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Original Research

Relationship between Erk1/2 signal pathway and nerve cell apoptosis rats with ischemic stroke

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Abstract: To investigate the relationship between the Erk1/2 signal pathway and neuronal apoptosis in ischemic stroke rats. Male SD(Sprague Dawley) rats (n = 24) were randomly divided into three groups, each containing 8 rats: sham-operated group, MCAO(Midle cerebral artery oclusion) group, and MCAO + U0126 intervention group (U0126 group). In *in vitro* trial, primary cortical nerve cells were divided into three groups: control group, OGD(Oxygen and glucose deprivation) group, and U0126 intervention group (U0126 group). *In vivo* protein expression levels of Erk1/2, p-Erk1/2 and Bcl-2 were determined using western blot. The expressions of Bcl-2, Bcl-xl and Bax were assayed using immunohistochemical staining. Nerve cell mortality in cerebral tissue was detected using TUNEL staining. In *in vitro* trials, cell apoptosis was assayed with flow cytometry and LDH release. The activity of caspase-3 was determined. Nerve cell apoptosis was determined using Hoechst33258 staining method. In *in vivo* trial, it was found that the protein expression level of p-ERK1/2 in cerebral tissue in the MCAO group was significantly increased, when compared with that of the sham-operated group, while the protein expression level of p-Erk1/2 in the U0126 group was significantly lower than that in the MCAO group. The expression levels of Bcl-2 and Bcl-xl in the MCAO group were significantly lower than the corresponding expression levels in the sham-operated group, while the expressions of Bcl-2 and Bcl-xl in the U0126 group were significantly lower than those in MCAO group. In MCAO group, the expression of Bax was significantly higher than that in the sham-operated group, while Bax expression was higher in U0126 than in MCAO group. There were significantly higher number of dead nerve cells in MCAO group than in the sham-operated group, while nerve cell mortality in U0126 group was significantly lower than in MCAO group. In *in vitro* trials, flow cytometry revealed significantly higher apoptosis of OGD-treated nerve cells, relative to the control group. Nerve cells exposed to U0126 and treated with ODR (Oxygen-dependent repressor) were significantly decreased in population, when compared with single OGD treatment group. The LDH release level of nerve cells treated OGD was significantly increased, when compared with that of the control group. However, LDH release level of nerve cells treated with OGD after U0126 intervention was significantly decreased, relative to the single OGD treatment group. The dilution of nerve cell nucleus after OGD treatment was significantly increased, when compared with that of the control group. For nerve cells treated with ODR after U0126 intervention, the nuclear dilution was significantly decreased, relative to that of nerve cell nucleus in the single OGD treatment group. The OGD treatment led to significant increase in nerve cell caspase-3 activity, relative the control group. However, the caspase-3 activity of nerve cells treated with ODR after U0126 intervention was significantly decreased, when compared with single OGD treatment group. The activation of Erk1/2 signal pathway during ischemic stroke promotes apoptosis of nerve cells. Based on these findings, it can be reasonably inferred that the ERK1/2 signal pathway may be an important target for treating ischemic stroke.

Key words: Erk1/2 signal pathway; Apoptosis of nerve cells; OGD; ODR; U0126.

Introduction

Ischemic stroke is one of the most common neurological diseases. It is characterized by irregular blood supply to the brain as a result of cerebral artery occlusion. Ischemic stroke is one of the major causes of increasing mortality and disability all over the world, especially in patients aged over 60 years (1). The pathophysiological mechanisms associated with cerebral ischemic injury are complex, and involve a variety of processes such as oxidative stress, apoptosis, as well as excitotoxicity and inflammatory mechanisms (2). Insufficient blood supply brings about neuronal damage in the ischemic area. In the reperfusion process after middle cerebral artery occlusion, the brain is re-oxygenated and infused with glucose, which lead to the generation of free radicals and reactive oxygen species, and apoptosis of cells in cerebral ischemia area, resulting in increased oxidative stress response (3). Cell death induced by ischemic stroke was initially thought to be caused almost entirely by the necrotic process in which ischemia leads to cellular changes, swelling of organelles, rupture of the plasma membrane, and release of intracellular substances. These features of cell death usually occur in a vast majority of cells in ischemic stroke. However, in the mid-1990s, a more complicated picture emerged which identified cells with apoptotic characteristics, including DNA fragments and presence of membrane-bound apoptotic bodies (4). The population of nerve cells with apoptotic characteristics usually peak 24 h or more after a stroke. They are distributed throughout the entire infarction area after transient or permanent occlusion of blood vessels, but are more obvious in tissues with less ischemia, and more common in the brain with

