Effect of β-patchoulene on cerebral ischemia-reperfusion injury and the TLR4/NF-κB signaling pathway

FU-BO ZHANG¹, JIAN-PING WANG¹, HONG-XIA ZHANG¹, GUI-MEI FAN¹ and XIN CUI²

Departments of ¹Neurology and ²Rheumatology, Cangzhou Central Hospital, Cangzhou, Hebei 061001, P.R. China

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Abstract. β-patchoulene (β-PAE), an active constituent of the Pogostemon cablin, is well known for its anti-inflammatory and antioxidative functions in various diseases. However, little is known about the impact of β-PAE on the cerebral ischemia-reperfusion (I/R) injury. The current study aimed to determine the neuroprotective effect of β-PAE and the underlying mechanisms on cerebral I/R injury. Following pretreatment with β-PAE (10 mg/kg body weight) by tail intravenous injection for 1 h, Sprague-Dawley rats were subjected to middle cerebral artery occlusion for 2 h and reperfusion for 24 h. The results indicated that pretreatment with β-PAE could diminish the infarct volume, decrease the brain water content, reduce the neurological deficit score and restore the mitochondrial membrane potential, compared with the untreated I/R injury group. Furthermore, cell apoptosis was markedly suppressed by β-PAE, and this effect was associated with the decreased apoptosis regulator BAX/apoptosis regulator Bcl-2 expression ratio and caspase-3 activity. In addition, β-PAE significantly inhibited the release of proinflammatory factors, including tumor necrosis factor-α, interleukin (IL)-1β and IL-6. Superoxide generation and malondialdehyde levels were reduced while the levels of glutathione peroxidase and superoxide dismutase were elevated following treatment with β-PAE, indicating the antioxidative role of β-PAE in cerebral I/R injury. Furthermore, the Toll-like receptor 4 (TLR4)/nuclear factor-κB (NF-κB) signaling pathway was inhibited by β-PAE, as demonstrated by the decreased TLR4 expression and nuclear translocation of p65, and increased IkBα level. Taken together, the results suggested that β-PAE may exhibit a neuroprotective effect on cerebral I/R injury in rats through inactivating the TLR4/NF-κB signaling pathway.

Introduction

Ischemic stroke, the leading cause of mortality and long-term disability among patients with stroke, accounts for 85% of all stroke cases (1). The occlusion of a blood vessel caused by the formation of thrombus or embolus in the brain are the most common processes resulting in ischemic stroke (2). For patients with ischemic stroke, the most effective therapy is to restore the blood circulation as soon as possible (3). However, reperfusion is associated with potential risks, including cerebral infarction, cerebral edema, nerve function deficit and cerebral microcirculatory damage, collectively known as cerebral ischemia/reperfusion (I/R) injury (4). Previous studies have indicated that cerebral I/R injury is accompanied by a series of mechanisms, including inflammation, oxidative stress and apoptosis, which serve roles in the subsequent disability and mortality (5,6). Therefore, alleviation of the inflammatory response, oxidative stress and cell apoptosis is one of the most important approaches to promote the recovery from the ischemic stroke.

β-patchoulene (β-PAE; C₁₅H₂₃) extracted from the Pogostemon cablin is an active natural tricyclic sesquiterpene (7). The results obtained in the current study demonstrated that β-PAE may exhibit a potent anti-inflammatory effect as previously reported (8). Furthermore, a previous study indicated that β-PAE may serve as an antioxidant in a mouse model (9). The above results indicated that β-PAE may be used for the treatment of cerebral I/R injury.

Toll-like receptor 4 (TLR4), a germline-encoded pattern recognition receptor, serves a role in the regulation of inflammation (10). As an important target gene of TLR4, nuclear factor-κB (NF-κB) regulates the production of inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6 at the transcriptional level (11). A previous study revealed that the TLR4/NF-κB signaling pathway was strongly activated during the development of cerebral I/R injury (12). Therefore, suppressing the activity of the TLR4/NF-κB signaling pathway represents a potential neuroprotective therapeutic strategy for the treatment of...
cerebral I/R injury. A previous study indicated that β-PAE may inhibit the NF-κB pathway in a number of ways (9). Therefore, β-PAE may serve a protective role in cerebral I/R injury by inactivating the NF-κB pathway.

