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***Basic Study***

**Inhibitory effect of maspinon neovascularization in diabetic retinopathy**

Qiu F *et al*. Maspinon neovascularization in DR

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**Abstract**

BACKGROUND

Diabetic retinopathy (DR) is a serious and potentially blinding complication of diabetes mellitus. Retinal neovascularization is one of the main pathological features of proliferative DR, and inhibiting retinal neovascularization is a research focus.

AIM

The aim was to evaluate the effect of intravitreal injection of recombinant human maspin on neovascularization in DR.

METHODS

An oxygen-induced retinopathy (OIR) mouse model was used to simulate neovascularization in DR. New born C57BL/6J mice were randomly divided to a normal control group, a maspin injection OIR group, and an OIR group. The mice in the maspin injection OIR group were injected with recombinant human maspin in the bilateral vitreous cavity on postnatal day P12, and those in the OIR group were injected with sterile phosphate buffered saline. The protein expression of vascular endothelial growth factor (VEGF) and hypoxia-inducible factor 1-alpha (HIF-1α) in the retina was measured by western blotting, and the mRNA expression of VEGF and HIF-1α was measured by real-time polymerase chain reaction. The vascular cell nuclei that broke through the inner limiting membrane (ILM) were counted in haematoxylin-eosin stained retinal sections.

RESULTS

It was found that the number of vascular cell nuclei breaking through the ILM was 31.8 ± 8.75 in the OIR group, which was significantly more than that in the normal control group (*P* < 0.001). The number of vascular cell nuclei breaking through the ILM was 6.19 ± 2.91 in the maspin injection OIR group, which was significantly less than that in OIR group (*P* < 0.01). The relative protein and mRNA expression of VEGF and HIF-1α was significantly lower in the retinas in the maspin injection OIR group than in those in the OIR group (*P* < 0.01).

CONCLUSION

Maspin inhibited neovascularization in DR by modulating the HIF-1α/VEGF pathway, which provides a potential and effective strategy for the treatment of DR.

**Key Words:** Maspin; Diabetic retinopathy; Neovascularization; Vascular endothelial growth factor; Hypoxia-inducible factor 1-alpha

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**Core Tip:** The aim of our study was to evaluate the effectiveness of intravitreal injection of recombinant human maspin on neovascularization in diabetic retinopathy. A mouse model of oxygen-induced retinopathy was used to simulate neovascularization in diabetic retinopathy. Maspin inhibited neovascularization in this model by modulating the hypoxia-inducible factor 1-alpha/vascular endothelial growth factor pathway, which provides a potential and effective strategy for the treatment of diabetic retinopathy.

**INTRODUCTION**

Diabetic retinopathy (DR) is a serious and potentially blinding complication of diabetes mellitus[1]. The prevalence of DR in patients with diabetes is 34.6% worldwide[2]. The global prevalence of diabetes is estimated to be 10.2% (578 million) by 2030 and 10.9% (700 million) by 2045[3], and the prevalence of DR is expected to rise accordingly. DR progresses in stages from a non-proliferative to a more vision-threatening proliferative (PDR) form. Retinal neovascularization is a key pathological feature of PDR, and inhibiting retinal neovascularization is a research focus[4].

Maspin is a member of the serine protease inhibitor (serpin)family. Studies have shown that maspin, which is a class II tumour suppressor gene, can induce tumour cell apoptosis, reduce the movement of tumour cells, and increase adhesion to inhibit tumour invasion and metastasis. Maspin can also directly induce endothelial cell apoptosis and inhibit the endothelial cell signalling pathway to inhibit the development of tumour angiogenesis. Recombinant maspin inhibits corneal endothelial neovascularization by inhibiting the expression of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor[5]. Recombinant maspin was found to inhibit tumour growth and angiogenesis in an animal model of prostate cancer[6]. Injection of adenovirus carrying maspin into the left ventricle was found to disrupt angiogenesis in developing and mature tumours[7]. Maspin is highly expressed during keratinocyte senescence and has antiangiogenic activity[8]. Maspin-mimetic nanostructures can inhibit angiogenesis in tubulogenesis assays with human umbilical vein endothelial cells and *in vivo* assays in the chick chorioallantoic membrane[9].

