

Original Research Article

Effect of bone morphogenetic protein-2 on diabetic retinopathy and its mechanism of action

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Abstract

Purpose: To investigate the effect of bone morphogenetic protein-2 (BMP-2) on human retinal vascular endothelial cells (RECs) and human retinal pigment epithelial cells (RPE) cultured in high glucose (HG) *in vitro*, and the underlying mechanism.

Methods: Cell counting kit-8 (CCK-8) was used to determine cell proliferation while Western blot was used to assay the expressions of extracellular matrix and angiogenesis-related factors, Expressions of cytokines and chemokines were assessed by quantitative real time polymerase chain reaction (qRT-PCR) and enzyme-linked immunosorbent assay (ELISA). Changes in Smad, ERK, JNK and p38MAPK signal pathway were measured by transfection and interference.

Results: The level of expression of BMP-2 in HG group was higher than that in normal glucose (NG) culture group. The expressions of angiogenesis-related factors i.e. vascular endothelial growth factor (VEGF) and intercellular cell adhesion molecule-1 (ICAM1), pro-inflammatory factors i.e. IL-6 and chemokine monocyte chemoattractant protein-1 (MCP1), increased significantly in HG group compared to NG and HG + BMP-2 groups. Phosphorylation of Smad1/5/8 and activation of ERK, JNK and p38MAPK signaling pathways were enhanced by BMP-2.

Conclusion: These results suggest that BMP-2 promotes angiogenesis and enhances the expressions of inflammatory cytokines via Smad signaling pathway.

Keywords: Diabetic retinopathy, Retina, Pigment epithelial cells, Vascular endothelial cells, Bone morphogenetic protein-2

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INTRODUCTION

Diabetic retinopathy (DR) is one of the predisposing factors to blindness among people in the age range of 26 - 75 years. It is also a major socio-economic burden in most countries [1]. The most important markers of vascular injury during DR are leukocytosis, high

permeability of blood vessels, and early-stage inflammatory response, followed by retinal neovascularization (RNV) [2]. Diabetic retinopathy-induced vision loss is due to the rupture of the blood-retinal barrier (BRB) which results in macular edema, retinal detachment, and intra-retinal and vitreous hemorrhage. Several growth factors have been shown to play

vital roles in the progression of DR. These are vascular endothelial growth factor (VEGF), angiopoietin, and insulin-like growth factor [3]. However, the potential molecular mechanisms involved in DR have not yet been fully understood.

Bone morphogenetic protein (BMP) contains a wide range of conserved growth factor groups, more than 30 members of which have been identified to date, and it is the largest member of the transforming growth factor beta (TGF β) superfamily [4]. Three members of this family i.e. BMP-2, BMP-4 and BMP-7 and their receptors (BMPR) play important roles in ocular biochemistry. Some researchers have suggested that BMP signaling may be involved in regulation of retinal vascular homeostasis and diabetes-induced vascular dysfunction such as atherosclerosis [5]. The BMP signaling pathway is involved in the regulation of endothelial cell tube formation and oxidative stress during angiogenesis. The association between BMP and angiogenesis was demonstrated by the discovery of BMPER, a precursor of BMP in endothelial cells, indicating that BMP-2 is an extracellular regulator needed for normal BMP signaling [6]. Recently, the role of BMPER in regulating protein levels after angiogenesis stimulation in oxygen-induced retinopathy (OIR) mouse models has been reported [7].

However, in spite of the fact that these evidence suggest that BMP plays a key role in the induction and maintenance of vascular inflammation and angiogenesis in DR, the underlying mechanisms remain relatively unknown. In addition, the function of BMP-2 in the pathogenesis of DR is not clear. Therefore, in the present study, the effect BMP-2 on retinal pigment epithelium (RPE) and retinal vascular endothelial cells (RECs) cultured in HG, and the mechanisms involved, were investigated *in vitro*. This was with a view to providing new scientific information that might enhance the prevention and treatment of DR.

EXPERIMENTAL

CCK8 cell viability assay

Cells were inoculated in 96-well plates at a density of 5×10^3 cells/mL. The plate was cultured at 37 ° C in a humidified incubator containing 5 % CO₂. After cell fusion, RECs cells were divided into three groups: NG culture group, HG group, and high glucose + BMP-2 group. Then, 10 μ L CCK-8 solution was mixed and added to each well in the 96-well culture plate and incubated for 1 hour. Absorbance was

determined at 450 nm in an ELISA monitor, and the results were recorded.