The current study used a rat model of middle cerebral artery occlusion (MCAO) to study the effect of β-PAE on cerebral I/R injury and investigate the underlying mechanisms.

Materials and methods

Experimental animals. A total of 32 specific pathogen free male Sprague-Dawley rats (age, 8 weeks; weight, 80-120 g) were obtained from Experimental Animal Center of Hebei Province (Shijiazhuang, China) and housed in a controlled environment at 25±3°C and 60% humidity, in a 12-h light/dark cycle with free access to food and water. The experiment was approved by The Ethics Committee of Cangzhou Central Hospital (Cangzhou, China). All rats were maintained in a specific pathogen free environment with free access to food and water for 7 days.

Group allocation and the animal model of focal cerebral ischemia. β-PAE was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany; purity >97%). All rats were randomly divided into four groups (n=8/group): Sham, β-PAE, I/R and I/R + β-PAE. Prior to surgery, rats in the β-PAE and I/R + β-PAE groups were pretreated with β-PAE (10 mg/kg body weight in normal saline) by tail intravenous injection. The dose of 10 mg/kg of β-PAE was selected based on previously published data. Liu et al (13) indicated that high dose (100 µmol/l) of β-PAE resulted in significant cytotoxicity to GES-1 cells. Furthermore, according to previous reports, 10 mg/kg of β-PAE may exert protective effects on a number of pathological processes, including acute lung injury (14), gastric ulcer (7) and inflammatory disorders (9). Following injection for 1 h, the focal cerebral ischemia injury model was generated in the I/R and I/R + β-PAE groups through thread embolism as previously described (15). Rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (30 mg/kg body weight; Sigma-Aldrich; Merck KGaA) prior to MCAO. Rats were euthanized by intraperitoneal injection of pentobarbital sodium (200 mg/kg body weight) prior to collecting the brain tissues. The external carotid artery and left common carotid artery were exposed via a midline neck incision. A 6-0 nylon monofilament was used to occlude the origin of the MCA and block the blood flow. After 2 h of occlusion, reperfusion was accomplished by filament withdrawal, and the incision was sutured. For sham and β-PAE group rats, external carotid artery and left common carotid artery were exposed without MCA occlusion. During surgery, the internal temperature of rats was maintained at 37±0.5°C using a heating pad. Rats were used for subsequent experiments after 24 h.

Measurement of the brain infarct volume. The cerebral infarct volume was assessed using the 2,3,5-triphenyltetrazolium chloride (TTC; Sigma-Aldrich; Merck KGaA) staining. Briefly, brain tissues were isolated from rats (n=8) and fixed in 4% buffered paraformaldehyde at 4°C for 20 min, embedded in paraffin and 3-µm-thick slides were cut. Following dewaxing, slides were stained with 2% TTC for 30 min at room temperature, followed by fixation with 10% formaldehyde neutral buffer solution overnight at 4°C. Red staining was observed in the normal healthy areas and the infract areas were unstained. Infarct volume was evaluated using the ‘Count/Size’ tool of Image-Pro software (version 6.0; Media Cybernetics, Inc., Rockville, MD, USA).

Brain water content. The brain of each rat was isolated gently and rapidly within <2 min. Each brain was weighted immediately using an electronic balance (Sartorius AG, Göttingen, Germany). Subsequently, brains were dried at 100°C for 24 h in an oven and weighed again. Brain water content was calculated using the following equation: (Wet weight-dry weight)/wet weight x100%.

Modified Neurological Severity Scores (mNSS). Neurological deficit was measured using mNSS as previously described (16). Higher scores indicated increased severity of the injury. The following scoring criteria were used: Score 0, normal behavior; no observable neurological deficits; score 1, failure to extend contralateral forelimbs; score 2, contralateral circling when walking; score 3, contralateral falling over when walking; score 4, inability to walk and no spontaneous motor activity.