The mouse model of oxygen-induced retinopathy (OIR) has much in common with human ischemic retinopathy and effectively simulates retinal neovascularization *in vivo*. The model is widely used to study neovascularization in DR[10,11]. At present, the effect of maspin on retinal neovascularization in animal models is not clear. In this study, we investigated whether maspin could inhibit retinal neovascularization in a mouse OIR model. Through the results of this study, we hope to find agents that inhibit neovascularization in DR and provide a theoretical basis for clinical treatments.

**MATERIALS AND METHODS**

***Ethical approval***

All mouse procedures were performed following the guidelines of the Chinese Ministry of Science and Technology Guidelines on the Humane Treatment of Laboratory Animals.

***Animal groups***

New born C57BL/6J mice were randomly divided into three groups of 25 each, a normal control group, a maspin injection OIR group, and an OIR group. The mice were housed in a specific pathogen free animal laboratory. On postnatal day P17, 10 mice (20 eyes) were randomly selected from each group for haematoxylin-eosin (HE) staining and 15 mice (30 eyes) were selected for RNA extraction from the left eye for real-time polymerase chain reaction (PCR) and protein extraction from the right eye for western blotting.

***Mouse OIR model***

The mouse OIR model was established as previous described[12]. On day P7 new-born C57BL/6J mice and their mothers were transferred to a constant hyper oxygen chamber with a volume fraction of 75% ± 2% and were then returned to normal air on day 12. Mice in the normal control group were housed in normal air.

***Maspin administration***

On day 12, 0.5 µL of 0.05 mg/mL recombinant human maspin was injected into the vitreous cavities of mice in the maspin injection OIR group mice with a microsyringe (Hamilton Company, Reno, NV, United States). In the OIR group, 0.5 µL of sterile phosphate buffered saline was injected.

***Histological analysis of retinal sections***

Ten mice (20 eyes) in each group were used to prepare tissue sections of eyeball specimens. The eyeballs were fixed in 4% paraformaldehyde for 24 h and embedded in paraffin. Sagittal sections parallel to the cornea and optic disc were prepared at a thickness of 6 µm. One of every six sections was selected at random; five sections were randomly selected from each eye. The sections were dewaxed and rehydrated in a graduated ethanol series for HE staining. The number of vascular cell nuclei that broke through the inner limiting membrane (ILM) was counted with an optical microscope, and the average number that broke through the ILM in each section was calculated. Vascular cell nuclei in the vitreous cavity that were not associated with the ILM were not counted).

***Western blotting***

Mouse retinal tissue was lysed in RIPA lysis buffer (Beyotime, Jiangsu, China) and the concentration of the extracted protein was determined with a bicinchoninic acid protein assay kit (Beyotime). Forty micrograms of total protein extract from each sample was separated by 5%–14% sodium dodecyl sulphate–polyacrylamide gel electrophoresis and electrophoretically transferred onto polyvinylidene fluoride membranes (Millipore, Bedford, MA, United States). The membranes were incubated with primary antibodies (anti-VEGF, 1:500 dilution, BIOSS, Beijing, China; anti-HIF-1α, 1:500 dilution, Proteintech, Rosemont, IL, United States). Proteins were analysed with an enhanced chemiluminescence kit (Beyotime). β-actin served as an internal control.

***Real-time PCR***

mRNA was extracted from mouse retinal tissue with RNA pure total RNA extraction kits (Bioteke, Jiangsu, China). cDNA was generated with super M-MLV transcriptase (Bioteke). The primers were as follows: HIF-1α, forward: 5′-AGT GTACCC TAA CTA GCC GA-3′, reverse: 5′-CAC AAA TCAGCA CCA AGC -3′; VEGF, forward: 5′-ACA CACCCA CCC ACA TAC ATA-3′, reverse: 5′-ACT CAA GTCCAC AGC AGT CAA-3′. Relative expression of VEGF and HIF-1α mRNAs was calculated by the comparative cycle threshold method. β-actin served as an internal control.

