anti-rabbit IgG (Wuhan Biosciences Co., Ltd., Hubei, China), rabbit anti-phosphoglyceraldehyde Dehydrogenase (GAPDH, Hangzhou Xianzhi Biotechnology Co., Ltd., Zhejiang, China), Light shift chemiluminescent EMSA kit (Thermo Scientific, USA), bicinchoninic acid (BCA) protein concentration determination kit, cell reactive oxygen species (ROS) detection kit (Biyuntian Biotechnology Institute, Hubei, China), diamino Diphenyldiethylenediamine (DAB) Chromogenic Kit (Beijing Solvay Technology Co., Ltd., China), apoptosis detection kit (Jiangsu Keyi Biotechnology Co., Ltd., Jiangsu, China), immunohistochemistry Kit (Beijing ZhongshanJinqiao Biotechnology Co., Ltd., China). Model IX51 inverted microscope, Eppendorf, BX53 fluorescence microscope (Olympus, Japan), Nikon E100 microscope (Nikon, Japan), microplate reader (American Thermo Electron Corporation, USA), and flow cytometry (BD Biosciences, USA).

Rat retinal vascular endothelial cells (RVECs) culture, identification and activity detection.

The RVECs were isolated and cultivated as previous described (Greenwood 1992). The rats were anesthetized by intraperitoneal injection of 10% chloral hydrate with 3.3% ketamine. The eyes were placed in a petri dish containing iced PBS gauze, and the retinal tissues were harvested and cut into pieces. They were digested with a type II collagenase for 30 min at 37 °C, and the digestive fluid was absorbed. The retinal tissues were then transferred to solution A consisting of NaCl, KCl, CaCl, MgCl2 and mannitol, and the osmotic pressure was adjusted to 310 mosmol. The retinal microvessels were cut into quadrants and placed in a glass-bottomed chamber. A coverslip was then placed over the retina, so that the microvessels adhered to the coverslip. The cells were sieved through two stainless steel mesh of 100 μm pore size, and the eluate was gathered. The eluate was centrifuged at 1500 r/min for 5 min at normal room temperature and the supernatant was removed. The precipitate after centrifugation was put to Dulbecco’s modified Eagle’s medium (DMEM) containing 100 × 10^3 U/L heparin sodium and fetal bovine serum. It was inoculated on a 24-well culture plate, and then placed in a 37 °C, 5% CO2 incubator and periodically exchanged. Retinal vascular endothelial cells were observed under an inverted microscope before the medium was changed. The labeled hybrid cells were
removed with a scraper. Following this, the cell viability was assessed using trypan blue staining.

The 4th generation rat RRVECs were inoculated on gelatin-coated coverslips at a density of 1 × 10⁵ cells/ml and cultured for 24 h. After the cells were adherent, the coverslips were removed and immunofluorescence staining was used to identify the cells with vWF and anti-CD31 antibody at 4 °C over night. In the negative control group, PBS was used instead of the primary antibodies. Cy3 and FITC as the secondary antibodies were added to the working solution at 37 °C for incubation, respectively. The color was inspected under a fluorescence microscope, and then, the photos were collected. Cy3 emits green fluorescence and FITC emits red fluorescence as a positive expression.

**Grouping of cells**

The RRVECs were divided into four groups, which are the normal control group, osmolarity control group, high glucose group and curcumin treatment group (High glucose + Curcumin). The glucose concentration in the normal control group was 5.5 mmol/L. The osmotic control group had 5.5 mmol/L glucose and 19.5 mmol/L mannitol to a final concentration of 25 mmol/L, and was added with glucose at a concentration of 25 mmol/L for 72 h in the high glucose group. In the treatment group, the cells were exposed to glucose at a concentration of 25 mmol/L for first 72 h and then treated with 30 μmol/L curcumin for 48 h.