Detection of mitochondrial membrane potential (MMP). Chondriosomes were extracted from the hippocampi in each group as previously described (17). Briefly, the hippocampus was isolated from each brain gently and homogenized using a Teflon glass homogenizer at 0°C in a mixed medium including mannitol, 220 mM; sucrose, 70 mM; 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid, 2 mM; EGTA, 0.2 mM and bovine serum albumin (BSA; Sigma-Aldrich; Merck KGaA), 0.5 mg/ml; pH 7.2. The samples were centrifuged twice and the mitochondrial sediment was washed twice with the isolation medium (Sangon Biotech Co., Ltd., Shanghai, China) for 10 min at 8,000 x g at 4°C. The mitochondrial pellet was re-suspended in cold isolation medium without EGTA. Mitochondrial permeability transition pore (MPTP) was detected using the MMP colorimetric method kit (cat. no. C2006; Beyotime Institute of Biotechnology, Haimen, China) with a microplate reader at a wavelength of 450 nm.

Flow cytometry assay. Rats (n=8) in each group were euthanized by intraperitoneal injection of pentobarbital sodium (200 mg/kg body weight) and subjected to perfusion prior to brain collection. Heparin (0.01%) was injected into the tail vein of the rats. Subsequently, brains were lavaged through a 300-µm nylon mesh and centrifuged at 3,000 x g. The samples were centrifuged twice and the mitochondrial sediment was washed twice with the isolation medium (Sangon Biotech Co., Ltd., Shanghai, China) for 10 min at 8,000 x g at 4°C. Mitochondrial permeability transition pore (MPTP) was detected using the MMP colorimetric method kit (cat. no. C2006; Beyotime Institute of Biotechnology, Haimen, China) with a microplate reader at a wavelength of 450 nm.
Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay. The mRNA expression levels of TNF-α, IL-1β and IL-6 were measured using RT-qPCR. Total RNA was extracted from brain tissues with TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The extracted RNA was reverse transcribed into cDNA using the ThermoScript™ RT-PCR system at 40˚C for 2 h (Invitrogen; Thermo Fisher Scientific, Inc., USA). The reaction was performed at 37˚C for 30 min, followed by 42˚C for 30 min and 85˚C for 5 min. Transcripts were quantified by qPCR using a Bio-Rad CFX96 real-time PCR detection system with SYBR-Green IQTM (Thermo Fisher Scientific, Inc.). The reaction was performed at 95˚C for 3 min, followed by 40 cycles of 95˚C for 12 sec and 60˚C for 40 sec. An internal control (GAPDH) was used to normalize the expression of the target genes. Quantification of TNF-α, IL-1β and IL-6 levels was conducted using the CFX96™ Real-Time PCR detection system (Bio-Rad Laboratories, Inc.). The primers were purchased from Sangon Biotech Co., Ltd. (Nanjing, China). The primers were designed using Primer Premier 5.0 software (PremierBioSoft International, USA) and were synthesized by Sangon Biotech Co., Ltd. (Nanjing, China). The primer sequences were as follows: TNF-α forward, 5'-ACA TTC CCA CTG ACC TTTGCTTC-3'; reverse, 5'-TCACCCATCCCA TCTTCTCCTC-3'; IL-1β forward, 5'-CAGCCAGCCAGTATC ACTCA-3' and reverse, 3'-AGGCCACAGGTATTTTGTGC-5'; and IL-6 5'-forward, GGGCGATCGGATTTGTGTGAT-3' and reverse, 3'-GGACCCCGACAGATCGGGT-5'. The relative expression of the test gene was calculated using the 2^ΔΔCq method (20).

Caspase-3 activity measurement. Caspase-3 colorimetric assay kit (cat. no. 610322; BD Biosciences, San Jose, CA, USA) was used to determine the caspase-3 activity in brain tissues according to the manufacturer's protocol. The results were evaluated using a microplate reader (Bio-Rad Laboratories, Inc.) at a wavelength of 405 nm.

Measurement of mitochondrial superoxide generation. Mitochondrial superoxide generation was measured by superoxide flashes in single mitochondria in living brain cells extracted from rats in each group as previously described (21). At least 20 brain cells/culture were evaluated in each group.

