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Aquaporin 4 expression and ultrastructure of the blood-brain barrier following cerebral contusion injury★

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Abstract
This study aimed to investigate aquaporin 4 expression and the ultrastructure of the blood-brain barrier at 2–72 hours following cerebral contusion injury, and correlate these changes to the formation of brain edema. Results revealed that at 2 hours after cerebral contusion and laceration injury, aquaporin 4 expression significantly increased, brain water content and blood-brain barrier permeability increased, and the number of pinocytotic vesicles in cerebral microvascular endothelial cells increased. In addition, the mitochondrial accumulation was observed. As contusion and laceration injury became aggravated, aquaporin 4 expression continued to increase, brain water content and blood-brain barrier permeability gradually increased, brain capillary endothelial cells and astrocytes swelled, and capillary basement membrane injury gradually increased. The above changes were most apparent at 12 hours after injury, after which they gradually attenuated. Aquaporin 4 expression positively correlated with brain water content and the blood-brain barrier index. Our experimental findings indicate that increasing aquaporin 4 expression and blood-brain barrier permeability after cerebral contusion and laceration injury in humans is involved in the formation of brain edema.

Key Words
neural regeneration; brain injury; cerebral contusion and laceration injury; aquaporin 4; blood-brain barrier; ultrastructure; brain edema; human; early stage; photographs-containing paper; neuroregeneration

Research Highlights
(1) Aquaporin 4 expression, brain water content, and blood-brain barrier index were upregulated at early stages following cerebral contusion and laceration injury in humans.
(2) Aquaporin 4 expression positively correlated with brain water content and blood-brain barrier permeability at early stages following cerebral contusion and laceration injury in humans.

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INTRODUCTION

Aquaporin 4 is the most important aquaporin in the brain and plays a regulatory role in water transport across the cell membrane[1]. In addition, aquaporin 4 is mainly distributed on the contact surface between the brain and liquid lacuna (such as blood vessels, the subarachnoid cavity and brain ventricles). Aquaporin 4 is closely associated with the reabsorption of cerebrospinal fluid, osmotic regulation, brain edema formation, and other physiological or pathological processes[2-5]. The blood-brain barrier is a dynamic adjusting interface that is present in the brain, spinal cord capillaries, and nerve tissue. This interface has a protective effect; once the barrier is damaged, capillary plasma protein and water leakage may increase brain extracellular fluid, leading to vasogenic brain edema[6-7]. Aquaporin 4 expression closely correlates with blood-brain barrier function[8]. Secondary brain edema following cerebral contusion and laceration injury is a leading cause of disease progression or death, and aquaporin 4 is found to participate in the formation of brain edema[8]. Existing studies have focused on the mechanism underlying brain edema formation after intracerebral hemorrhage using mostly animal models[9]. Moreover, studies are confined to immunohistochemical detection of aquaporin 4 expression and the blood-brain barrier index, and there is little evidence regarding the mechanism involved in the formation of brain edema following cerebral contusion and laceration injury. To further understand the effect of brain edema at early stages after cerebral injury in humans, we aimed to observe changes in aquaporin 4 expression and to the ultrastructure of the blood-brain barrier at varying time points following contusion and laceration injury. In addition, we investigated the relationship between aquaporin 4 expression and brain edema formation.

RESULTS

Aquaporin 4 expression was upregulated after cerebral contusion and laceration injury

Immunohistochemical staining showed that aquaporin 4 was expressed in astrocytes and ependymal cells, especially in gliocytes that were directly in contact with the capillary and pia mater, as well as pericytes. Aquaporin 4-positive cells were only distributed in the cell membrane, while no positive staining was found in the cytoplasm or nucleus. Aquaporin 4 expression was scarcely seen in normal brain tissue, and both the number of aquaporin 4-positive cells and the number of blood vessels began to increase at 2 hours after brain contusion, and reached a peak at 12 hours (Figure 1). Compared with the control group, aquaporin 4 expression significantly increased at each time point in the cerebral contusion and laceration group ($P < 0.01$; Table 1).