Western blotting

The different cell groups were harvested with centrifugation and lysed in protease-inhibitor containing lysis buffer. A portion of total protein from each lysate was subjected to SDS-PAGE, followed by transference to polyvinylidene difluoride (PVDF) membrane. Non-specific binding was blocked by incubating the membranes with non-fat milk for 1 h at room temperature. The PVDF membranes were thereafter incubated overnight with the appropriate primary antibodies at 4 °C. Then, incubation with horseradish-conjugated secondary antibody was carried out. The level of expressions of the various proteins were determined using infrared imaging. Protein expressions were normalized to that of β -actin which was used as control.

Real-time PCR analysis

After RNA extraction and cDNA reverse transcription, RT-PCR was performed. Repeated denaturation and annealing were extended for 40 cycles for amplification [8]. Finally, the threshold cycle (CT) values and the melting curve parameters were calculated, and the levels of expression were estimated using the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

Data are expressed as mean \pm standard deviation (SD). One-way ANOVA was used for comparison between groups, while Least Significant Difference (LSD) was used for multiple comparisons. All statistical analyses were done with SPSS version 13.0. Values of $p < 0.05$ were considered statistically significant.

RESULTS

High glucose induced expression and secretion of BMP-2 in hREC and ARPE-19 cells

The results in Figure 1 show that BMP-2 expression was significantly increased in the HG group ($p \leq 0.05$).

BMP-2 promoted hREC cell proliferation under high glucose conditions

As shown in Figure 2, cell viability in the HG group was significantly lower than that in the NG group. Cell viability increased gradually in

response to increases in the concentration of BMP-2. The cell viability of HG + BMP-2 group was significantly higher than that of the HG group ($p < 0.05$).

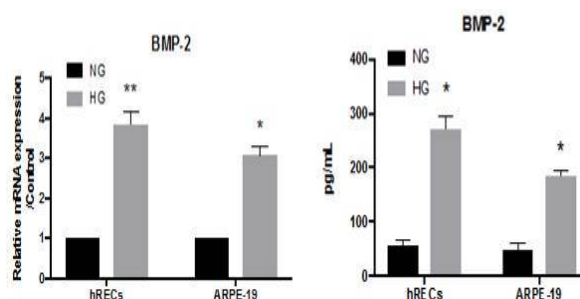


Figure 1: Effect of high glucose on the expression of BMP-2 in the cells

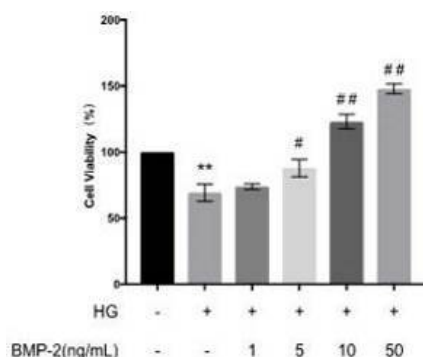


Figure 2: Effect of high glucose and BMP-2 treatments on cell viability

BMP-2 increased the expressions of angiogenic factors in hREC cells under high glucose conditions

As shown in Figure 3, the expressions of VEGF and ICAM-1 in the HG group were significantly higher than those in the control group, and the expressions of VEGF and ICAM-1 in the HG + BMP-2 group were markedly higher than those in the pure HG group ($p < 0.05$).

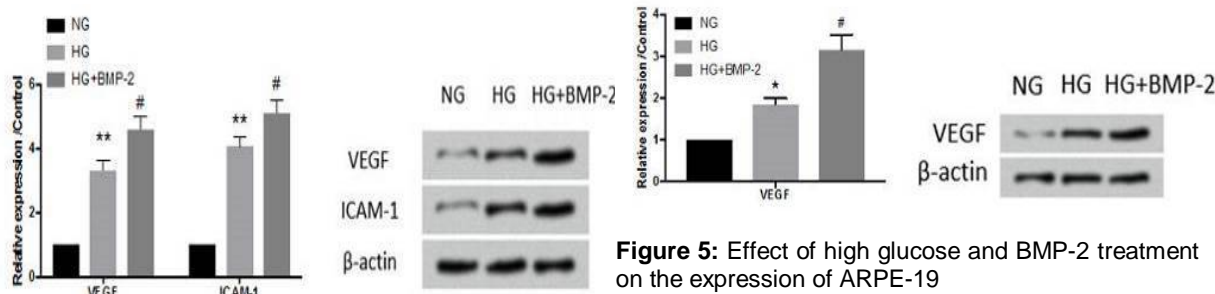


Figure 3: Effect of high glucose and BMP-2 treatments on expressions of VEGF and ICAM-1