transient ischemia lasting for 2 h (5). Changes following cerebral ischemic injury include increased intracellular calcium ions and oxidative stress, which are considered as critical and potential factors for nerve cell apoptosis. Cerebral ischemia triggers on a series of molecular cascade responses that makes enables the brain to adapt to ischemic injury. The Erk1/2 signal pathway is an important link in these molecular cascade responses (6). Studies have shown that the Erk1/2 signal pathway is activated in the forebrain or in focal cerebral ischemia, and other studies have found that this signal pathway promotes neuronal survival (7, 8). Research has shown that oligodendrocytes are targets of ischemic injury. This may be associated with Erk1/2 which is known to be involved in the regulation of ischemic neuron injury and survival. The Erk1/2 pathway exits in neurons and in oligodendrocytes of ischemic brain. In previous studies, this signal pathway was thought to play an important role in ischemic stroke, but its effect on apoptosis of nerve cells in ischemic stroke, and the underlying mechanism remain unclear(9).

Therefore, this study aimed to investigate the relationship between ERK1 / 2 signaling pathway and neuronal apoptosis in ischemic stroke.

Materials and Methods

Experimental animals

This study used male Sprague-Dawley (SD) rats weighing 280 to 320 g. The rats were purchased from the animal center of the Chinese Academy of Sciences (CAS), and were maintained at temperatures between 20 to 24°C, with humidity in the range of 60 to 70 % under 12 h light/12 h dark cycle. The rats were provided feed and drinking water *ad libitum*.

Establishment of ischemic stroke model rats in vivo and in vitro

This study used middle cerebral artery (MCAO) in the left brain to induce focal cerebral stroke in the rats. The rats were fasted for 10 h before surgery and anaesthetized with intraperitoneal injection of chloral hydrate at a dose of 300 mg/kg. Surgical nylon sutures (diameter = 0.26mm; Beijing Shadong Biotech Co., Ltd, Beijing) were used to block middle artery in the left brain. The sham-operated group was similar to the MCAO group, except that the left middle cerebral artery was not occluded. Rats in the U0126 intervention group were given U0126 (5 μl, 0.2 μg/μl) Sigma, America), or carrier (0.1 PBS containing 0.4% dimethyl sulfoxide) 30 min before ischemia via intraventricular injection. In the whole operation process, a heating pad was used to maintain the rectal temperature of the rats at 37°C. The final point of the trial was 48 h post-surgery. In *in vitro* trial, primary cortical neurons (OGD) were cultured and used to treat the cells. Twenty-four hours later, the cells were treated with U0126 (10μM), followed by treatment with OGD. The cells were collected 48 h after the treatment.

Experiment group

A total of 24 rats were randomly divided into three groups, with 8 rats per group: sham-operated group, MCAO group, and MCAO+ U0126 intervention group (U0126 group), using random number table. For *in vitro* studies, primary cortical neurons were divided into three groups: control group, OGD group and U0126 intervention group (U0126 group).

Western blot assay

The total proteins around the infarction and cerebral cortex in rats in the sham-operated group were extracted using protein lysates. The protein samples were separated using 12 % SDS-PAGE and transferred to polyvinylidene fluoride film in transfer buffer solution containing 0.1% SDS (Roche, Germany). Subsequently, 5 % skim milk powder was added to block non-specific binding. Then, primary antibodies for ERK1/2 (1:1000), p-ERK1/2 (1:1000) and Bcl-2 (1:1000) were added to the membrane and incubated overnight at 4°C, with shaking on the shaker during the incubation. After three washes in TBST, the secondary antibody, peroxidase-conjugated goat anti-rabbit IgG (1:3000) was added, and incubation was carried out for 2 h at room temperature. Finally, chemiluminescent HRP substrates were used to observe the protein bands. Image J software was used to quantitatively analyze strip strength. The expressions were quantified relative to that of GAPDH (1:5000) which was used as internal reference. All the antibodies were products of American CST Company.