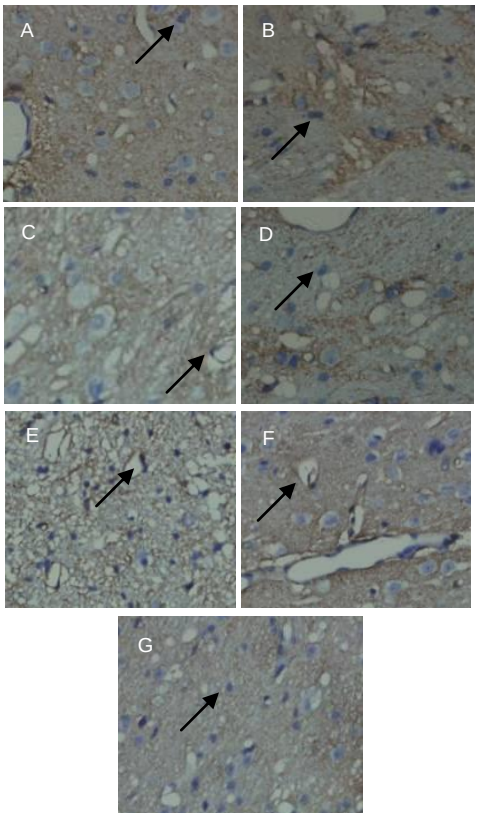


Figure 1 Aquaporin 4 expression (arrows) at different time points following cerebral contusion and laceration injury (immunohistochemical staining, light microscope, × 400).

(A) Control group, aquaporin 4 was expressed in glial cells and the endothelium.

(B) At 2 hours after contusion and laceration injury, the number of aquaporin 4-positive cells began to increase.

(C) At 6 hours after contusion and laceration injury, the number of aquaporin 4-positive cells increased, and vacuoles were microscopically visible, indicating the formation of brain edema.

(D) At 8 hours after contusion and laceration injury, aquaporin 4 expression significantly increased, staining was apparent, the gap between tissues was widened, and edema was aggravated.

(E) At 12 hours after contusion and laceration injury, staining was more obvious, and the number of aquaporin 4-positive cells and brain edema reached its peak.

(F) At 24 hours after contusion and laceration injury, staining weakened, and the number of aquaporin 4-positive cells decreased.

(G) At 72 hours after contusion and laceration injury, staining weakened further, and the number of aquaporin 4-positive cells significantly decreased.

Table 1 Changes in aquaporin 4 expression (absorbance), brain water content (%), and blood-brain barrier index after cerebral contusion and laceration injury at different times

Group	Aquaporin 4 expression	Brain water content	Blood-brain barrier index
Control	1.25±0.05	77.46±0.89	0.32±0.01
Cerebral contusion and laceration injury 2 hours	1.29±0.01[a]	78.57±0.68[a]	0.49±0.03[a]
Cerebral contusion and laceration injury 6 hours	1.40±0.03[a]	79.50±0.67[a]	0.68±0.01[a]
Cerebral contusion and laceration injury 8 hours	1.57±0.04[a]	80.48±0.49[a]	0.78±0.01[a]
Cerebral contusion and laceration injury 12 hours	1.65±0.05[a]	82.60±0.72[a]	0.88±0.01[a]
Cerebral contusion and laceration injury 24 hours	1.30±0.03[a]	78.34±0.37[a]	0.54±0.01[a]
Cerebral contusion and laceration injury 72 hours	1.26±0.01[a]	77.47±0.66[a]	0.32±0.01[a]

Data are expressed as mean ± SD of ten samples in the control group and at each time point in the experiment group. [a]$P < 0.01$, *vs.* control group using the two sample *t*-test. Brain water content = (wet weight – dry weight)/wet weight × 100%; blood-brain barrier index = cerebrospinal fluid albumin/serum albumin.

Brain water content increased after cerebral contusion and laceration injury

After contusion and laceration injury, brain water content significantly increased at 2 hours ($P < 0.01$), continued to increase at 6 and 8 hours ($P < 0.01$), and reached a peak at 12 hours ($P < 0.01$), after which it gradually decreased (Table 1).

Blood-brain barrier permeability was enhanced after cerebral contusion and laceration injury

The blood-brain barrier index can reflect the integrity of the blood-brain barrier[10]. Our findings showed that the blood-brain barrier index increased following cerebral contusion and laceration injury, and reached a peak at 12 hours. Compared with the control group, the blood-brain barrier index significantly increased in the cerebral contusion and laceration group at each time point ($P < 0.01$; Table 1).