BMP-2 inhibited the expression of ZO-1 in the extracellular matrix of ARPE-19 cells and promoted the expression of SMAα and MMP2

Figure 4 shows that the expression of cell ZO-1 in the HG group was significantly lower than that in the NG group. Moreover, the expression of ZO-1 in HG + BMP-2 group was lower than that in HG group. These results demonstrated that the migration ability in the HG + BMP-2 group was lower than that in the pure HG group ($p < 0.05$). In contrast, the expressions of SMA α and MMP2 in HG group was markedly higher than that in NG group, and the expression of HG + BMP-2 group was significantly higher than that in HG group ($p < 0.05$). These results indicate that the expression of ZO-1 was inhibited by BMP-2, while the expressions of SMA α and MMP2 were enhanced by BMP-2.

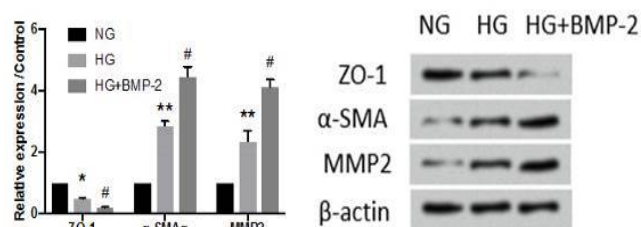


Figure 4: Changes in extracellular matrix proteins after high glucose and BMP-2 treatment

BMP-2 enhanced the expression of ARPE-19 cytokine in hyperglycemia

Figure 5 shows that the expression of cytoangiogenic factor VEGF in the HG group was markedly higher than that in the NG group. Moreover, the expression of VEGF in the HG + BMP-2 group was remarkably higher than that in the simple HG group ($p < 0.05$). These results suggest that the expression and secretion of angiogenesis factor VEGF were enhanced by BMP-2.

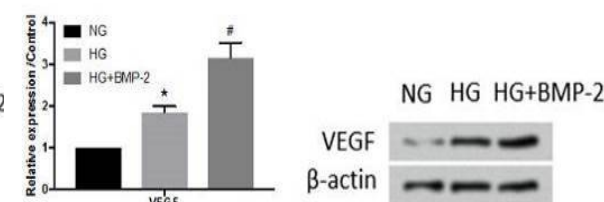


Figure 5: Effect of high glucose and BMP-2 treatment on the expression of ARPE-19

BMP-2 increased the expressions of hREC and ARPE-19 under high glucose

Figure 6 shows that the expressions of pro-

inflammatory cytokines IL-6 and MCP-1 in the HG group were significantly higher than those in the NG group. Moreover, the expressions of IL-6 and MCP-1 in the HG + BMP-2 group were significantly higher than those in the simple HG group ($p < 0.05$).

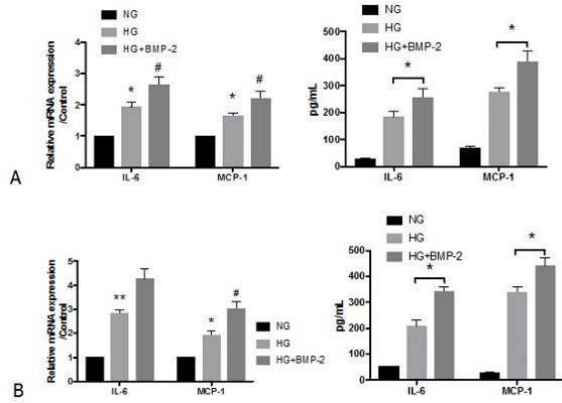


Figure 6: Effect of high glucose and BMP-2 treatment on expression levels of pro-inflammatory factors. A: hREC cell; B: ARPE-19 cell