Immunohistochemistry

Animal experiment protocols were approved by the Ethics Committee for the Use of Experimental Animals of Yan'an University. At 48 h post-surgery, the rats were euthanized , and left brain cortex samples were collected (n=8), and fixed in 10 % neutral formalin buffer for 24 h. The tissues were treated, embedded in paraffin and cut into 10 μm slices using a cryostat. The slices were then subjected to immunohistochemistry. In essence, the slices were dewaxed and hydrated, and were first blocked with 8 % goat antiserum for 1 h at room temperature, and washed thrice with PBS after discarding the antiserum. Then, the slices were incubated with anti-Bcl-2 antibody (1:200), anti-Bcl-xl antibody (1:200) and anti-Bax antibody (1:200) for 24 h at 4°C, after which the primary antibodies were discarded, and the slices were washed thrice with PBS, followed by incubation with secondary antibody at 37 °C for 30 min. Then, the sheets were mounted and examined under a microscope.

TUNEL staining

The dUTP nick end labeling (TUNEL staining) step of the terminal deoxynucleotidyl transferase was carried out in accordance with the method of Lee *et al*. Sections (n=8) were stained using an *in situ* apoptosis assay kit (Roche, Germany). The TUNEL staining was performed from each selected rat section. Paraffin sections were dewaxed, hydrated and incubated in proteinase K(purchased from Sigma Aldrich) solution for 15 min. After washing in the equilibration buffer, each TdT reaction mixture was incubated in the dark for 1 h at room temperature. Then, it was incubated with stop solution at 37 °C for 30 min to terminate the reaction. Thereafter, the section was washed with PBS and cut into slices which were further incubated for 30 min. After washing with PBS, the slices were counterstained with 4'-6-diamino-2-phenylindole (DAPI ,purchased

from Sigma Aldrich) for 20 min. The number of neurons in the pyramidal cell layer (length was 400 μm) was counted at 37°C. The CA1 region was analyzed using a fluorescence microscope equipped with Image Pro software (Media Cybernetics, USA). The number of neuronal deaths was determined by comparing the number of TUNEL-positive neurons with the number of DAPI-positive neurons. The number of TUNEL positive cells from three random 1x1 mm^2 regions was calculated.

Determination of cell apoptosis and LDH release

Eight h ours **(**8h) after cell treatment with OGD, trypsin (2.5g/L) was used to digest the OGD-treated neurons. The digest was subjected to centrifugation at 1000 rpm for 10 min at room temperature. Then, the cells were re-suspended and incubated with Annexin V-FITC/propidium iodide (PI) for 30 min at 4 °C. The degree of cell apoptosis was quantitatively measured using flow cytometry Facscanto (Becton Dickinson) as described earlier. The degree of cell injury was measured using LDH release. According to previous studies, LDH assay kits(purchased from Haimen, China) in culture medium were used to detect release of LDH. After OGD treatment for 8 h, the amount of LDH released was measured spectrophotometrically at 492 nm (BioRad) after subtracting the background absorbance at 620 nm.

Assay of caspase-3 activity and Hoechst33258 staining

The enzymatic activity of caspase-3 was measured using a caspase-3 fluorescence assay kit (R&D, USA) according to the kit instructions. The medium was aspirated, washed with PBS, and the neurons on the plate were scraped off, and centrifuged at 4 °C for 10 min to precipitate the cells. The supernatant was gently discarded and dissolved (on ice for 10 min) in a lysate containing 20 mM Tris-HCl (pH 7.2), 1% Triton-X, 100 mM NaCl, 1 mM dithiothreitol. The cell lysate was cultured and then centrifuged at 4 °C for 10 min at 13000 g. Protein levels in the supernatant were quantified using BCA Protein Assay Kit (Pierce Manufacturing Inc., USA). Then, 100 μg of each protein sample was loaded into a 96-well plate and incubated with 10 ml of caspase-3 substrate provided in the kit for 2 h at 37 °C in the dark. Fluorescence was measured using a fluorescent microplate reader at excitation and emission wavelengths of 400 and 505 nm, respectively. The activity of caspase-3 was expressed as increase in the fluorescence intensity of the treated sample relative to the control group. After appropriate treatment, the cells were fixed with 4 % paraformaldehyde for 15 min at room temperature, washed 3 times with PBS, stained with Hoechst 33258 staining solution for 5 min, and then observed under a fluorescence microscope (Olympus, Japan). Apoptosis was quantified based on the appearance of split or concentrated nuclei.