**Morphological changes in RRVECs by transmission electron microscopy**

The cellular morphological changes in each group were detected by the transmission electron microscopy (TEM). The cells were collected via centrifugation (800 rpm, 5 min), and rinsed with PBS and immobilized with 1% paraformaldehyde and then with 2% glutaraldehyde for 12 h. Afterwards, the fixed cells were treated with 1% osmium tetroxide for 3 h, dehydrated through an ethanol gradient, and put in Araldite. Ultrathin sections were colored with both lead citrate trihydrate and uranyl acetate, and then observed by the TEM (Tecnai, FEI). The EDX ChemSTEM analysis system was used to take TEM images at magnifications of 2500×.

**Detection of intracellular superoxide by ROS assay**

The reactive oxygen species (ROS) levels in RRVEC were tested by flow cytometry. A single suspension of cells was prepared from each group of cells, inoculated evenly in a 6-well plate at the cell density of 2 × 10⁵ cells/well, and cultivated at 37 °C in 5% CO₂ saturation humidity all night. The cells were then digested with 0.25% trypsin without ethylenediaminetetraacetic acid. The digestion was stopped and the digested cells were centrifuged. The supernatant was quitted and the cells were rinsed twice with PBS. Following the instructions for the operation of the DCFH-DA cell ROS kit, the flow cytometry was performed.

**Flow cytometry analysis**

Flow cytometry was used to analyze the apoptosis of RRVECs in each group. Each group of cells was cultivated in a single suspension of cells from each sample, and incubated at normal room temperature for a quarter of an hour in dark environment. Solutions of Annexin (5 μL) and propidium iodide (1 μL) were added to cell suspension and incubated in the dark at room temperature for 15 min. After the incubation, 400 μL of annexin binding buffer was put, and finally it was examined by flow cytometry.

**Immunohistochemistry**

The expression level of NF-κB p65 in RRVECs was detected using immunohistochemical staining described previously (Bhat et al. 2015). The cells were seeded on a plate with 6-well cover glass. After 24 h, the serum-free medium was replaced and the culture was continued for 24 h. The slides were removed, washed with PBS and the polyclonal antibody rabbit anti-human NF-κB p65 was added. The procedure was performed according to the fungal avidin-peroxidase ligation immunohistochemistry kit. The cytoplasm showing yellow or brown-yellow staining was considered positive. Ten high-power field of each slice was inspected and taken photographs under a microscope. The images were analyzed using a digital image analysis system. The relative expression level of the target protein was figured by the average value of the positive cells.

**Detection of NF-κB Activity by EMSA**

The Electrophoretic Mobility Shift Assay (EMSA) was implemented using an NF-κB EMSA Kit (Thermo Scientific, USA). The nucleoproteins in the RRVEC were extracted with a Nucleoprotein and Cytoplasmic Protein Extraction Kit according to the production protocol. The following DNA sequences were used to synthesize in EMSA experiment: consensus oligonucleotides of NF-κB p65: 5′-AGT TGA GGG GAC TTT CCC AGGC-3′, 3′-TCA ACT CCC CTG AAA GGG TCCG-5′. The NF-κB/DNA binding activity was identified with Light Shift Chemiluminescent EMSA kit. The detailed procedure of EMSA has been already described as before[(Kitaoka et al. 2003; Juvekar et al. 2012)].
Western blotting

The expression of NF-κBp65, phosphorylated NFκBp65, IKB-α, IL-1β, Bax and Bcl-2 in each group of RRVECs was detected by Western blot. The total protein was abstracted and the target proteins were explored. Protein samples and standard proteins diluted in PBS were respectively added to 96-well plates. Two parallel wells were put for each standard sample. Three parallel wells were put for the sample to be proofed, and 20 μL samples was added to each well. Two parallel wells with PBS were blank controls. The liquid mixture of BCA protein with a ratio of 50:1 was added to each well in a 96-well plate, and cultivated for half an hour at 37 °C in dark environment. The microwell plate was read at a wavelength of 568 nm with a microplate reader. The linear regression equation was calculated according to the standard protein concentration and the corresponding value. Based on the value of the protein sample, the regression equation was used to figure out the sample protein concentration. The extracted protein sample and five-time protein loading buffer were placed in boiling water for 10 min to perform protein denaturation. Electrophoresis and transfer were completed according to the previous literature method. The membrane was blocked and the primary antibody was added to cultivate overnight at 4 °C (GAPDH: 1:1000; NF-κBp65:1:10,000; pNF-κBp65:1:1000; IKB-α:1:1000; IL-1β:1:1000; Bcl-2: 1:1000; Bax: 1:2000). After washing the membrane 5–6 times, the matching secondary antibody was added. After the membrane was rinsed, the films with chemiluminescence were developed, fixed, and rinsed. The film gray value was analyzed by bandscan. And that was repeated three times.

