

# dl-3n-butylphthalide reduces oxygen-glucose deprivation-induced endothelial cell damage by increasing PGC-1 $\alpha$

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**Abstract.** – **OBJECTIVE:** Animal experiments verified that dl-3-n-butylphthalide (NBP) can protect vascular endothelial cells from ischemic damage and promote vascular proliferation in ischemic stroke treatment, but the underlying mechanism has not been fully clarified. This study aimed to investigate the effects of NBP on peroxisome proliferators-activated receptor- $\gamma$  coactivator-1 $\alpha$  (PGC-1 $\alpha$ ) expression in endothelial cells exposed to oxygen-glucose deprivation (OGD) and to clarify the related molecular mechanism.

**MATERIALS AND METHODS:** SV40-transformed aortic rat endothelial cell line was cultured and subjected to OGD in the presence or absence of NBP. The cell viability was evaluated by using thiazolyl blue tetrazolium bromide (MTT) method. The cellular endothelial nitric oxide synthase (eNOS) activity was measured by using eNOS activity assay. The nuclear changes were assessed with Hoechst 33342 fluorescent dye. The immunofluorescence analysis and Western blotting assay were conducted to evaluate the protein expression.

**RESULTS:** We found that NBP could significantly prevent endothelial cells from OGD-induced injuries, in terms of cell morphology and cell viability. Both immunofluorescence analysis and Western blot findings confirmed that the NBP treatment further enhanced PGC-1 $\alpha$  expression during OGD, which was prevented in the presence of selective endothelial nitric oxide synthetase (eNOS) inhibitor N5-(1-Iminoethyl)-L-ornithine-HCL (L-NIO). Furthermore, we found that NBP could protect the eNOS activity about by 40% during OGD and did not influence the eNOS protein level in the spectrophotometric-based analysis.

**CONCLUSIONS:** NBP maintained the endothelial PGC-1 $\alpha$  expression via regulating eNOS activity during the exposure to OGD; therefore, it

presented its protective function to cell viability and vascular proliferation.

*Key Words:*

NBP, Endothelial injury, Hypoxia, eNOS, PGC-1 $\alpha$ .

## Introduction

Cerebral microcirculation system is essential for maintaining the brain functions after ischemic stroke<sup>1,2</sup>. Therefore, to understand the mechanism of the endothelial damage following ischemia may lead to better strategies in post-stroke intervention or development of new drugs to minimize or prevent endothelial cells from ischemic injury.

The transcriptional co-activator, peroxisome proliferators-activated receptor- $\gamma$  coactivator-1 $\alpha$  (PGC-1 $\alpha$ ), was first identified through its functional interaction with the nuclear receptor peroxisome proliferators-activated receptor- $\gamma$  (PPAR $\gamma$ ) in brown adipose tissue (BAT)<sup>3</sup>. PGC-1 $\alpha$  and its family members are preferentially expressed in tissues with high oxidative capacity, where they serve critical roles in the regulation of mitochondrial functional capacity and cellular energy metabolism<sup>4</sup>. Recently, PGC-1 $\alpha$  was also found to be present in vascular endothelial cells. The endogenous endothelial PGC-1 $\alpha$  protein plays a crucial protective role in the transcriptional regulation of the mitochondrial antioxidant defense system in vascular endothelial cells<sup>4,5</sup>. Furthermore, some researchers found that PGC-1 $\alpha$  had a critical function in an angiogenic pathway during hypoxia. It seemed stimulating vascular endothelial growth

factor (VEGF) expression<sup>6</sup>. Therefore, studies of PGC-1 $\alpha$  could lead to new strategies for vascular protection in the treatment of ischemic disease.

The precise regulatory mechanism of PGC-1 $\alpha$  has not been identified yet. The experiment data obtained from cell type studies have all proved that the endogenous nitric oxide can increase PGC-1 $\alpha$  and promote mitochondrial biogenesis in mammals<sup>7</sup>, and long-term (>24 h) treatment of endothelial cells with nitric oxide (NO) donors up-regulates PGC-1 $\alpha$  expression<sup>8</sup>. Gutsaeva et al<sup>9</sup> reported that a representative hypoxia preconditioning stimulated mitochondrial biogenesis in the subcortex of mouse brain in part by up-regulation of PGC-1 $\alpha$ . The process was NO dependent and mediated by the neuronal nitric oxide synthase (nNOS) isoform. But it is still unclear how PGC-1 $\alpha$  expresses in vascular endothelial cells during hypoxia, and whether it has a relationship with endothelial nitric oxide synthase (eNOS).

The 1-3-n-butylphthalide (1-NBP) was extracted as a pure component from seeds of *Apium graveolens* Linn. Afterward, dl-3-n-butylphthalide (dl-NBP) was synthesized and received the approval by the State Food and Drug Administration (SFDA) of China for clinical use in stroke patients in 2002<sup>10</sup>. Butylphthalide (NBP) can inhibit platelet aggregation and reduce the thrombus formation<sup>11</sup>, decrease the area of cerebral infarct<sup>12</sup>, and improve the energy metabolism in mice with cerebral ischemia<sup>13</sup>. However, the effect of NBP on the prevention of the endothelial cells against the ischemic damage should be studied in further investigation. We speculated that NBP might protect the endothelium from ischemic attack by regulating PGC-1 $\alpha$  expression, which was possibly mediated by the eNOS.

Therefore, in this study, we investigated the PGC-1 $\alpha$  expression in vascular endothelial cells during oxygen-glucose deprivation (OGD) and the possible relative regulation mechanisms. Moreover, we investigated how eNOS and PGC-1 $\alpha$  interacted in the protective function of NBP contributing to the survival of vascular endothelial cells during OGD, in order to detect new treatment targets of NBP and provide theoretical evidence for clinical cerebral vascular diseases treatment.