**Results**

β-PAE alleviates cerebral I/R injury. The structure of β-PAE is presented in Fig. 1A. To investigate the effect of β-PAE on cerebral I/R injury, rats with cerebral I/R were treated with β-PAE. The results indicated that treatment with β-PAE had no impact on the infarction volume (Fig. 1B), brain water content (Fig. 1C), neurological function (Fig. 1D) and MMP (Fig. 1E) of rats without I/R injury. However, the elevated infarct volume, brain water content, mNSS and MMP induced by I/R injury were significantly suppressed by treatment with β-PAE (Fig. 1). These results indicated that β-PAE markedly attenuate the cerebral I/R injury.

β-PAE decreases the I/R-induced cell apoptosis. To measure the effect of β-PAE on the apoptosis of brain cells, cell apoptosis rate and the expression levels of associated proteins were measured. Cell apoptosis rate (Fig. 2A), Bax/Bcl-2 ratio (Fig. 2B) and caspase-3 activity (Fig. 2C) were not altered in the β-PAE group compared with the sham group. However, cell apoptosis, Bax/Bcl-2 ratio and caspase-3 activity were significantly decreased in the I/R + β-PAE group compared with the I/R group (Fig. 2). These results indicate that β-PAE may markedly decrease brain cell apoptosis in rats with cerebral I/R injury.

β-PAE reduces the inflammatory response and oxidative stress. To determine whether β-PAE was associated with the inflammatory response and oxidative stress induced by I/R injury in brain tissues, the relevant inflammatory factors and oxidative stress parameters were evaluated using the CFX96™ real-time PCR detection system with SYBR-Green IQTM (Thermo Fisher Scientific, Inc.). The reaction was performed at 95˚C for 3 min, followed by 40 cycles of 95˚C for 12 sec and 60˚C for 40 sec. An internal control (GAPDH) was used to normalize the expression of the target genes. Quantification of TNF-α, IL-1β and IL-6 levels was conducted using the CFX96™ Real-Time PCR detection system (Bio-Rad Laboratories, Inc.). The primers were purchased from Sangon Biotech Co., Ltd. (Nanjing, China). The primers were designed using Primer Premier 5.0 software (PremierBioSoft International, USA) and were synthesized by Sangon Biotech Co., Ltd. (Nanjing, China). The primer sequences were as follows: TNF-α forward, 5'-ACA TTC CCA CTG ACC TTTGCTTC-3'; reverse, 5'-TCACCCATCCCA TCTTCTCCTC-3'; IL-1β forward, 5'-CAGCCAGCCAGTATC ACTCA-3' and reverse, 3'-AGGCCACAGGTATTTTGTGC-5'; and IL-6 5'-forward, GGGCGATCGGATTTGTGTGAT-3' and reverse, 3'-GGACCCCGACAGATCGGGT-5'. The relative expression of the test gene was calculated using the 2^ΔΔCq method (20).

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**Measurement of mitochondrial superoxide generation.** Mitochondrial superoxide generation was measured by superoxide flashes in single mitochondria in living brain cells extracted from rats in each group as previously described (21). At least 20 brain cells/culture were evaluated in each group.

**Determination of the oxidative damage parameters.** Brain tissues isolated from rats were homogenized in normal saline and centrifuged at 3,000 x g at 4˚C for 10 min. Supernatants were collected and the levels of malondialdehyde (MDA; cat. no. A003-1), superoxide dismutase (SOD; cat. no. A001-1-1) and glutathione peroxidase (GSH-Px; cat. no. A005) were determined according to the manufacturer's protocol of the corresponding detection kits (Nanjing Jiancheng Bioengineering Institute) using the UV/Vis Spectrophotometer (Beijing Lab Tech Instrument Co., Beijing, China). The absorbance was measured at a wavelength of 532, 420 and 340 nm for MDA, SOD and GSH-Px, respectively.