BMP-2 influenced the expressions of angiogenesis and inflammatory factor hRECs through Smad-dependent and non-smad-dependent pathways, while BMP-2 influenced angiogenesis and inflammatory factor expression in ARPE-19 cells through Smad-dependent pathways

The results of Western blot are shown in Figure 7 and Figure 8. The expression levels of Smad, ERK, JNK and MAPK in the HG group were significantly higher than the corresponding expression levels in the NG group ($p < 0.05$). However, the expressions of these proteins were significantly lower than those in the HG + BMP-2 group in both hRECs and ARPE-19 cells ($p < 0.05$). These results suggest that BMP-2 promoted the phosphorylation of smad1/5/8 and the activation of ERK, JNK and p38MAPK signaling pathway in both hRECs and ARPE-19 cells (Figure 7 A and B, Figure 8 A and B).

With Smad protein knockout, there were significant reductions in the expressions of pro-angiogenic factors and inflammatory factors. However, in hRECs cells, the effect of BMP-2 was not completely offset. These results demonstrate that BMP-2 influenced angiogenesis and expression of inflammatory factors through a mechanism that is partially dependent on the Smad signaling pathway in hRECs cells (Figure 7 C to E). In contrast, knockout of Smad protein markedly decreased the expressions of angiogenic factors and inflammatory factors in ARPE-19 cells, but there was no significant difference between HG + BMP-2 group and HG group. Thus, BMP-2 affected angiogenesis and inflammatory factor expression in ARPE-19 cells through a mechanism that completely depends on the Smad signaling pathway (Figure 8 C to E).

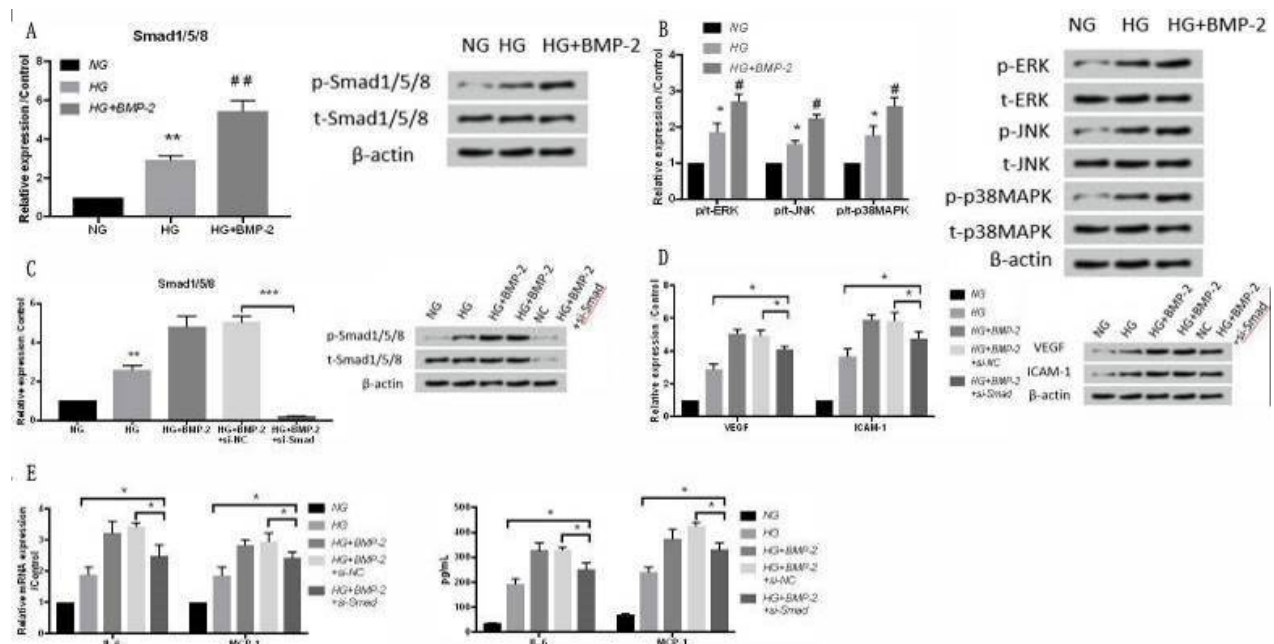


Figure 7: Changes in hREC cell signaling pathway proteins after high glucose and BMP-2 treatments