## Materials and Methods

### Materials

NBP was provided as a generous gift by Shijiazhuang Pharmaceutical Group Ouyi Pharma

Co., Ltd. (Shijiazhuang, China). It was dissolved in dimethyl sulfoxide (DMSO, Beyotime Biotech., Shanghai, China) before dilution with the cell culture medium. The final concentration of treatment was 0.01, 0.1, 1.0, 10  $\mu$ mol/l, and the final concentration of DMSO per well was 0.2%.

### Cell Culture

The SV40-transformed aortic rat endothelial cell line (SVAREC), obtained from Shanghai Bomai Company (Shanghai, China), were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Gibco BRL Co. Ltd., Grand Island, NY, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco BRL Co. Ltd., Grand Island, NY, USA) at 37°C with 5% CO<sub>2</sub> and 95% air (v/v) at 90% humidity.

This study was approved by the First Affiliated Hospital, Sun Yat-Sen University, Guangzhou, China.

### Cell Viability Assay

Cellular viability was assessed by using the mitochondrial assay kit (BioChain Institute, Inc., San Francisco, CA, USA) according to the manufacturer instructions. After OGD treatment thiazolyl blue tetrazolium bromide (MTT) labeling reagent at a final concentration of 0.5 mg/ml was added into each well and incubated for 4 h to allow the formation of purple formazan crystal. Then, the cells were washed and 200  $\mu$ l DMSO was added to each well to dissolve the formazan by pipetting up and down several times. Finally, the spectrophotometric absorbance of the solubilized purple formazan crystals was measured using a microplate reader (Mode: Multiskan MCC type 355, Thermo Fisher Scientific, Waltham, MA, USA) at an absorbance wavelength of 570 nm. The results of the OGD groups were normalized and expressed as the percentage of the average optical density reading of the sham-OGD group (normal control).

### eNOS Activity Assay

The cellular eNOS activity was measured by using the Nitric Oxide Synthase Assay Kit (Nanjing Jiancheng Tech. Co, Ltd., Nanjing, China) according to the protocol. The sample solution or distilled water (blank control) was mixed with the solvents (the kit provided) by steps. The cell-permeable fluorescent probe DAF-FM DA (3-amino, 4-aminomethyl-2', 7'-difluorescein, diacetate), contained in the solvents, can cross the cell membrane, bind NO (evolving from L-Arginine under

the catalysis of Nitric Oxide Synthase), and produce a fluorescent compound Benzotriazole derivative. So, by using the selective eNOS inhibitor N<sup>5</sup>-(1-Iminoethyl)-L-ornithine-HCL (Sigma-Aldrich, St. Louis, MO, USA), the eNOS activity can be determined by measuring the optical density through the colorimetric method.

### **Nuclear Morphology Analysis**

Nuclear changes from non-treated and treated cells were assessed with Hoechst 33342 fluorescent dye (Sigma-Aldrich, St. Louis, MO, USA). Hoechst was added to the culture medium at a concentration of 10  $\mu$ g/ml and incubated in the dark at 100% humidity for 10 min at 37°C. The nuclear morphology was visualized under fluorescent microscopy ( $\times$ 400 magnification). The cell death was identified by the presence of highly condensed chromatin or fragmented nuclei.

### **Immunofluorescence Assay**

For immunofluorescence analyses, the cells grown on glass plates (which were inserted in culture dishes) were fixed with freshly prepared 4% formaldehyde (Beyotime Biotech., Shanghai, China) in Phosphate-Buffered Saline (PBS; pH 7.4) for 20 min, washed with PBS, permeabilized with 1% Triton X-100 (Beyotime Biotech., Shanghai, China) for 30 min, and then rinsed with PBS. Following the blockade of the nonspecific binding sites by incubation with blocking buffer (5% goat serum in PBS) for 30 min, the cells were incubated for 1 h with the primary rabbit anti-rat PGC-1 $\alpha$  antibody (diluted 1:500 in 3% BSA/PBS, Santa Cruz Biotech., Santa Cruz, CA, USA). These bindings were further detected by incubation with FITC-conjugated goat anti-rabbit secondary antibody (diluted 1:1000 with 3% BSA/PBS, Santa Cruz Biotechnology, CA, USA). After washing with PBS for 3 times, the slides were mounted and analyzed on an Olympus inverted fluorescence microscopy (Mode: BX51, Olympus, Tokyo, Japan). The fluorescence pictures were taken with identical exposure settings. For nuclear counterstaining, the cells were incubated in 10  $\mu$ g/ml 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, St. Louis, MO, USA) for 10 min after immunostaining. For negative control, the sections stained in the absence of primary antibody showed no signals (data not shown). The mean fluorescence intensity (MFI) was calculated with the software ImagePro Plus (IPP, Media Cybernetics, Inc., Warrendale, PA, USA). The results were obtained from five independent experiments performed in triplicate.