Ultrastructural changes after cerebral contusion and laceration injury

Neurons in the control group exhibited large and round nuclei, a clearly visible nucleoli and cell membrane, and chromatin of uniform density. Cellular organelles such as mitochondria, and the rough endoplasmic reticulum and Golgi apparatus all appeared normal. Cells exhibited normal neurite structure, and a microvascular endothelial cell layer and basal layer.

At 2 hours after cerebral contusion injury, the number of pinocytotic vesicles and capillary endothelial cells increased, and swelling and edema of foot processes was observed in capillary astrocytes, which were still connected to the capillary basement membrane. The cell body of neurons began to condense slightly, however, the capillary basement membrane remained intact.

At 6–8 hours, the capillary basement membrane thickened and swelling of mitochondria was visible. At 12 hours, swelling of endothelial cells was apparent, the basement membrane began to dissolve, and nerve fibers demyelinated.

At 24–72 hours, astrocytes began to shrink and neurite swelling was evident. In addition, electron microscopy revealed that a large number of vacuoles and severe edema in gliocyte foot processes, with a loss of nuclear and cytoplasmic protein, mitochondrial swelling, disappearance and cavitation, as well as capillary basement membrane breakage and disappearance. A small amount of neurons and glial cells ruptured. Neutrophil infiltration was also apparent between brain tissues (Figure 2).

Correlation between aquaporin 4 expression, blood-brain barrier permeability and brain water content

Pearson correlation analysis revealed that changes in aquaporin 4 expression coincided with changes in brain water content, showing a significant positive correlation ($r = 0.912$, $P < 0.01$); the blood-brain barrier index also changed with brain water content, showing a significant positive correlation ($r = 0.877$, $P < 0.01$); aquaporin 4 expression positively correlated with the blood-brain barrier index ($r = 0.908$, $P < 0.01$; Figure 3).

DISCUSSION

Aquaporin 4 is widely distributed in the central nervous system[11], and participates in brain edema formation and elimination[12-15]. Aquaporin 4 is specific and highly selective for water molecules, and plays an important role in regulating pore size for water selective transport[16-17]. Aquaporin 4 contributes to the brain edema that occurs in a variety of disorders[1-2, 18].

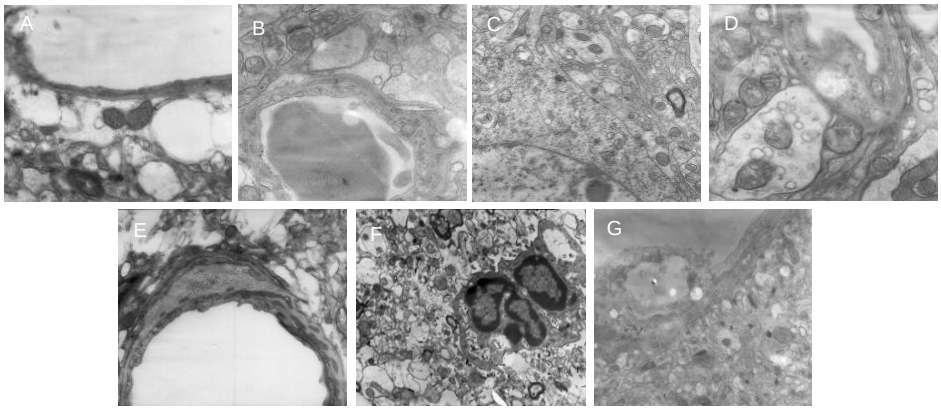


Figure 2 Ultrastructural changes in the blood-brain barrier after cerebral contusion and laceration injury in brain tissue at different times (transmission electron microscope, × 40 000).

(A) Control group, normal blood-brain barrier showed normal neurites, microvascular endothelial cell layer and basal layer structure. Mitochondria, rough endoplasmic reticulum and Golgi bodies were visible.

(B) At 2 hours after contusion and laceration injury, the number of pinocytotic vesicles in capillary endothelial cells increased significantly, the number of lamellipodia increased, astrocytic foot process began to swell, and the capillary basement membrane remained intact.