**Statistical analysis.** Data are presented as the mean ± standard deviation. One-way analysis of variance followed by Bonferroni's post hoc test was used to analyze the differences between groups using SPSS software (version 17.0; SPSS, Inc., Chicago, IL, USA OR SPSS). P<0.05 was considered to indicate a statistically significant difference.
oxidative damage parameters were detected. Results suggested that there were no marked differences in the expression levels of inflammatory factors, including TNF-α, IL-1β and IL-6, and oxidative damage parameters, including superoxide generation,
β-PAE inactivates the TLR4/NF-κB signaling pathway. To further investigate the underlying mechanisms of the effect of β-PAE on cerebral I/R injury, the activity of the TLR4/NF-κB signaling pathway was measured using western blotting. There was no difference in the TLR4 and IkBα expression in the cytoplasm and nucleus. No difference of the NF-κB p65 expression in the nucleus was observed between the β-PAE group and I/R + β-PAE group (Fig. 4). However, the total protein expression of TLR4 and nuclear expression of NF-κB p65 were suppressed while the total protein expression of IkBα and cytoplasmic protein expression of NF-κB p65 were increased in the I/R + β-PAE group compared with the I/R group. These results suggested that the TLR4/NF-κB pathway may be inactivated following treatment with β-PAE.

Discussion

Stroke imposes a significant socio-economic burden globally (22). At present, restoration of the blood supply is the only effective therapy available for patients with ischemic stroke (3). However, brain cell damage induced by cerebral ischemia may be further aggravated when the blood supply is recovered in the brain tissue, resulting in cerebral I/R injury (23). Uncontrolled cerebral I/R injury eventually exacerbates cerebral infarction, cerebral edema and nerve injury (24). Therefore, it is necessary to develop effective therapeutic methods to ameliorate cerebral I/R injury.

A number of studies indicated that numerous extracts from Chinese herbs serve positive roles in the treatment of I/R injury, including salvianolic acid B (25), trans-polydatin (26), vitexin (27) and triptolide (28). β-PAE, an active ingredient of Pogostemon cablin, exhibits a broad spectrum of pharmacological functions, including anti-inflammatory and antioxidative activities (4,13). Previous studies indicated that oxygen supply and neuroinflammation are the major causes of cell death and cerebral edema during the development of cerebral I/R injury (29). Therefore, β-PAE may be useful in the treatment of cerebral I/R injury. In the current study, pretreatment with β-PAE effectively reduced the infarct volume, decreased the brain water content, mitigated the neurological impairment and recovered the MMP.

Apoptosis serves an important role in the progression of cerebral I/R injury (30). A previous study indicated that the expression level of caspase-3, Bax/Bcl-2 ratio and rate of cellular apoptosis were elevated during cerebral I/R (31). Furthermore, increased caspase-3 levels and Bax/Bcl-2 ratio resulted in elevated chromatin condensation, mitochondrial membrane permeabilization, DNA fragmentation and cell apoptosis (32). Therefore, preventing the apoptosis of nerve cells is considered to be a suitable therapeutic target for limiting the volume of the cerebral infarct after stroke (33). In the current study, pretreatment with β-PAE markedly reduced cell apoptosis, Bax/Bcl-2 ratio and caspase-3 activity.

Ischemic hypoxia results in an increased number of free radicals during reperfusion, and such reperfusion stimulating extensive oxidative damage is considered one of the major reasons for brain cell damage and death in I/R injury (34). Tan et al (30) observed that oxidative stress was induced by cerebral I/R injury as indicated by suppressed SOD and GSH-Px levels, and increased MDA level (35). Numerous researchers have demonstrated the anti-oxidative effect of β-PAE. According to Wu et al (5), β-PAE inhibited MAD expression in LPS-induced lung injury (8). A previous study revealed that pretreatment with β-PAE at doses of 10, 20 and 40 mg/kg markedly enhanced the SOD, CAT and GSH activity, and significantly suppressed the MDA level in ethanol-induced gastric injury (13). In the current study, superoxide generation and MDA levels increased while the GSH-Px and SOD activities decreased in rats with cerebral I/R injury. However, pretreatment with β-PAE reversed these alterations.