### **Western Blot Assay**

The endothelial cells (ECs) were lysed with a buffer that contained 10 mmol/l Tris (Beyotime Biotech., Shanghai, China), pH 7.4, 100 mmol/l NaCl, 1 mmol/l ethylenediaminetetraacetic acid (EDTA, Beyotime Biotech., Shanghai, China), 1 mmol/l ethylene glycol-bis ( $\beta$ -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA, Sigma-Aldrich, St. Louis, MO, USA), 1 mmol/l NaF, 20 mmol/l Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 2 mmol/l Na<sub>3</sub>VO<sub>4</sub>, 0.1% sodium dodecyl sulfate (SDS, Sigma-Aldrich, St. Louis, MO, USA), 0.5% sodium deoxycholate, 1% Triton-X 100 (Beyotime Biotech., Shanghai, China), 10% glycerol, 10  $\mu$ g/ml leupeptin, 60  $\mu$ g/ml aprotinin, and 1 mmol/l phenylmethanesulfonyl fluoride (PMSF). Cell debris was removed by centrifuging (12000  $\times$ g) for 10 min at 4°C. The samples (20  $\mu$ g) were treated with 5 $\times$  Laemmli's sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (0.35 mol/l Tris-Cl, pH 6.8, 15% SDS, 56.5% glycerol, 0.0075% bromophenol blue), followed by heating at 100°C for 5 min, and then subjected to 8% SDS-PAGE gel for electrophoresis. The proteins were then transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) with a semidry transfer unit (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were then blocked by use of 5% non-fat milk in Tris-Buffered-Saline and Tween-20 (TBST-20) buffer (0.1% Tween 20, pH 7.4) for 1 h at room temperature and incubated with anti-PGC-1 $\alpha$  antibody (Cell Signaling Technology, Beverly, MA, USA), and anti-eNOS antibody (Abcam Biotechnology, Cambridge, MA, USA) overnight at 4°C. The membranes were then incubated with the secondary antibody (horseradish peroxidase-conjugated anti-mouse/rabbit immunoglobulin antibody). The immunoreactive bands were detected by an enhanced chemiluminescence kit (Millipore, Billerica, MA, USA). The anti- $\beta$ -actin antibody (Sigma-Aldrich, St. Louis, MO, USA) was employed as a loading control. The Western blots were quantified densitometrically.

### **Oxygen-Glucose Deprivation (OGD) Treatment of Cultures**

We used OGD, an *in vitro* model that best mimics *in vivo* cerebral ischemia. Briefly, 24 h after SUVRECs were seeded in different culture plates, the culture medium was changed to the glucose-free RMPI 1640 containing either NBP or selective eNOS agonist/inhibitor or both in different groups. Then, the cells were placed in-

to an anaerobic chamber that was flushed with 5% CO<sub>2</sub> and 95% N<sub>2</sub> (v/v). The cell cultures within the anaerobic chamber were kept in a humidified incubator at 37°C for various time intervals in different experiments. To terminate the OGD, the culture medium was changed to normal medium and the plates were returned to a 5% CO<sub>2</sub>/95% air incubator. In the normal control groups, the cell cultures were subjected to the same experimental procedures only with medium, but without the exposure to the glucose-free RPMI 1640 or anoxia.

### **Statistical Analysis**

The data were expressed as mean  $\pm$  SEM and analyzed by using the statistical package SPSS Version 18.0 for Windows (SPSS, Inc., Chicago, IL, USA). The Tukey's post-hoc test was used to validate the ANOVA for pairwise comparison measurement data among groups. A statistical significance was defined when  $p < 0.05$ .

## **Results**

### **eNOS-NO was Essential for PGC-1 $\alpha$ Expression in Vascular Endothelial Cells**

Firstly, we detected the expression of PGC-1 $\alpha$  in normal vascular endothelial cells. The Western blot results showed that PGC-1 $\alpha$  protein was constitutively expressed in vascular endothelial cells. However, when the cells were pretreated with the L-NIO (N5-(1-Iminoethyl)-L-ornithine-HCL, at a concentration of 1.0  $\mu$ M), a selective eNOS inhibitor, the levels of PGC-1 $\alpha$  expression were significantly decreased compared to that in untreated control cells (Figure 1A,  $p < 0.05$ ). This result suggested that the eNOS activity is a critical maintainer for PGC-1 $\alpha$  expression in normal vascular endothelial cells.

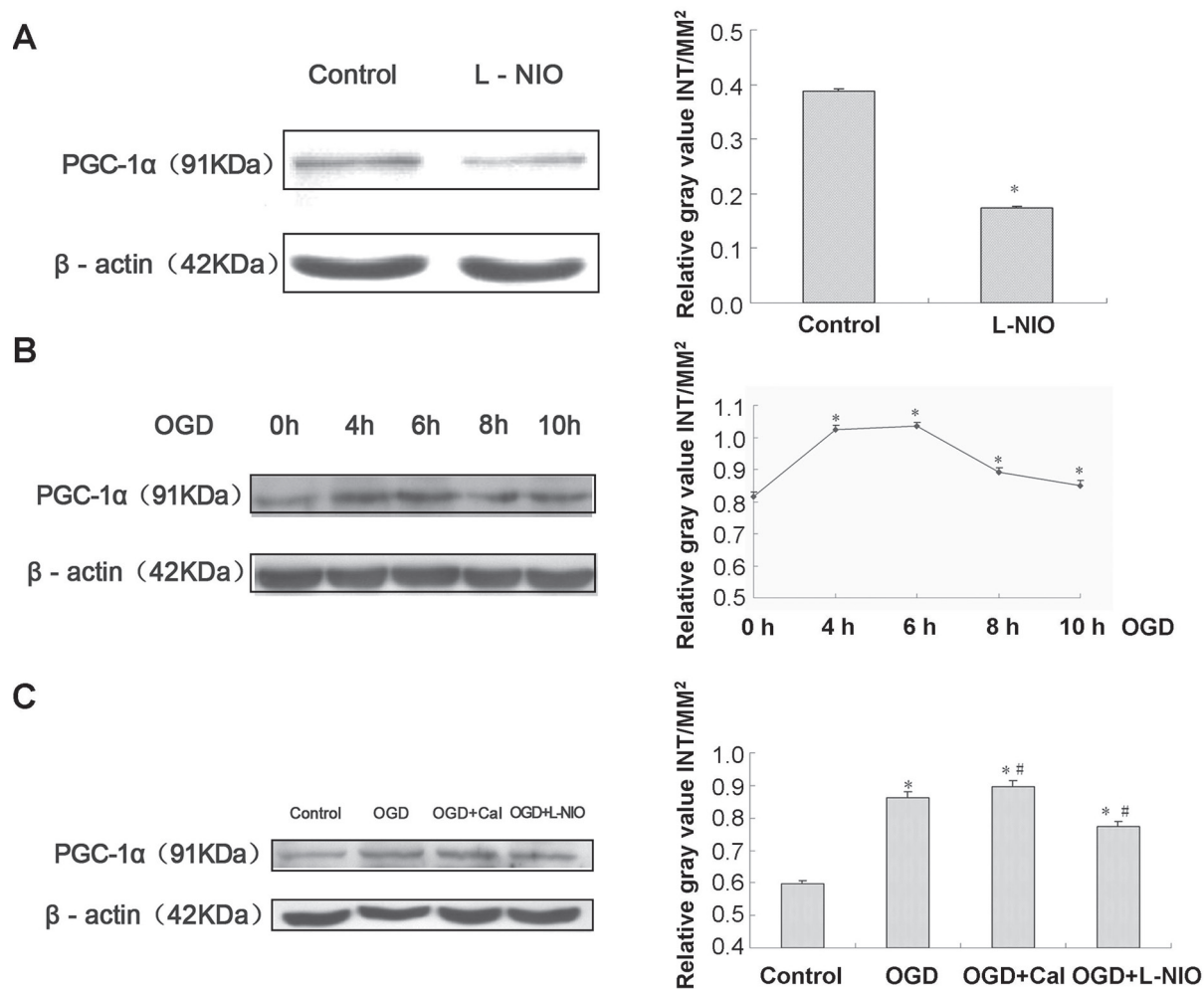
We further evaluated PGC-1 $\alpha$  expression in vascular endothelial cells during OGD. The kinetics analysis demonstrated that the cellular PGC-1 $\alpha$  protein expression levels were increased at the beginning of OGD, then expeditiously increased to a peak at 6 h, and then showed a marked decrease (Figure 1B). More significantly, the selective eNOS agonist CaI treatment (Calcium Ionophore, at the concentration of 0.05  $\mu$ M) maintained PGC-1 $\alpha$  expression levels during OGD even after 6 h. On the contrary, the eNOS inhibitor L-NIO (at the concentration of 1.0  $\mu$ M) remarkably decreased the PGC-1 $\alpha$  (Figure 1C,  $p < 0.05$ ).