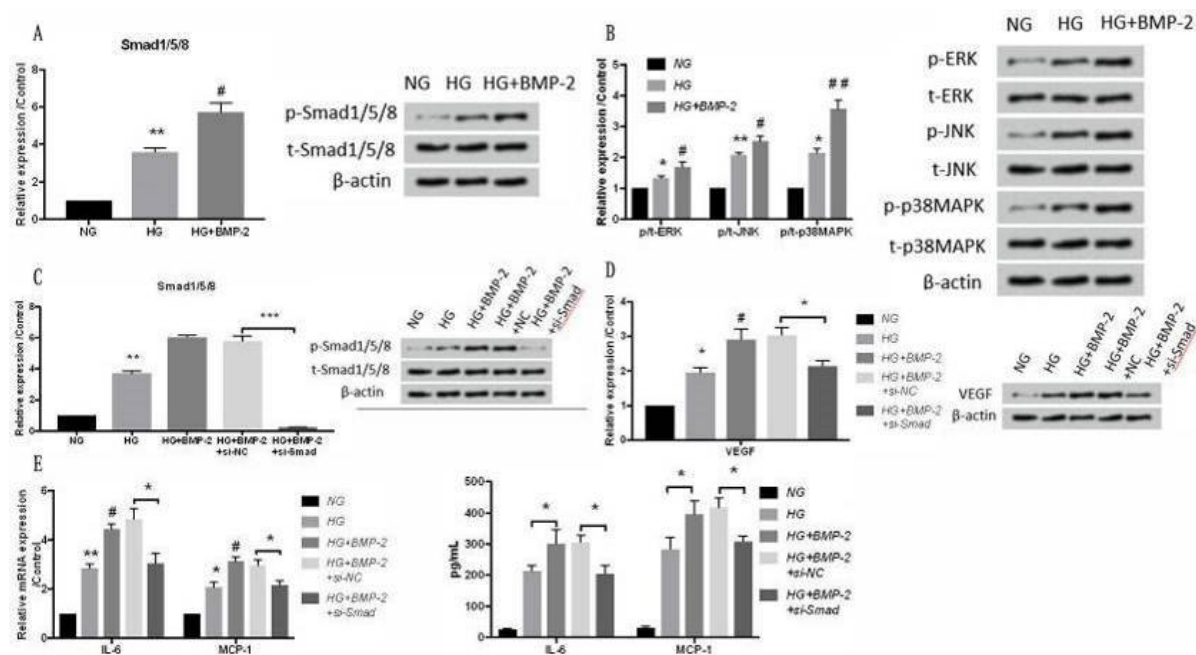


Figure 8: Effect of high glucose and BMP-2 treatments on ARPE-19 signaling pathway proteins

DISCUSSION

Diabetic retinopathy (DR) is a serious complication of diabetes mellitus (DM) and a major cause of visual impairment and blindness in adults [9]. The clinical diagnosis of DR is based on abnormal performance of retinal vascular lesions. Accordingly, DR is divided into two stages: non-proliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR) [10].

The most common cause of vision loss in DR patients is diabetic macular edema (DME) [11]. It appears at any stage of DR, resulting in loss of vision or distortion of visual images. The current specific treatment strategies for DR include intravitreal drug therapy, laser photocoagulation and vitreous surgery, all of which are aimed at managing microvascular complications [12]. At present, the primary treatment strategy for any stage of DR is the application of anti-VEGF drugs. Traditional laser treatment may partially enhance stable vision, while anti-VEGF treatment can improve vision and reduce adverse eye reactions. The poor therapeutic effect of anti-VEGF may be due to the participation of other VEGF-related molecular signaling pathways in its pathogenesis. Thus, it is important to investigate the potential mechanisms of DR and provide a new insight for the development of new methods of DR treatment.

It has been reported *in vivo* and *in vitro* that high glucose induces apoptosis of pericytes [13,14].