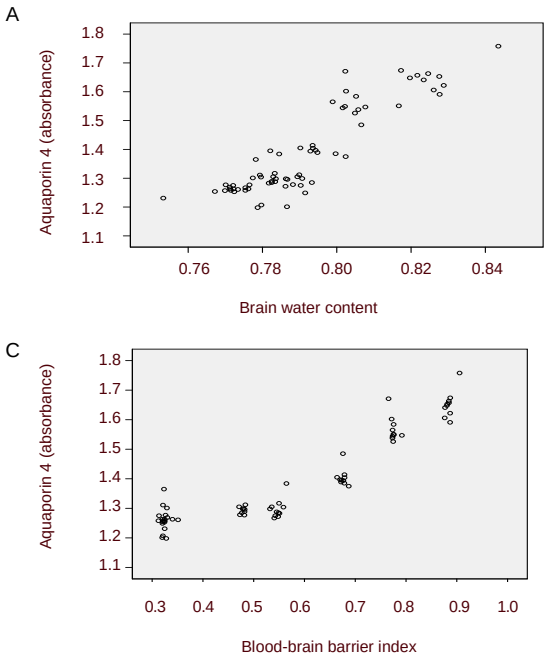
(C) At 6 hours after contusion and laceration injury, the number of pinocytotic vesicles increased, mitochondrial swelling was visible, and the capillary basement was thickened and still remained intact.

(D) At 8 hours after contusion and laceration injury, the capillary basement membrane was thickened, and the integrity was damaged, swollen mitochondria were visible in foot processes and endothelial cells.

(E) At 12 hours after contusion and laceration injury, exudate leaked between endothelial cells and pericytes, the basement membrane began to dissolve, and the nerve fiber synaptic boundary was not clearly visible, and in some cases had disappeared.

(F) At 24 hours after contusion and laceration injury, an uneven density of exudate was observed, and neutrophils had infiltrated.

(G) At 72 hours after contusion and laceration injury, a large number of vacuoles and serious edema was visible in glial cell foot processes, with the performance of glial loose, loss of nuclear and cytoplasmic protein, mitochondrial swelling, disappearance and cavitation, as well as capillary basement membrane breakage and disappearance.



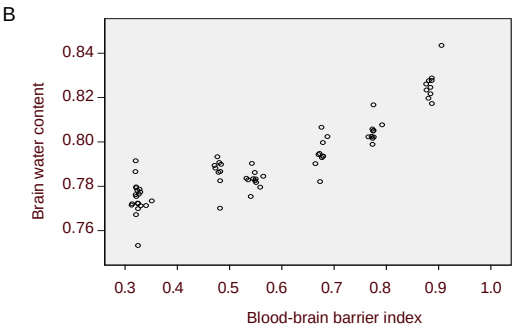


Figure 3 Pearson correlation analysis of aquaporin 4 expression, blood-brain barrier index and brain water content.

(A) A significant positive correlation between aquaporin 4 and brain water content (r = 0.912, P < 0.01) .

(B) A significant positive correlation between brain water content and blood-brain barrier index (r = 0.877, P < 0.01).

(C) A significant positive correlation between aquaporin 4 and blood-brain barrier index (r = 0.908, P < 0.01).

In this study, we observed the dynamic change of aquaporin 4 expression after cerebral contusion and laceration injury in humans using immunohistochemistry. Our results revealed that aquaporin 4 expression and the number of blood vessels increased at 2 hours. At the same time, brain water conten and the blood-brain barrier index also increased. The above indices reached their peak at 12 hours and then gradually declined. Along with changes in brain water content and the blood-brain barrier index, aquaporin 4 expression altered, indicating a positive correlation. Our experimental findings indicate that at early stages of contusion and laceration injury, aquaporin 4 is involved in brain edema formation and recession. Aquaporin 4 expression increased following injury, resulting in aggravation of brain edema, which is consistent with previous reports[8-9, 19-20].

Aquaporin 4 is closely associated with the blood-brain barrier[21] and contributes to maintain blood-brain barrier function. However, whether aquaporin 4 gene deletion can lead to damage of the blood-brain barrier remains controversial. Saadoun *et al* [22] believed that gene knockout of rat aquaporin 4 would not alter blood-brain barrier integrity. In contrast, Zhou *et al* [23] found that gene knockout of rat aquaporin 4 could alter blood-brain barrier integrity. According to the studies of Wolburg *et al* [24], aquaporin 4 is the most important constituent of the blood-brain barrier, and blood-brain barrier damage is closely related to the upregulation of aquaporin 4 expression. In this study, we focused on the blood-brain barrier index and electron microscopy observations of the blood-brain barrier to monitor ultrastructural changes. Our results demonstrated that after contusion and laceration of brain tissue, the blood-brain barrier index increased and blood-brain barrier structure slightly changed at early stages after injury. During this time, the capillary basement membrane remained intact. After several hours, the capillary basement membrane thickened, endothelial cells began to swell, the basement membrane began to dissolve, and blood-brain barrier disruption occurred at 24–72 hours. This result is evidence that the blood-brain barrier was damaged at early stages of brain contusion and laceration injury, and that it positively correlated with the expression of aquaporin 4. We speculate that aquaporin 4 may be closely related to blood-brain barrier integrity and function, which is consistent with the findings of Higashida *et al* [25].