Statistical analysis

Each experiment was repeated at least three times. The data are presented as mean ± S.E.M. One-way ANOVA was used for comparison between groups. The comparison between the groups was performed using Turkey test. Statistically significant difference between mean values was assumed at $p < 0.05$.

Results

Levels of sERK1/2 pathway-relevant protein expressions in cerebral cortex tissue of rats

The relative expression level of p-Erk1/2 in MCAO rats was significantly higher than that in the sham operation group $p < 0.01$), while the relative expression level of p-Erk1/2 in MCAO rats after U0126 treatment was significantly lower than that in single MCAO group ($p < 0.01$; Figures 1A and 1B) . The relative expression level of Bcl-2 in the MCAO group was significantly lower than that in the sham operation group, while the expression level of Bcl-2 in MCAO group treated with U0126 before surgery was significantly higher than that in the MCAO group ($p < 0.05$; Figures 1A and 1C). There were no significant differences in Erk1/2 protein expression levels amongst the three groups (Figure 1A). These results suggest that the Erk1/2 pathway is activated in MCAO-operated rats, and that this activation can be blocked by U0126.

Levels of Bcl-2, Bcl-xl and Bax in rat cerebral cortex

The level of Bcl-2 in brain tissue around the cerebral infarction in MCAO rats was significantly lower than that in the sham operation group, while the level of Bcl-2 in the MCAO group before U0126 treatment was significantly higher than that in MCAO surgery group alone ($p < 0.05$; Figures 2A and 2B). The Bcl-xl level in the brain of MCAO group was significantly lower than

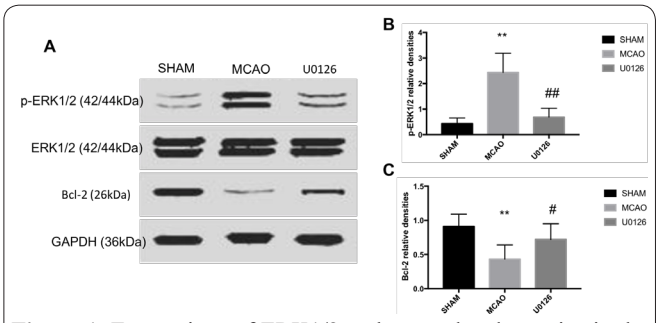

Figure 1. Expressions of ERK1/2 pathway-related proteins in the cerebral cortex of SHAM, MCAO and U0126 groups. A: Western blot analysis of protein electrophoresis. B-C: quantification of the A protein band.**,P<0.01,vs SHAM group; #,p<0.05,vs MCAO group; ##,p<0.01,vs MCAO group.

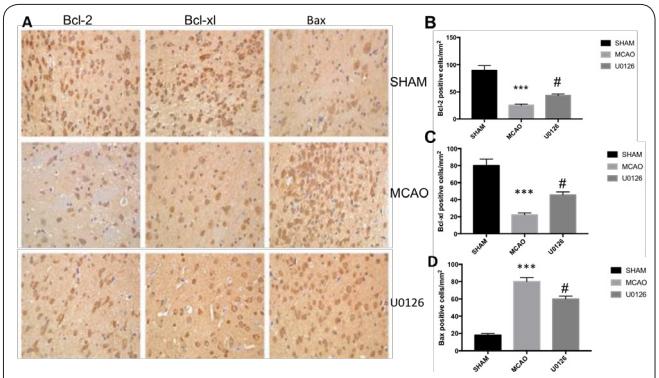

Figure 2. Results of immunohistochemical staining of Bcl-2, Bcl-xl, and Bax in rat cerebral cortex. A: Immunohistochemical staining of cerebral cortex tissue; B-D: quantification of proportion of positive expressions.***,P<0.001,vs SHAM group; #,p<0.05,vs MCAO group.

that in the sham operation group ($p < 0.001$). However, Bcl-xl level in the MCAO group after U0126 intervention was significantly higher than that in the MCAO group alone ($p < 0.05$; Figures 2A and 2C). The Bax level in brain tissue around the cerebral infarction area in MCAO rats was significantly higher than that in control group ($p < 0.001$), while Bax level in U0126 group was significantly lower than that in the MCAO group ($p < 0.05$; Figures 2A and 2D).