***Statistical analysis***

The results were reported as means ± SD. All assays were repeated at least three times. SPSS software (Version 20.0, IBM, Armonk, NY, United States) was used for the statistical analysis. Between-group differences were compared by analysis of variance, and *P* value < 0.05 were considered to be statistically significant.

**RESULTS**

***Number of preretinal neovascular nuclei***

In the normal control group, the ILM was smooth, with vascular cell nuclei breaking through the ILM in only a few places (Figure 1A). Many pathologic neovascular tufts broke through the ILM in the OIR group (Figure 1B and C). The number of vascular cell nuclei in the maspin injection OIR group was significantly decreased compared with that in the OIR group (Figure 1D and E). In the normal control group, 0.52 ± 0.10 vascular cell nuclei broke through the ILM in each tissue section. The number that broke through the ILM in the OIR group was 31.8 ± 8.75, which was significantly higher than that in the normal control group (*P* < 0.001). In the maspin injection OIR group, 6.19 ± 2.91 vascular cell nuclei broke through the ILM in each tissue section, which was significantly less than that in OIR group (*P* < 0.01) and more than that in the normal control group (*P* < 0.01; Figure 1F).

***VEGF and HIF-1α protein expression in the retina***

The western blot results found that relative protein expression of VEGF and HIF-1α in retinas from the OIR group was significantly higher than that in the normal control group (both, *P* < 0.001). The expression of VEGF and HIF-1α protein in retinas from the maspin injection OIR group was significantly lower than that in the OIR group (*P* < 0.01), and higher than that in the normal control group (*P* < 0.01; Figure 2A, B, and C).

***Expression of VEGF and HIF-1α mRNA in the retina***

The relative mRNA expression of VEGF and HIF-1α mRNA in retinas from the OIR group was significantly higher than that in the normal control group (both *P* < 0.001). The relative mRNA expression of VEGF and HIF-1α mRNA in retinas from the maspin injection OIR group was significantly lower than that in the OIR group (*P* < 0.01), and higher than that in the normal control group (*P* < 0.01; Figure 2D and E).

**DISCUSSION**

Our previous studies showed that maspin inhibited high glucose-induced angiogenesis in human retinal microvascular endothelial cells[13]. The inhibitory effect of maspin on retinal neovascularization *in vivo* has not been reported. Intravitreal injection is the main method of studying and treating DR[4,14-16]. The mouse OIR model is widely used to simulate neovascularization and to study the prevention of neovascularization in ischemic retinal diseases such as DR. The retinal blood vessels of 7-d-old mice are not yet mature. Inhalation of high concentration oxygen stimulates retinal blood vessels to undergo reversible spasmodic changes[17]. Continuous hyperoxia causes small vessel occlusion and areas of retinal nonperfusion. After the mice returned to normal air on day P12, the central avascular retina was in a state of hypoxia, leading to both normal vessel regrowth and the formation of extraretinal pathological neovascularization[12,18]. On day P17, retinal neovascularization reached the most advanced stage[19-22]. The model shares many characteristics with DR, and the number of neovascular sites can be measured by counting the nuclei that break through the ILM in HE-stained retinal tissue sections[19,21,22]. We found that significantly fewer nuclei broke through the ILM in the maspin injection OIR group than in the OIR group, indicating that maspin inhibited the development of neovascularization in the DR model.