### **NBP Protected Against OGD-Induced Cell Injury**

We previously reported that OGD induced cell death in human umbilical vein endothelial cells<sup>14</sup>. The results shown in Figure 2 expanded this observation. There was a significant loss in cell viability after 4 h (71% viability), 6 h (45% viability), 8 h (38% viability), and 10 h (22% viability) following exposure to OGD compared with untreated controls (Figure 2A). But treated with NBP, the cell viability values (after 6 h OGD) were  $51.5\% \pm 0.5\%$ ,  $60.6\% \pm 0.8\%$ ,  $71.3\% \pm 0.6\%$ , and  $69.8\% \pm 0.8\%$  at NBP concentration of 0.01, 0.1, 1.0, and 10  $\mu$ mol/l, respectively (Figure 2B). So, NBP at 1.0  $\mu$ mol/l may already reach a saturated state, and the concentrations beyond 1.0  $\mu$ mol/l will not increase the protective efficiency. The untreated cells exhibited normal morphology (Figure 2C, control), whereas cells exposed to OGD 6 h showed typical cell injury (Figure 2C, OGD). The immunofluorescence pictures of the nuclear Hoechst staining showed that normal nuclei were round shaped and had nice boundary and staining. But, after OGD 6 h, there were visible crenation of nuclei, condensation, and fragmentation and a great decrease of cell viability ( $44.55\% \pm 0.4\%$ ). However, NBP could significantly relieve the morphological changes (Figure 2D), and the cell viability could be markedly rescued by NBP 1.0  $\mu$ M ( $71.3\% \pm 0.6\%$ ) (Figure 2B).

### **NBP Promoted PGC-1 $\alpha$ Expression and eNO Mediated NBP-Induced PGC-1 $\alpha$ Expression in Endothelial Cells Exposed to OGD**

To further investigate the effects of NBP on PGC-1 $\alpha$  expression, we incubated endothelial cells with NBP (1.0  $\mu$ M), and then processed to OGD. We found that NBP treatment significantly enhanced PGC-1 $\alpha$  expression, compared to that in untreated OGD group, indicating that NBP further increased PGC-1 $\alpha$  levels, which was largely abolished in the presence of L-NIO (1.0  $\mu$ M). (Figure 3A, B,  $p < 0.05$ ). The results in Figure 3C showed that NBP (1.0  $\mu$ M) or L-NIO (1.0  $\mu$ M) for 6 h had no effect on normal cells. As we proved, NBP (1.0  $\mu$ M) could markedly rescue cell viability during OGD. But the treatment of the cells with eNOS inhibitor L-NIO (1.0  $\mu$ M) may severely impair the protecting effects of NBP (Figure 3C, cell viability descent to  $47.86\% \pm 3.8\%$ ,  $p < 0.05$ ).