Following cerebral contusion and laceration, aquaporin 4 expression was upregulated, which is a protective response. However, whether aquaporin 4 expression is directly or indirectly involved in damage to the blood-brain barrier and brain edema formation remains unclear. Shi *et al* [26] found that aquaporin 4 gene deletion aggravated NMDA-induced cortical damage, increased the density of degenerating neurons in the damaged zone, and raised the permeability of the blood-brain barrier when compared with wild-type mice. Therefore, aquaporin 4 may play a protective role in brain injury induced by NMDA. Up-regulation of aquaporin 4 expression resulted in blood-brain barrier damage and formed vasogenic edema. In contrast, if the blood-brain barrier is not damaged, cytotoxic brain edema may not lead to an increase in aquaporin 4 expression[27]. Aquaporin 4 is an important factor for the onset of cerebral edema, especially for blood-brain barrier disruption, which causes vasogenic edema formation. We hypothesized that the mechanism of edema formation may depend on phosphorylation of the protein kinase C pathway[28-29], which regulates the expression of vascular endothelial growth factor, increases the permeability of the blood-brain barrier[30], increases K^+ siphon, which results in aggravation of electrolyte disorder[31], and induces intracellular Ca^{2+} overload[32], thus causing cerebral edema. Therefore, aquaporin 4 inhibitors (acetazolamide[33-34] and TGN-020[35]), which inhibit aquaporin 4 overexpression, may alleviate blood-brain barrier damage, prevent cerebral edema and reduce cerebral injury[36-38]. Although significant advancements in the treatment of cerebral edema have been achieved, a specific target therapy and a specific aquaporin 4 inhibitor are yet to be identified, and the exact molecular mechanism of aquaporin 4 is not currently understood. Further studies are required to conclusively determine its mechanism of action to better control cerebral edema, and decrease brain injury morbidity and mortality.

In summary, this study analyzed the relationship between aquaporin 4 expression, blood-brain barrier index and ultrastructural changes in blood-brain barrier following cerebral contusion and laceration injury. Our results have direct clinical relevance as the brains were directly harvested from injured patients, thus providing direct and reliable clinical evidence for the treatment of cerebral edema after brain injury. However, we only observed changes in aquaporin 4 expression and the ultrastructure of the blood-brain barrier at early stages of head injury (72 hours). Further investigations are required as data before 2 hours and after 72 hours were not able to be collected. Due to patient admission times and the preparation time for surgery, craniocerebral injury operations were generally performed within 2–72 hours

after injury, and no cases were observed prior to 2 hours and after 72 hours. In addition, we did not detect and compare aquaporin 4 expression in brain tissue from the same patient at different times after injury owing to individual differences (such as gender, age and physical fitness) and operation limitations. Therefore, it is necessary to expand the collection time, improve research methods, reduce individual differences, and clearly detail observation times in order to collect more reliable evidence.

MATERIALS AND METHODS

Design

An open randomized controlled experiment.

Time and setting

Experiments were performed in the Molecular Biology Laboratory, Deyang People's Hospital and the Neural Biological Laboratory of the Third Military Medical University of Chinese PLA from February 2011 to January 2012.

Materials

Brain tissue specimens

Seventy patients with cerebral contusion and laceration injury, confirmed by craniocerebral CT, and with surgical indications (CT confirmed the midline shifted greater than 1 cm; supplementary Figure 1 online), aged 30–40 years, were involved in this study and agreed to receive surgery. Patients with great variations in age, operation mode and disease conditions were excluded. Among the patients, 40 were men and 30 were women, with a mean age of 31.34 ± 4.25 years. The site of injury included the frontal lobe in 52 cases, temporal lobe in 8 cases, and cerebellum in 10 cases. Cerebral contusion and laceration group: brain tissue specimens harvested from a 3-cm area in the center of the injury area in 60 patients; control group: non-functional normal brain tissue from 10 patients (frontal or temporal pole brain tissue resected due to internal decompression during operation).
The cerebral contusion and laceration group was further assigned into six subgroups according to the operation time, namely 2, 6, 8, 12, 24, and 72 hours.