VEGF promotes the division and proliferation of vascular endothelial cells and increases vascular permeability[23] and is a key angiogenic factor that induces retinal neovascularization[24]. Clinical studies have found that inhibiting VEGF effectively inhibited retinal neovascularization; anti-VEGF therapy has become the main method of treating DR and other retinal neovascular conditions[25-27]. Under hypoxic conditions, HIF-1α is produced in the nucleus and binds to the HIF-1α binding site on the target gene to initiate transcription and promote angiogenesis[28]. HIF-1α can regulate the expression of VEGF, and is active in maintaining energy metabolism and angiogenesis of tumour cells. The activation of VEGF transcription and maintenance of VEGF mRNA stability in hypoxic tissues is mainly regulated by HIF-1α. VEGF is one of the target genes of HIF-1α[29]. HIF-1α and VEGF are abnormally upregulated in PDR, and HIF-1α regulates the expression of VEGF and promotes retinal neovascularization[30-32]. Interfering RNA targeting VEGF and HIF-1a was effective in inhibiting retinal neovascularization[33,34]. Inhibiting VEGF and HIF-1α is an effective method to treat DR[35,36]. Our previous studies showed that maspin could inhibit HIF-1α and VEGF expression in HG-treated human retinal microvascular endothelial cells. In this study, the retinal expression of VEGF and HIF-1α in was significantly lower in the maspin injection OIR group than that in the OIR group, suggesting that maspin may inhibit neovascularization in DR by modulating the HIF-1α/VEGF pathway. In our study, we observed the inhibitory effect of one dose of recombinant human maspin on retinal neovascularization in OIR on day 17. The effect of different doses of maspin on retinal neovascularization in OIR at different times is planned in future research.

**CONCLUSION**

In conclusion, our study showed that maspin inhibited neovascularization in DR by modulating the HIF-1α/VEGF pathway, providing a potential and effective strategy for the treatment of DR.

**ARTICLE HIGHLIGHTS**

***Research background***

Diabetic retinopathy (DR) is a serious and potentially blinding complication of diabetes mellitus.

***Research motivation***

We used an experimental animal model to find a more effective strategy for the treatment of DR.

***Research objectives***

The study aim was to evaluate the effect of intravitreal injection of recombinant human maspin on neovascularization in DR.

***Research methods***

An oxygen-induced retinopathy (OIR) model in mice was used to simulate neovascularization in diabetic retinopathy. On postnatal day P12, 0.5 µL of 0.05 mg/mL recombinant human maspin was injected into the vitreous cavity of maspin injection OIR group mice. The protein and mRNA expression of vascular endothelial growth factor (VEGF) and hypoxia-inducible factor 1-alpha (HIF-1α) in the retina were assayed. The numbers of vascular cell nuclei that broke through the inner limiting membrane were counted.

***Research results***

The results revealed that intravitreal injection of maspin inhibited neovascularization and reduced protein and mRNA expression of VEGF, HIF-1α in the retinal tissue of OIR model mice.

***Research conclusions***

Maspin inhibited neovascularization of DR by modulating the VEGF/HIF-1α pathway, providing a potential and effective strategy for the treatment of DR.

***Research perspectives***

Retinal neovascularization is one of the main pathological features of PDR. Inhibiting retinal neovascularization is a research focus.

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**Footnotes**

**Institutional animal care and use committee statement:** The study was reviewed and approved by Shenyang Fourth People’s Hospital Institutional Review Board.