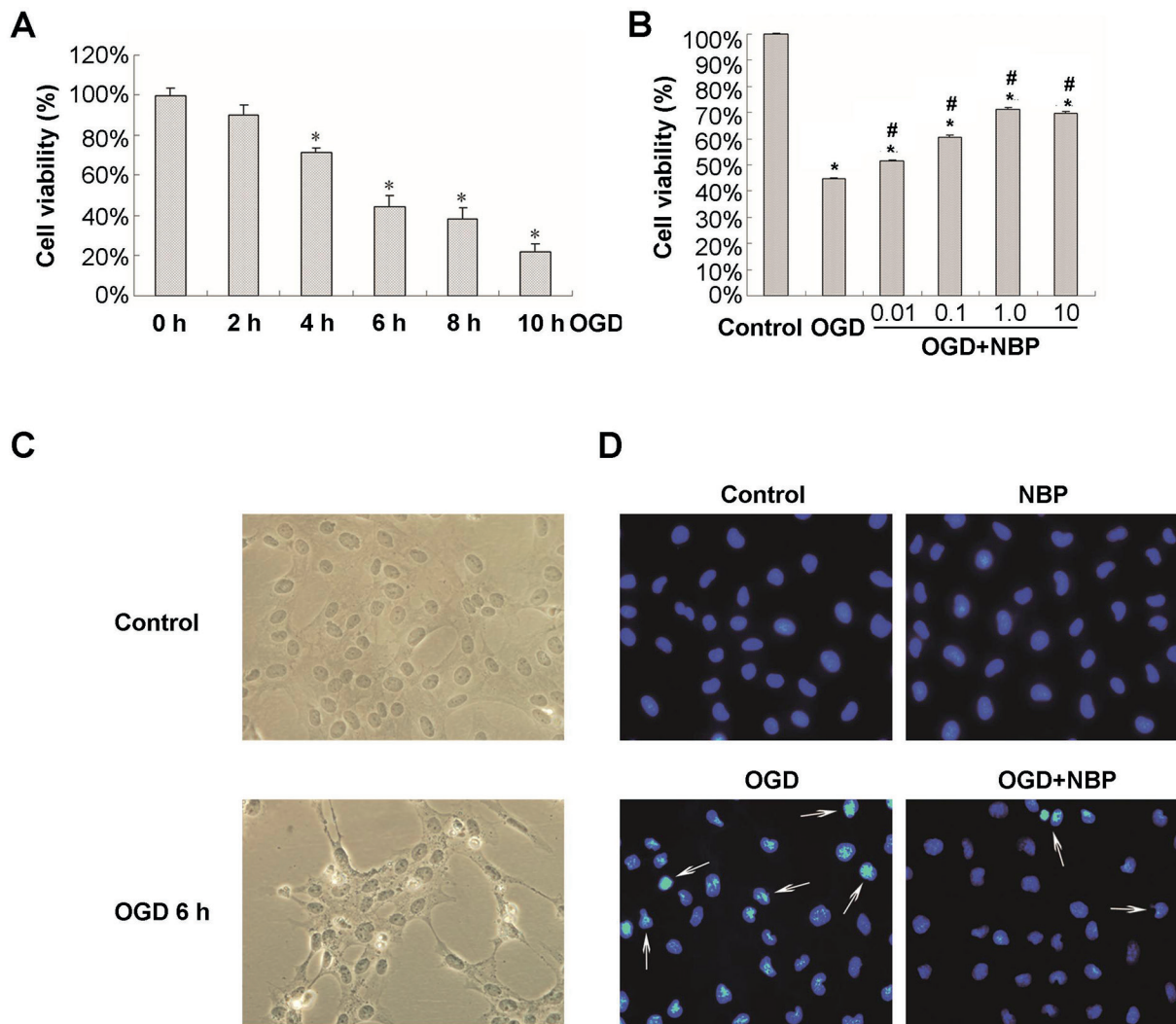


**Figure 1.** eNOS-NO is essential for PGC-1 $\alpha$  expression in vascular endothelial cells. **A**, SVARECs were pretreated with selective eNOS inhibitor L-NIO 1.0  $\mu$ M for 6 h then, the PGC-1 $\alpha$  protein decreased. **B**, The cells were subjected to OGD processing for 0, 4, 6, 8, 10 h, respectively. The trend graph shows the time-dependent protein expression of PGC-1 $\alpha$ . **C**, The cells were subjected to OGD 6 h processing and treated with CaI 0.05  $\mu$ M or L-NIO 1.0  $\mu$ M respectively, at the same time. The PGC-1 $\alpha$  protein (91 kDa) was quantitatively assessed by Western blots.  $\beta$ -actin protein (42 kDa) expression was used as the internal control. Data represent mean  $\pm$  SEM of three independent experiments. \* $p$ <0.05 compared with normal control. # $p$ <0.05 compared with OGD group.

**NBP Illustrated No Influence on eNOS Protein Levels but It Protected eNOS Activity Against OGD**

We studied the eNOS protein and activity in endothelial cells during OGD in the presence or absence of NBP treatment. As shown in Figure 4A, the eNOS protein levels were increased to a peak at OGD 4 h, and then showed a significant decrease at OGD 6 h ( $p$ <0.05). But NBP (1.0  $\mu$ M) could not increase the protein levels of eNOS after OGD 6 h as it did on PGC-1 $\alpha$  levels (Figure 4B). We further investigated the effects of NBP on eNOS activity in vascular endothelial cells. eNOS activity in SVARECs was very low (0.0036

$\pm$  0.0006 U/ml) in the normal controls. Both NBP (1.0  $\mu$ M) and CaI (0.05  $\mu$ M) treating for 4 h could promote eNOS activity in SVARECs, which were  $0.0071 \pm 0.00035$  U/ml and  $0.0089 \pm 0.00063$  U/ml ( $p$ <0.05), respectively. After OGD 4 h, the eNOS activity increased almost ten-fold ( $0.0313 \pm 0.00015$  U/ml). The same as eNOS agonist CaI, NBP (1.0  $\mu$ M) exposure markedly enhanced the eNOS activity after OGD 4 h ( $0.0437 \pm 0.00013$  U/ml) ( $p$ <0.05), which was abrogated by the NOS inhibitor L-NIO (1.0  $\mu$ M; decreased to  $0.0294 \pm 0.00022$  U/ml) (Figure 4C,  $p$ <0.05), suggesting that the eNOS activity is critical for NBP-mediated PGC-1 $\alpha$  regulation.



**Figure 2.** NBP protects against OGD-induced cell injury. **A**, Cells were exposed to OGD processing, and viability was determined using MTT assay at the indicated times. **B**, The cells were exposed to different NBP concentration (0.01, 0.1, 1.0, and 10  $\mu$ M) during OGD 6 h. Cell viability was measured by the MTT assay. **C**, The morphology of normal SVARECs and cells suffered OGD 6 h under microscopy ( $\times 400$  magnification). **D**, Cells were stained with Hoechst (10  $\mu$ g/ml), and nuclei were visualized under fluorescent microscopy ( $\times 400$  magnification). Arrows indicate the nuclei showing chromatin condensation and fragmentation. Data represent mean  $\pm$  SEM of six independent experiments. \* $p < 0.05$  compared with normal control. # $p < 0.05$  compared with OGD group.