Since pericytes are responsible for providing the supporting structure of capillaries, the loss of capillary walls can lead to local exudation of capillaries. This process is linked to the formation of microaneurysm which appears as an initial clinical symptom of DR. Furthermore, pathological changes such as decreases in pericytes, apoptosis of endothelial cells and thickening of basement membrane manifest in the pathogenesis of DR and blood-retina barrier (BRP) damage [15]. Studies have shown that the regulation of leukocyte migration and activation of chemokines are associated with the pathogenesis of DR [16]. It has been reported that the levels of chemokines secreted by macrophages such as monocyte inflammatory protein-1 α (MIP-1 α), MIP-1 β and MCP-1 are significantly increased in diabetic patients. In diabetic mice, it has been shown that MCP-1 deficiency might result in decreased retinal vascular leakage [17]. In addition, the expressions of serum inflammatory factors, for example tumor necrosis factor α (TNF- α), interleukin 6 (IL-6), IL-8 and IL-1 β are significantly upregulated in diabetic patients, and it has been demonstrated that the increased levels reflect the severity of DR [18].

Microglia are activated by high-glucose stress, which results in increased secretions of TNF- α , IL-6, MCP-1, and VEGF. Consistent with the results of this study, the expressions of the angiogenesis-related factors VEGF and ICAM1 in the HG group were significantly higher than those in the NG culture group. Moreover, the expression levels of angiogenesis-related factors

in HG + BMP-2 group were higher than their corresponding expression levels in the HG group. Results from qRT-PCR and ELISA showed that the expression levels of the pro-inflammatory cytokine IL-6 and the chemokine MCP1 in HG group were significantly higher than those in NG group, but were lower than those in the HG + BMP-2 group.

Retinal neurodegeneration is an initial stage in the development of DR [19]. In animal models, apoptosis of retinal nerve cells manifest in diabetic rats 30 days after diabetes induction [20]. Increasing evidence suggest that retinal neurodegeneration is likely to be an independent pathological basis of DR [21]. In diabetic patients, internal retinal thinning was checked out, but without DR or mildest DR (microaneurysm) [22]. In the present study, under the condition of HG, investigations were carried out on the effect of BMP-2 on the biological functions of hREC and ARPE-19 cells. The results indicate that in hREC cells, the proliferation of cells and expressions of angiogenesis and inflammatory factors were enhanced by BMP-2 partially through the Smad signaling pathway, while the expressions of angiogenesis and inflammatory cytokines were upregulated by BMP-2 entirely through the Smad signaling pathway in ARPE-19 cells. Therefore, further studies on the molecular mechanism of retinal neurodegeneration and the signal mechanism related to BMP-2 will be very valuable in the development of early treatment strategy for DR.

The application of corticosteroids seems more significant in the treatment of DME, particularly in patients with refractory DME and more serious adverse reactions of anti-VEGF treatment. As effective anti-inflammatory drugs, corticosteroids target a wide range of mediators involved in the pathogenesis of DME. These include VEGF, TNF- α , chemokine, leukocyte stagnation and phosphorylated tight junction protein. Unresponsiveness to anti-VEGF is mostly likely the result of a series of cytokines in DME cases. Corticosteroids are very efficacious anti-inflammatory drugs which target a wide range of mediators participating in the pathogenesis of DME, such as VEGF, TNF- α , leukocyte stasis and phosphorylated human occludin proteins [23,24]. Interestingly, vitreous implants may induce dislocation of the anterior chamber, but this could be treated by injecting the implant with balanced saline solution [25].

Intravitreal corticosteroid therapy is effective for DME. In particular, lower frequency intraocular injections and sustained release of corticosteroids reduce costs and achieve better

patient compliance. However, since the effectiveness of corticosteroid therapy for PDR has not been defined, it should be applied as first-line therapy only when anti-VEGF drugs present a high risk [26]. At the same time, as one of the most important pro-inflammatory cytokines in the vitreous tissue of DR patients, IL-6 has been studied as a prospective target for the anti-inflammatory treatment of DR [27].

CONCLUSION

The results obtained in this study indicate that BMP-2 promotes angiogenesis and inflammatory cytokine expressions via a mechanism that partially depends on the Smad signaling pathway. These findings have beneficial potentials in the management of DR.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. All authors read and approved the manuscript for publication. Zaohe Sun and Guangming Wan conceived and designed the study. Shenzhi Liang and Cheng Qian collected and analyzed the data, while Zaohe Sun wrote the manuscript.

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