The TLR4 signaling pathway is associated with the occurrence and development of the ischemic stroke (36). Interaction between TLR4 and MyD88 results in the activation of NF-κB (37). NF-κB, a ubiquitous transcription factor participating in the inflammatory and immune responses, is inhibited by IkBα proteins (38). The activation of NF-κB leads to the synthesis of inflammatory cytokines, including IL-1β, IL-6 and TNF-α. A previous study indicated that proinflammatory responses exacerbate cerebral I/R injury, especially at the early stage of this condition (39). β-PAE exhibits well-established anti-inflammatory abilities and is widely used in the treatment of numerous inflammatory diseases (7,8). According to Chen et al (8), increased secretion of TNF-α, IL-6 and IL-1β induced by LPS was markedly suppressed by pretreatment with β-PAE, and this effect may have occurred due to the inactivation of the NF-κB signaling pathway in the bronchoalveolar lavage fluid. In addition, in the carrageenan-induced paw edema, pretreatment with β-PAE inhibited the release of TNF-α, IL-1β, IL-6, prostaglandin E2 and nitric oxide in a dose-dependent manner (9). Furthermore, the overexpression of TNF-α, IL-6 and IL-1β was significantly suppressed by β-PAE through the inactivation of the NF-κB signaling pathway in ethanol-induced gastric injury in rat model (13). The current study revealed that the activity of the TLR4/NF-κB signaling pathway was decreased following pretreatment with β-PAE due to the inhibition of TLR4 expression, enhanced IkBα activity and suppressed nuclear translocation of NF-κB p65 in brain tissues with I/R injury. In addition, the expression levels of TNF-α, IL-1β and IL-6 were suppressed following pretreatment with β-PAE.

In conclusion, to the best of our knowledge, the current study is the first to reveal that β-PAE may induce neuroprotective effects on cerebral I/R injury. The protective function was demonstrated by the decreased infarct volume, reduced brain water content, alleviated neurological dysfunction, restored MMP and decreased cell apoptosis rates. The underlying mechanism may include the inhibition of oxidative stress via suppressing superoxide generation and MDA level, and increasing the levels of GSH-Px and SOD. Furthermore, the release of proinflammatory factors, including TNF-α, IL-1β and IL-6 was suppressed by β-PAE through the inactivation of the TLR4/NF-κB signaling pathway. The present results
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Figure 3. β-PAE reduces the inflammatory response and oxidative stress of rats with the I/R injury. (A) Western blotting was used to analyze the expression levels of TNF-α, IL-1β and IL-6 in the brain tissue. (B) The mRNA levels of TNF-α, IL-1β and IL-6 were measured using reverse transcription-quantitative polymerase chain reaction. (C) Superoxide flashes were measured to evaluate mitochondrial superoxide generation in brain tissues. (D) Oxidative damage parameters of brain cells, including MDA, GSH-Px and SOD levels were measured using the corresponding detection kits. Experiments were repeated at least three times, and data are presented as the mean ± standard deviation. *P<0.05, **P<0.01 vs. sham group; ¤P<0.05, ¤¤P<0.01 vs. the I/R group. β-PAE, β-patchoulene; I/R, ischemia-reperfusion; TNF, tumor necrosis factor; IL, interleukin; MDA, malondialdehyde; GSH-Px, glutathione peroxidase; SOD, superoxide dismutase.

Figure 4. β-PAE inactivates the TLR4/NF-κB signaling pathway. (A) Expression of TLR4 and IκBα was assessed by western blot analysis of protein samples extracted from the brain tissue. (B) The mRNA levels of TLR4 and IκBα were measured in nuclear and cytoplasmic protein samples via western blotting. Experiments were repeated at least three times, and data are presented as the mean ± standard deviation. *P<0.05 vs. sham group; **P<0.01 vs. the I/R group. β-PAE, β-patchoulene; I/R, ischemia-reperfusion; NF-κB, nuclear factor-κB p65; TLR4, Toll-like receptor 4.
suggest that β-PAE may be further developed as a potential therapeutic agent in the treatment of cerebral I/R injury.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions
FBZ constructed the middle cerebral artery occlusion model and performed western blotting; JPW and HXZ were involved in the in evaluation of Modified Neurological Severity Scores, infarct volume and statistical analysis. GMF conducted the mitochondrial membrane potential assay. XC conducted reverse transcription-quantitative polymerase chain reaction. XC was responsible for the design of the present study and drafting the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
The present study was approved by The Ethics Committee of Cangzhou Central Hospital.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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