## Discussion

When an ischemic stroke happens, ischemia and hypoxia are profound metabolic challenges with potentially catastrophic consequences. The acute and chronic adaptive responses to hypoxia include the initiation of the gene transcription for the proteins involved in angiogenesis, anaerobic glucose metabolism, and oxygen transport. These responses sustain  $O_2$  supply to tissues and enhance cell survival during  $O_2$  deprivation<sup>15</sup>. The transcriptional co-activator PGC-1 $\alpha$  is a potent modulator

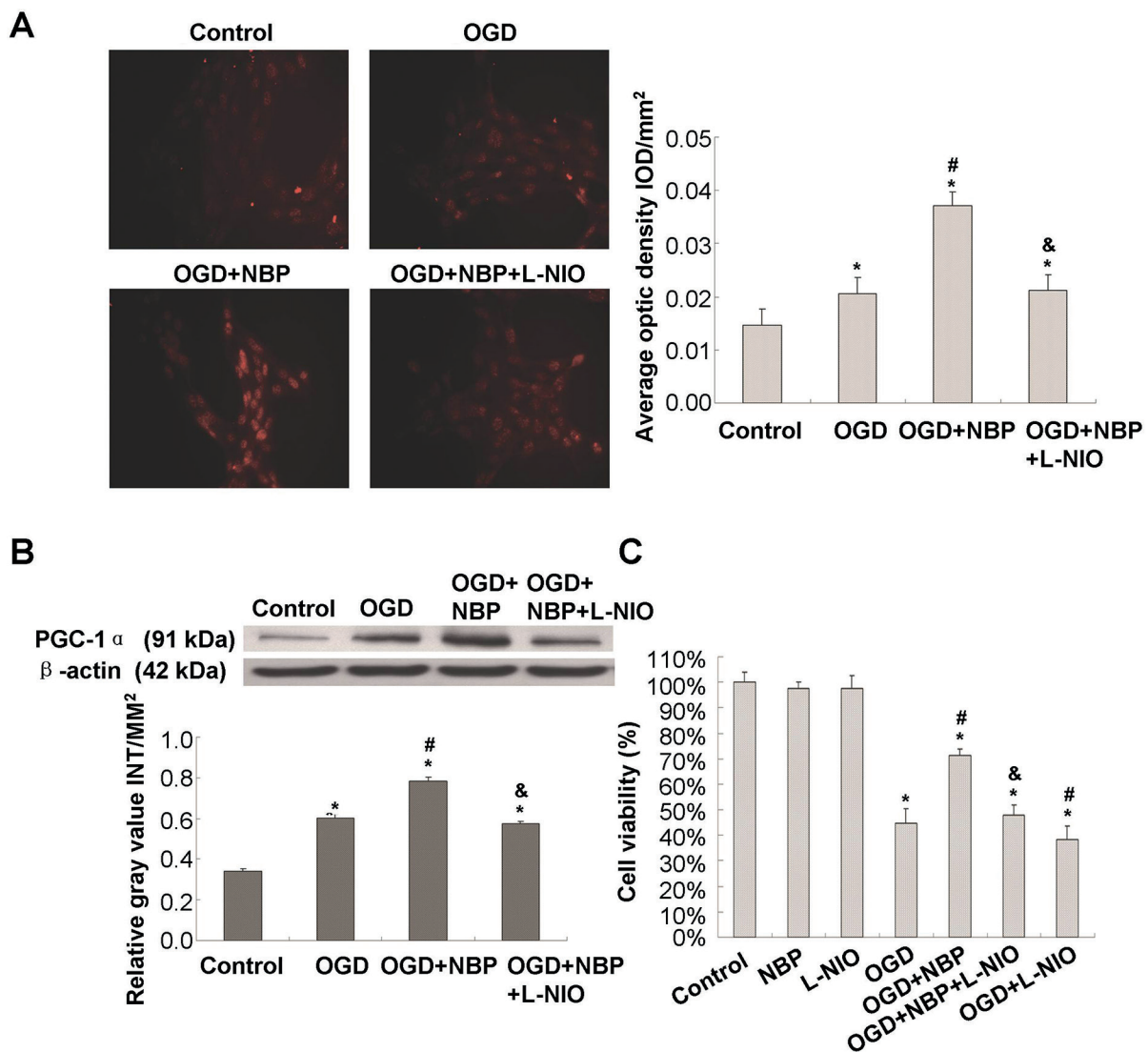
of oxidative metabolism in numerous settings<sup>16,17</sup>. It is present in vascular endothelial cells. The endogenous endothelial PGC-1 $\alpha$  protein plays a crucial protective role in the endothelium<sup>5</sup>.

In the present study, we investigated the expression of PGC-1 $\alpha$  in vascular endothelial cells during OGD, its possible regulation mechanism, and the role of NBP in the protection of the microcirculation after stroke. Our results demonstrated that the PGC-1 $\alpha$  expression in our system increased following the onset of OGD and reached the peak at 6 h later. This finding is in agreement

with previous observations indicating that PGC-1 $\alpha$  can be induced by a lack of nutrients and oxygen<sup>6</sup>.