Statistical methods

Statistical software SPSS20.0 was used for statistical analysis. The data were distributed by the Shapiro–Wilk test, and the rate of the cellular apoptosis was indicated as a percentage. Other experimental data were shown as the mean ± standard deviation (x±s); the average homogeneity between groups was tested using the variance by Levene test, and analysis of variance multiple comparisons (ANOVA) with Bonferroni’s multiple comparison test was performed to analyze the data in more than two groups of variables. p < 0.05 was considered to be statistically significant.

Results

The isolated RRVECs were patina-like monolayer adherent growth after one week, when inspected under an inverted microscope. The cultivated cells were strongly positive for vWF and CD31 expression which are endothelial cell specific markers (Fig. 1a–c).

The ultrastructure changes in RRVECs were perceived by transmission electron microscopy to detect whether curcumin could alleviate the damage caused by high glucose concentration. As were illustrated in Fig. 1d–g a distinct thickening of retinal capillary basement membrane, swollen cell and shrunken nucleus in the high glucose group were observed compared with the normal control group. However, with the treatment of curcumin, an obvious reduction in retinal capillary basement membrane thickness and other abnormal structure including swollen cell and shrinkage of cellular nucleus were displayed in RRVECs.

Figure 2a and b exhibited that the relative levels of ROS of RRVECs were significantly different among the control group, osmotic control group, high glucose group and curcumin treatment group (p ≤ 0.001). Compared with the control group, the relative content of ROS in RRVECs was significantly increased in the high glucose group (p ≤ 0.001). Compared with the high glucose group, the relative content of ROS in the RRVEC of the curcumin treatment group was significantly decreased, and the difference was statistically significant (p ≤ 0.001).

Annexin V-FITC/PI double staining results represented that the apoptosis rate of RRVECs in the control group, high glucose group and curcumin treatment group was statistically significant (p ≤ 0.001). Compared with the control group, the high glucose group showed significantly elevating RRVECs apoptosis rate (p ≤ 0.001). Furthermore, after the curcumin treatment with 30umol/L in RRVECs, the cell apoptosis was reduced dramatically (p < 0.05) (Fig. 2c, d).

The NF-κB signaling was examined by immunohistochemistry, Western blot and electrophoretic mobility shift assay (EMSA). As illustrated in the Fig. 3a–d, the expression of NF-κB p65 protein was positive in the cytoplasm of RRVECs in the control group (Fig. 3a). The positive expression of NF-κB p65 protein was found in the nucleus and cytoplasm of RRVECs in both high glucose group and curcumin treatment group (Fig. 3c, d). As compared with the control group, the expression of NF-κB was significantly increased in high glucose group (p < 0.01). However, the expression of NF-κB was declined sharply in the curcumin treatment group compared to the high glucose group (p < 0.05). (Fig. 3e). The results of the EMSA and Western blot tests demonstrated a similar results in immunohistochemistry. The binding activity of transcription
factor NF-κB was modulated by the treatment of curcumin, as is shown in Fig. 4a. In the high glucose group, the activity of NF-κB binding DNA was arised. However, when the RRVECs were incubated with the curcumin, the NF-κB activity was declined. Furthermore, the activity of NF-κBp65 was detected in RRVECs by Western Blot test. As shown in Fig. 4b and c, when RRVECs were exposed to 25 μmol/L of glucose, both the expression of NF-κB and phosphorylated NF-κB were increased significantly, compared with the normal control group. With the treatment of curcumin, the expression of NF-κB and pNF-κB were declined significantly compared with the high glucose group, respectively ($p < 0.05$, $p < 0.01$) (Fig. 4c).