**Conflict-of-interest statement:** The authors declare that they have no conflicting interests.

**Data sharing statement:** No additional data are available.

**ARRIVE guidelines statement:** The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

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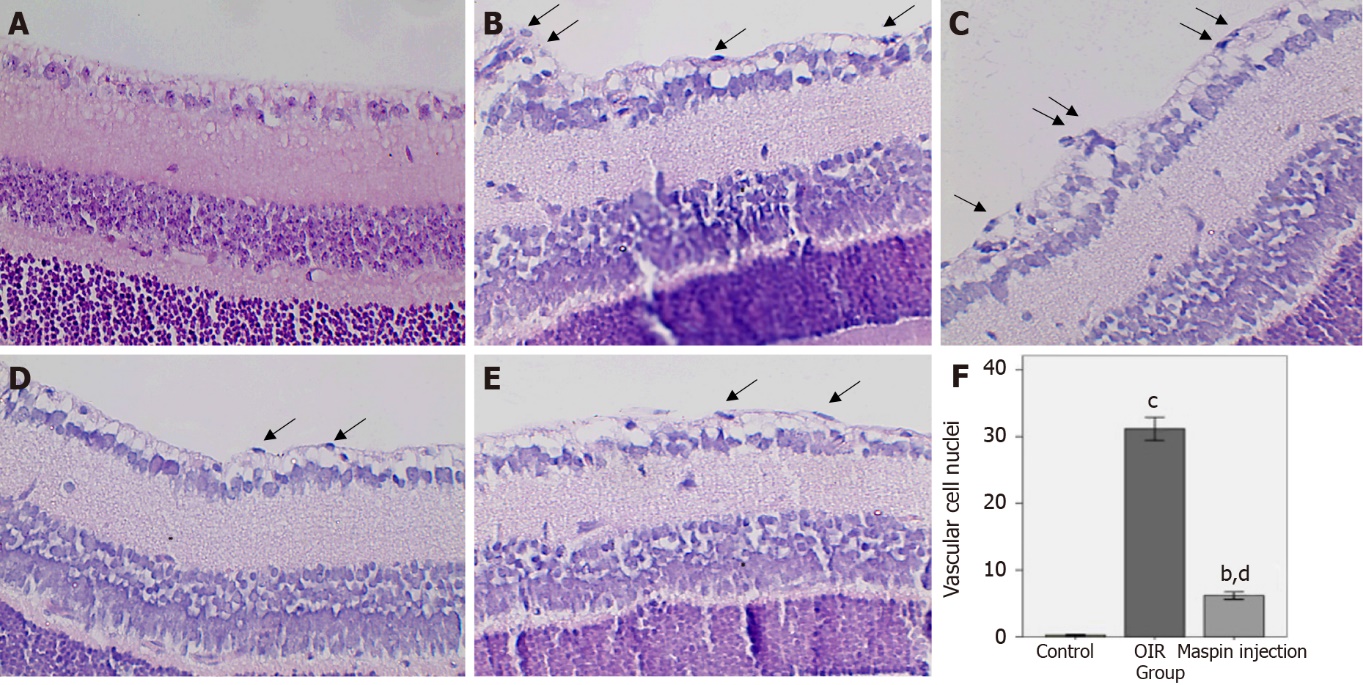
Grade C (Good): 0