The signaling mechanisms about PGC-1 $\alpha$  upstream regulation have been poorly understood. NO could be a positive regulator of mitochondrial biogenesis through the transcriptional induction of PGC-1 $\alpha$ <sup>7</sup>, and the long-term exposure to NO might be an important positive regulator of the expression of PGC-1 $\alpha$  and the mitochondrial ROS

detoxifying system<sup>8</sup>. Gutsaeva et al<sup>9</sup> demonstrated that the hypoxic preconditioning elicited subcortical mitochondrial biogenesis by a novel mechanism that requires nNOS regulation of PGC-1 $\alpha$ . In this study, we successfully demonstrated, *in vitro*, that eNOS-NO was vital to the expression of PGC-1 $\alpha$  in vascular endothelial cells under either anoxia or non-anoxia condition. Selective eNOS inhibitor reduced the PGC-1 $\alpha$  protein level compared to normal vascular endothelial cells or cells

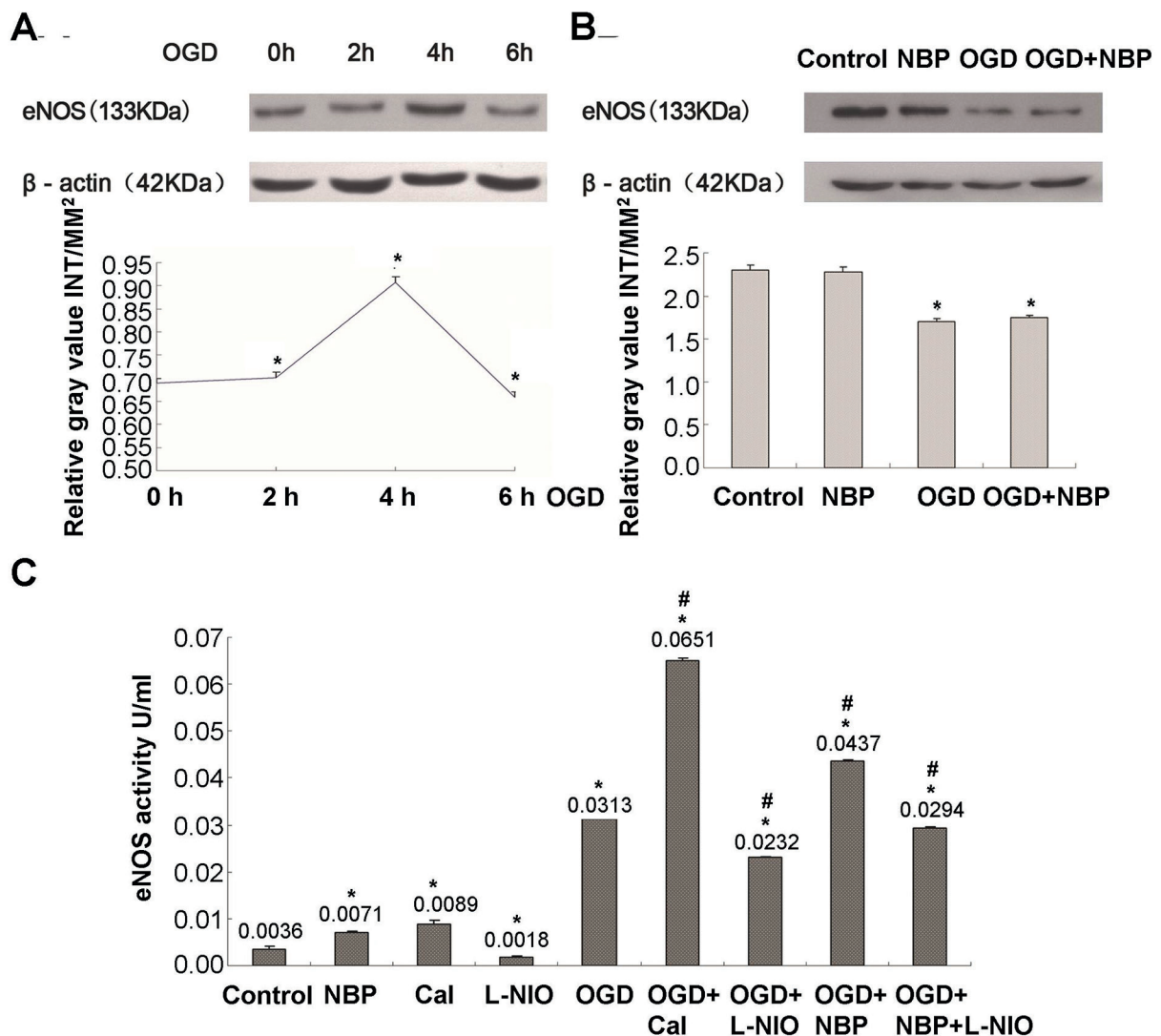


**Figure 3.** NBP augments PGC-1 $\alpha$  expression in endothelial cells exposed to OGD, but the promotion function is prevented by eNOS inhibitor. The cells were treated with NBP 1.0  $\mu$ M or L-NIO 1.0  $\mu$ M or both, then subjected to OGD 6 h. **A**, Photomicrograph under fluorescent illumination ( $\times 400$  magnification) showing the expression of PGC-1 $\alpha$ , it is overlapped with the stain of nuclei. **B**, The PGC-1 $\alpha$  protein (91 kDa) was quantitatively assessed by Western blots.  $\beta$ -actin protein (42 kDa) expression was used as the internal control. **C**, The treatment of cells with eNOS inhibitor L-NIO 1.0  $\mu$ M may severely impair the protecting effects of NBP. Cell viability was measured by the MTT assay. Data represent mean  $\pm$  SEM of at least three independent experiments. \* $p < 0.05$  compared with normal control. # $p < 0.05$  compared with OGD group. & $p < 0.05$  compared with OGD+NBP group.

under OGD, and special eNOS agonist increased the expression of PGC-1 $\alpha$  during OGD.