The results of the Western blot analysis demonstrated that in high glucose group, the levels of IKb-α, IL-β and Bax were dramatically increased, whereas, the expression of Bcl-2 was reduced in RRVECs, compared with the control group. When it was treated with curcumin, the expression level of IKb-α, IL-β and Bax were fallen off, whereas the Bcl-2 expression increased(Fig. 4d, e).

**Discussion**

Gupta et al. (2011) demonstrated that oral administration of curcumin to diabetic rats increased the expression of antioxidant enzymes and superoxide dismutase in rat retina after 16 weeks. It restrained the expression of TNF-α and VEGF and reduced the capillary basement membrane thickness. However, the study did not involve the possible cellular pathophysiological mechanisms.

This study revealed several important findings regarding roles of curcumin in protecting retinal vascular endothelial cell exposed to high glucose. First, the morphological changes were detected that curcumin ameliorated the abnormal change of cellular structure in RRVECs with high glucose. Second, curcumin decreased...
the level of ROS, phosphorylated NF-κB and NF-κB in RRVECs, which were elevated in high glucose environment. Third, with high glucose, the NF-κB in the cytoplasm of RRVECs was activated to enter into the nucleus and exerted transcriptional activity. And curcumin could reverse it. Furthermore, IL-1β which is the binding site of NF-κB was declined when RRVECs was treated with curcumin.

Using transmission electron microscopy, the morphological distinction of cellular abnormality were inspected in RRVECs exposed to the high glucose. It demonstrated that a vital thicking in retinal capillary basement membrane was found in high glucose group, compared to normal control RRVECs and osmolarity control group. The nucleus and cytoplasm of RRVECs at high glucose displayed cellular swelling and cellular nucleus shrinking. After curcumin treatment, it was demonstrated that curcumin ameliorated the abnormal change of cellular structure in RRVECs.

Our study also showed that the production of intracellular ROS can be significantly decreased by curcumin, when the RRVECs was exposed to high glucose. This suggests that curcumin can obviously resist the oxidative stress caused by high glucose. And the results of flow cytometry test showed that curcumin had a significant inhibitory effect on the apoptosis of RRVECs induced by oxidative stress.

The combined analysis of immunohistochemistry, EMSA, and Western blot tests of NF-κB showed that the level of phosphorylated NF-κB and NF-κB were significantly elevated, when RRVECs exposed to the high glucose. However, after treatment with curcumin, the level of phosphorylated NF-κB and NF-κB were distinctly decreased. Immunohistochemical staining displayed that when the RRVECs were

![Fig. 2 Comparison of relative ROS content and apoptosis rate of RRVECs by flow cytometry in each group. a The relative ROS content flow chart; b ROS content of RRVECs histogram; c cell apoptosis flow chart; d cell apoptosis histogram *p < 0.05; **p < 0.01](image-url)
induced by high glucose, the expression of NF-κB p65 in the nucleus of the cells was significantly enhanced, while the expression of NF-κB p65 in the cytoplasm was decreased. It was demonstrated that NF-κB in the cytoplasm of RRVECs entered into the nucleus and exerted transcriptional activity. With the treatment of curcumin, the expression of NF-κB p65 was decreased in the cytoplasm of RRVECs, meanwhile, the NF-κB in cytoplasm were inhibited from entering to the nucleus. The Western blot assay had the consistent results in the expressions of NF-κB and pNF-κB. The results indicated that curcumin could block the signal of NF-κB which may decrease the oxidative stress response of high glucose-induced RRVECs. The results of EMSA presented the same results as well. In the high glucose group, the activity of NF-κB/DNA binding was increased, whereas, after curcumin treatment, the NF-κB activity was obviously declined. These results demonstrated that oxidative stress induced by high glucose could activate NF-κB as well as the phosphorylated NF-κB, and curcumin could suppress the activity of NF-κB by anti-oxidative stress to protect the RRVECs exposed to high glucose.