Effect of MCAO surgery and U0126 on neuronal apoptosis in rat cerebral cortex

The degree of neuronal apoptosis in the brain tissue around the cerebral infarction in MCAO rats was significantly higher than that in the sham operation group ($p < 0.05$), and the number of apoptotic neuronal cells in rats injected U0126 prior to MCAO surgery was significantly decreased, when compared to MCAO group without U0126 ($p < 0.05$; Figures 3A -3G). The population of TUNEL-positive nerve cells in the MCAO group were significantly higher than that in the control group, but was decreased in the U0126 group.

Primary neuronal apoptosis and LDH release

There was significantly higher level of apoptosis in ischemic nerve cells treated with OGD than in the control group ($p < 0.01$). After U0126 intervention, the percentage of apoptosis in neurons treated with OGD was significantly decreased ($p < 0.01$; Figure 4A). As shown in Figure 4B, the LDH release level in OGD-treated nerve cells was significantly higher than that in the control group, and the degree of LDH release in ODR-treated neurons after U0126 intervention was significantly lower than that in the OGD-treated group ($p < 0.01$).

Primary nerve cell Hoechst33258 staining and caspase-3 activity assay

The nuclear concentration of nerve cells after OGD treatment was significantly higher than that of the control group ($p < 0.01$). However, after U0126 intervention, the nuclear concentration in ODR-treated nerve cells was significantly lower than that of the OGD-treated group ($p < 0.01$; Figures 5A and 5B). The activity of caspase-3 in OGD-treated neurons was significantly higher than that in the control group ($p < 0.01$). In contrast, caspase-3 activity in ODR-treated neurons after U0126 intervention was significantly lower than that in the OGD-treated group ($p < 0.05$; Figure 5C).

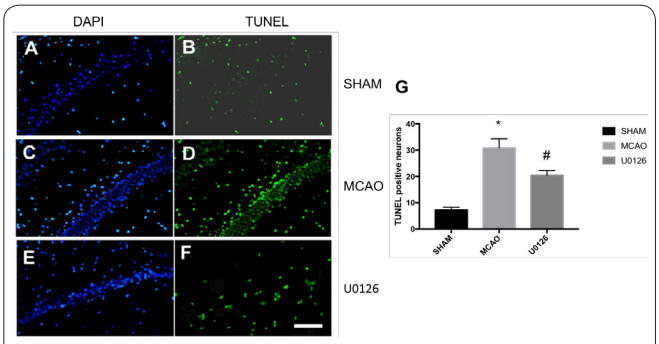

Figure 3. TUNEL staining of rat cerebral cortex. A-F: TUNEL and DAPI staining; G: Quantification of the number of TUNEL-positive cells in A-F.*,P<0.05,vs SHAM group; #,p<0.05,vs MCAO group.

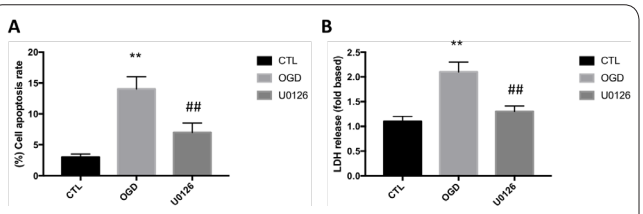

Figure 4. Degree of neuronal apoptosis after OGD treatment *in vitro*. A: Flow cytometry quantitative analysis of neuronal apoptosis. B: Quantitative analysis of LDH release .**,P<0.01,vs SHAM group; ##,p<0.01,vs MCAO group.

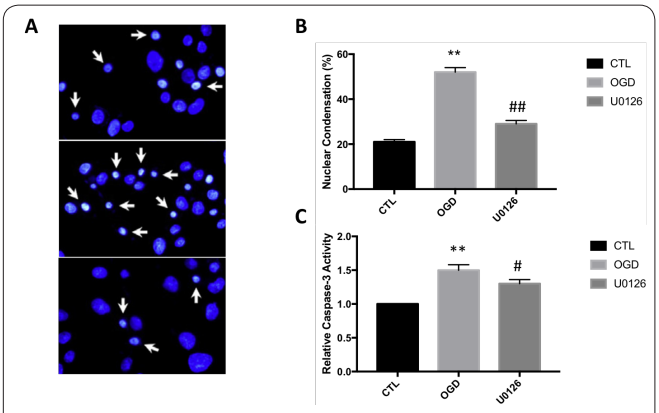

Figure 5. Results of neuronal Hoechst33258 staining and caspase-3 activity assay. A: Hoechst33258 stained cell morphology. B: Quantitative analysis of relative proportion of nuclear concentration; C: quantitative analysis of relative caspase-3 activity.**,P<0.01,vs SHAM group; #,p<0.05,vs MCAO group; ##,p<0.01,vs MCAO group.