We previously reported that OGD induced cell death in human umbilical vein endothelial cells<sup>14</sup>. The results in this study expanded the observation. There was a significant loss in cell viability after OGD 6 h (45% viability). This moderate cell injury level provides adequate space for drugs to reveal their therapeutic effect. We chose OGD 6 h to observe the

protective effect of NBP. After testing different concentration of NBP, we found that NBP at 1.0  $\mu$ mol/l may already reach a saturated state of the protective efficiency. From microphotographs and immunofluorescence pictures, we could clearly see cell morphology change after OGD. NBP 1.0  $\mu$ M could significantly alleviate nuclear condensation and fragmentation, further proving the protective effect of NBP on the endothelium.



**Figure 4.** NBP has no influence on eNOS protein level but it protects eNOS activity against OGD. **A**, Cells were subjected to OGD processing for 0, 2, 4, 6 h, respectively. Trend graph show the time-dependent protein expression of eNOS. **B**, Cells were treated with NBP 1.0  $\mu$ M, then subjected to OGD 6 h processing. The eNOS protein (133 kDa) was quantitatively assessed by Western blots.  $\beta$ -actin protein (42 kDa) expression was used as the internal control. **C**, Cells were co-incubated with NBP 1.0  $\mu$ M or L-NIO 1.0  $\mu$ M or Cal 0.05  $\mu$ M, then exposed to OGD 4 h processing or not. After that the eNOS activity of cultured endothelial cells were evaluated according to the assay. The data represent mean  $\pm$  SEM of three independent experiments. \* $p$ <0.05 compared with normal control. # $p$ <0.05 compared with OGD group. \* $p$ <0.05 compared with OGD+NBP group.



Our previous study found that NBP significantly attenuated OGD and induced a decrease in the SOD activity in vascular endothelial cells<sup>14</sup>. Similarly, Dong et al<sup>18</sup> also found that NBP could significantly attenuate the impaired activity of SOD in a model of focal cerebral ischemia. However, we do not know whether this effect is due to NBP's direct or indirect action on SOD. It is known that PGC-1 $\alpha$  is involved in the transcriptional regulation of the mitochondrial antioxidant defense system (such as Mn-superoxide dismutase, Uncoupling Protein 2 and Peroxiredoxin V) in vascular endothelial cells<sup>5</sup>. The expression of PGC-1 $\alpha$  increases the cellular levels of the mitochondrial antioxidant proteins, reduces the accumulation of ROS, prevents mitochondrial dysfunction and apoptotic cell death. The suppression of endogenous PGC-1 $\alpha$  expression results in the downregulation of the mitochondrial detoxification machinery<sup>4</sup>. Our results from immunofluorescence and Western blot demonstrated that NBP could significantly enhance OGD induced PGC-1 $\alpha$  expression in endothelial cells. The cells preconditioned with NBP had a much higher survival rate during OGD (MTT results). Therefore, we would suggest that the enhancement of NBP on OGD-induced PGC-1 $\alpha$  level may partially explain how NBP provides its protective action on SOD. The understanding of the mechanism of PGC-1 $\alpha$  regulation may have important implications as they may potentially lead to new drug development targeting to PGC-1 $\alpha$  regulation in stroke treatment.

NBP could also prevent brain damage from chronic cerebral hypoperfusion by increasing the expression of vascular endothelial growth factor<sup>19</sup>. Our previous study confirmed that NBP could prevent cold-induced ischemic stroke via improvement of cerebral micro-vessels and protection of vascular endothelial cells in stroke-prone reno-vascular hypertension rats<sup>12</sup>. However, the mechanism of NBP on vascular proliferation remains unclear. It has been recently proved that PGC-1 $\alpha$  could powerfully regulate the vascular endothelial growth factor expression and angiogenesis in cultured muscle cells and skeletal muscle *in vivo*<sup>6</sup>. PGC-1 $\alpha$ <sup>-/-</sup> mice show a striking failure to reconstitute blood flow in a normal manner to the limb after an ischemic insult, whereas the transgenic expression of PGC-1 $\alpha$  in skeletal muscle is protective. Our results demonstrated that NBP could significantly increase OGD induced PGC-1 $\alpha$  expression in endothelial cells, which, we speculate, may partly contribute to vascular proliferation after stroke.

Given that NBP could increase PGC-1 $\alpha$  expression during OGD, the eNOS-NO played a vital role in PGC-1 $\alpha$  expression regulation, and NBP protective function was extremely attenuated after eNOS was inhibited. Therefore, we believed that there is an underlying interaction between NBP and eNOS. As the crucial endogenous protective factor of endothelial cells, eNOS-NO increased early after hypoxia (2-4 h), enhanced its activity until 6 h, and then, the protein level of eNOS-NO descended<sup>20,21</sup>. Similarly, in the present study, the eNOS protein expression reached the peak value after OGD 4 h, and then the protein decreased. The eNOS activity mostly increased after OGD 4 h. Although NBP 1.0  $\mu$ M could not increase the protein level of eNOS after OGD 6 h as it did on PGC-1 $\alpha$  level, it enhanced eNOS activity both during OGD and normal condition. Again, as long as the eNOS inhibitor L-NIO was added, the enhancement function of NBP was abolished. Therefore, our researches indicated that NBP was trying to protect the elevated activity of eNOS, rather than its increased protein level. These could partly explain why NBP can promote the expression of PGC-1 $\alpha$  during OGD. However, the concrete mechanism of how NBP acts on eNOS activity needs further investigation. We are now trying to establish the PGC-1 $\alpha$  knockout vascular endothelium cell line, in order to further investigate the important role of PGC-1 $\alpha$  in the protective effect of NBP in ischemic damage.

## Conclusions

We demonstrated that endogenous eNOS-NO is vital to the expression of PGC-1 $\alpha$  in normal endothelial cells and in cells during OGD. The role of NBP in the increase of the expression of PGC-1 $\alpha$  during OGD depends on eNOS activity. Active eNOS is crucial for NBP to present its protective function in cell viability, anti-oxidative damage ability, and vascular proliferation.

## Conflict of interest

The authors declare no conflicts of interest.

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