Grade D (Fair): 0

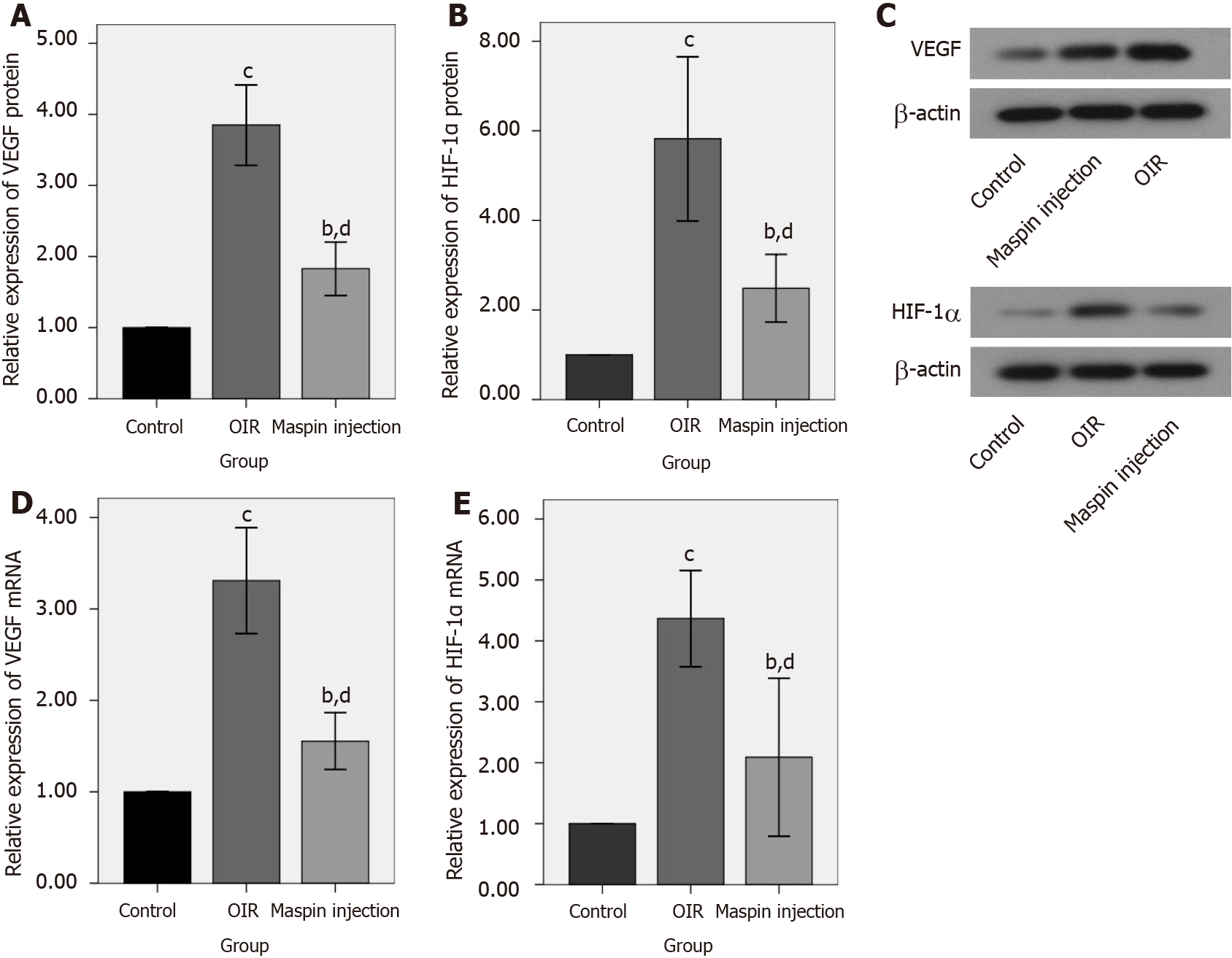
Grade E (Poor): 0

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**Figure Legends**



**Figure 1 Retinal neovascularization was determined by counting the vascular cell nuclei (arrow) breaking through the inner limiting membrane on postnatal day 17.** A: No nuclei were detected in the normal control group; B, C: There were many pathologic neovascular tufts beyond the inner limiting membrane (ILM) in the oxygen-induced retinopathy (OIR) group; D, E: Fewer vascular cell nuclei broke through the ILM in the maspin injection OIR group than in the OIR group but more than that in the normal control group. Magnification ×400; F: Data are means ± SD. b*P* < 0.01 *vs* normal control group; c*P* < 0.001 *vs* normal control group; d*P* < 0.01 *vs* the OIR group. OIR: Oxygen-induced retinopathy.



**Figure 2 Maspin downregulated vascular endothelial growth factor and hypoxia-inducible factor 1-alpha expression in the maspin injection oxygen-induced retinopathy group.** A, B, C: Relative protein expression of vascular endothelial growth factor (VEGF) and hypoxia-inducible factor 1-alpha (HIF-1α) were determined by western blotting; D, E: Relative mRNA expression of VEGF and HIF-1α were assayed by real-time polymerase chain reaction. Data are means ± SD. b*P* < 0.01 *vs* normal control group; c*P* < 0.001 *vs* normal control group; d*P* < 0.01 *vs* oxygen-induced retinopathy group. VEGF: Vascular endothelial growth factor; HIF-1α: Hypoxia-inducible factor 1-alpha; OIR: Oxygen-induced retinopathy.



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