Bcl-2 and Bax regulated by NF-κB are the suppressor of apoptosis and pro-apoptosis gene, respectively. The promoter region of Bcl-2 gene which contains a binding site of NF-κB, and NF-κB can directly upregulate or promote the expression of Bcl-2 through other pathways (Wang et al. 2014, 2015; Mohammadi et al. 2016). To study the specific molecular mechanism of curcumin in anti-oxidative stress, we observed the expression of Bcl-2 and Bax in RRVECs exposed to high glucose.

We detected that the protein expression of Bax was dramatically increased, but the Bcl-2 protein expression was diminished after the RRVECs were induced by high glucose. With the treatment of curcumin, the expressions of NF-κB and Bax were declined obviously and the expression of Bcl-2 was raised apparently. The consistency of the expressions of NF-κB p65, Bcl-2 and Bax protein in our study revealed that RRVECs induced by high glucose could activate NF-κB pathway, which resulted in the decline of the expression of Bcl-2 protein and enhancement of the expression of Bax protein. However, curcumin could suppress this process.

IL-1β, which is managed by the transcription factor NF-κB is a vital pro-inflammatory cytokine participated in the pathogenesis of oxidative stress. The binding sites of NF-κB have been recognized in the promoter of IL-1β. Activating the NF-κB pathway can induce the IL-1β expression lead to the recruitment of inflammatory cells, and increase of the activity of inflammatory mediators (Hiscott et al. 1993; Scholz et al. 2013; Caradonna et al. 2018). The inactive form of IL-1β (IL-1β 31KDa) must be cleaved into the active form (IL-1β 17KDa) and then release from the cell. Moreover, in this study, it was demonstrated that the IL-1β 17KDa was increased significantly in RRVECs incubated with high glucose. However, when it was treated with curcumin, the expression of IL-1β 17KDa was declined.

The results of this study indicate that curcumin can inhibit the production of ROS and control the occurrence of apoptosis in RRVEC induced by high glucose. The possible mechanism might be that the suppression of NF-κB

Fig. 3 The detection of NF-κB signal activity and protein expression of RRVECs in each group by immunohistochemistry. a NF-κB protein expression detected by immunohistochemical staining in normal RRVECs; b NF-κB protein expression detected by immunohistochemical staining in osmotic control group; c NF-κB protein expression detected by immunohistochemical staining in RRVECs exposed to high glucose concentration; d NF-κB protein expression detected by immunohistochemical staining in the curcumin treatment group; e histogram of relative NF-κB expression in each group. *p<0.05, **p<0.01
expression leads to the increased expression of Bcl-2, and consequently, the cellular apoptosis is alleviated. Therefore, it can be suggested that curcumin can protect the retinal vascular endothelial cells by inhibiting NF-κB pathway and antagonizing oxidative stress. The more precise and profound mechanism remains to be further confirmed by extensive molecular biology studies.

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Author contributions In this work, JH, YL, and QY conceived the study and designed the experiments. JH, YL, QY, YC, and TN performed the experiments. YC and GX contributed to the data collection; JZ, TN, XJ, WZ, and YY performed the data analysis and interpreted the results. JH and WL wrote the manuscript; WL contributed to the critical revision of the article.

Declarations

Conflict of interest Jiang Huang, Quanyoung Yi, Yuhong You, Yao Chen, Tongtong Niu, Yi Li, Ji Zhang, Xiaoyan Ji, Guoxu Xu, Weijie Zou, Fangfang Ji and Weifeng Luo declare that they have no conflict of interest.

Ethics approval All animal experiments were conducted following the guidelines of China Council on Animal Care and Use. The study was approved by the Ethical Committee of the Second affiliated Hospital of Soochow University.

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