Discussion

Ischemic stroke is characterized by impairment of blood circulation to the brain due to occlusion of the cerebral arteries. It is a widespread neurological disorder (2). Apoptosis is an important feature of cerebral ischemic stroke which plays a key role in the pathogenesis of advanced infarction. After ischemia or reperfusion (24 to 72 h), activated caspase-2 is up-regulated, leading to cellular DNA break and nuclear condensation (10). The Bcl-2 protein is an important factor in the intrinsic pathway of apoptosis, and it regulates apoptosis of vascular cells. The Bcl-2 protein family is composed of the anti-apoptotic protein Bcl-2 and the pre-apoptotic protein Bim, which play an important role in the mitochondrial apoptosis pathway (11). It has been reported that apoptosis may be negatively correlated with the expression of Bcl-2 (12). In the present study, it was found that the protein expression levels of Bcl-2 and Bcl-xl in ischemic stroke rats were significantly lower than those in the control group, suggesting increased neuronal apoptosis in ischemic stroke rats. This increase was alleviated by specific inhibitors of the Erk1/2 pathway, suggesting that increased neuronal apoptosis induced by ischemic stroke is likely to be associated with the Erk1/2 pathway. The ERK pathway, being an important member of the MAPK signal family, is primarily involved in apoptosis, cell proliferation and differentiation (13). Previous studies have shown that Erk1/2 is essential for the recovery of myelin and the transition from oligodendrocyte precursors to mature oligodendrocytes (14). In addition, it has been revea-

led that inhibition of P-Erk1/2 suppresses the transition and myelin recovery during development (15). The Erk signal pathway is involved in the sequential promotion of Raf, Erk, p38MAPK and other related proteins (16). The activation of Erk1/2 is important in several neuronal death models. Studies have shown that Erk1/2 was overexpressed in ischemic stroke, and that inhibition of Erk1/2 pathway reduced focal infarct volume and brain damage in ischemic stroke mice (17).

Phosphorylated Erk1/2 may cause neuronal damage after ischemia. Inhibition of pErk1/2 following ischemic stroke may provide neuroprotection against cerebral ischemic injury (18). However, it has also been suggested that phosphorylation of Erk after ischemic stroke may support neuronal survival (19). Therefore, in the present study, an attempt was made to investigate whether Erk1/2 activation was associated with neuronal protection or neuronal death. *In vivo* studies revealed that Erk1/2 was activated in the brain tissue of ischemic stroke rats, and the expression of phosphorylated Erk1/2 was significantly increased. At the same time, there was an increase in the population apoptotic neuronal cells during ischemic stroke. Furthermore, there was a decrease in the number of apoptotic cells following U0126 intervention for specific inhibition of the Erk1/2 pathway. In *in vitro* experiments, a similar phenomenon was revealed. In *in vitro* simulated neuronal ischemic stroke, the population of apoptotic neuronal cells was significantly increased, as revealed through LDH release assay, as well as Hoechst33258 staining and caspase-3 activity assays. The inhibition of Erk signaling by U0126 attenuated caspase-3 activation and apoptotic cell death. Based on the results of *in vitro* and *in vivo* experiments, it can be reasonably speculated that the Erk1/2 pathway is activated during ischemic stroke, thereby promoting neuronal apoptosis.

The results obtained in the present study suggest that the activation of the Erk1/2 signal pathway during ischemic stroke promotes neuronal apoptosis. Based on these findings, it can be speculated that the Erk1/2 signal pathway may be an important target for the treatment of ischemic stroke.

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None.

Conflict of interest
There are no conflict of interest in this study.

Author's contribution
All work was done by the author s named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Ning Shi; Xiaorong Gao, Haijun Li, Zhiyan Dong, Pengxuan Zhang, Rui Liu, Yidong Xue, Ning Shi collected and analysed the data; Xiaorong Gao and Haijun Li wrote the text and all authors have read and approved the text prior to publication.

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