Cerebrospinal fluid and venous blood sampling

Cerebrospinal fluid was surgically collected through puncture or lumbar puncture for the determination of cerebrospinal fluid albumin. Elbow vein blood was extracted for serum albumin detection.

All involved patients and their relatives agreed to the experiment and signed the informed consent. The experimental disposals complied with the *Helsinki Declaration* ethical standard.

Methods

Brain water content determination

Brain tissue 150 mg were weighed for wet weight, placed in a 100°C constant temperature box for 24 hours, and then weighed for dry weight. Brain water content was calculated according to the following formula: (wet weight – dry weight)/wet weight × 100%, to represent the degree of brain edema[39].

Immunohistochemical detection of aquaporin 4 expression

Brain tissue specimens at 1 mm thick were fixed with 4% (w/v) paraformaldehyde for 6 hours and rinsed for 12 hours, followed by gradient ethanol dehydration, chloroform, ethanol xylene and waxing, and soft paraffin embedding into blocks. Specimens were cut into slices using an automatic microtome, at 5 μm thickness. After conventional dewaxing, slices were incubated with 3% (v/v) H_2O_2 for 10 minutes at room temperature, blocked with sheep serum for 20 minutes, and incubated with rabbit anti-aquaporin 4 polyclonal antibody (1:50; Abnova, Walnut, CA, USA) at 4°C overnight. Sections were then incubated with biotinylated-conjugated goat anti-rabbit IgG (1:100; Abnova) for 1 hour at room temperature, and with SABC (Abnova) at 37°C for 20 minutes. Diaminobenzidine (Sigma, St. Louis, MO, USA) was used for coloration, followed by routine dehydration, transparency and mounting. Finally, slices were observed under a light microscope (Olympus, Tokyo, Japan). Positive staining was observed as yellow fine particles in the cell membrane. Using the Image-Pro-Plus image analysis system (Media Cybernetics, Inc. Bethesda, MD, USA), the staining of aquaporin 4 was semi-quantitatively analyzed using five randomly selected cerebral cortical slices from each specimen, and five cerebral cortical areas on each slice.

Blood-brain barrier index determination

Cerebrospinal fluid and serum were harvested to determine cerebrospinal fluid and serum albumin levels using a CX7 automatic biochemical analyzer (Beckman Coulter, Inc, Fullerton, CA, USA). According to the formula formulated by Wang[40], the blood-brain barrier index was calculated as follows: blood-brain barrier index = cerebrospinal fluid albumin/serum albumin.

Blood-brain barrier ultrastructure

Normal brain tissue and brain tissue fixed at each time

point (1.0 mm × 1.0 mm × 1.0 mm) were fixed in glutaraldehyde and 1% (w/v) osmium tetroxide, dehydrated in acetone, embedded with 618 epoxy resin *in situ*, and prepared into ultrathin sections (60 nm) for double lead staining. Sections were observed under a transmission electron microscope (JEM-2000; Sanyo, Tokyo, Japan).

Statistical analysis

Quantitative data were expressed as mean ± SD and analyzed using SPSS 13.0 software (SPSS, Chicago, IL, USA). Values between groups were compared using the two samples *t*-test and the correlation between two variables was analyzed using Pearson correlation analysis. A *P* value less than 0.05 was considered a significant difference, and less than 0.01 an extremely significant difference.

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Author contributions: Xinjun Li and Hong Xu had full access to the study concept and design. Xinjun Li was responsible for data analysis and statistical processing, drafted the manuscript, and collected and integrated the data. Yangyun Han and Hong Xu participated in article validation. All authors provided data and approved the final version of the paper.

Conflicts of interest: None declared.

Ethical approval: This study was approved by the Ethics Committee of Deyang People's Hospital in China.

Author statements: The manuscript is original, has not been submitted to or is not under consideration by another publication, has not been previously published in any language or any form, including electronic, and contains no disclosure of confidential information or authorship/patent application disputations.

Supplementary information: Supplementary data associated with this article can be found in the online version, by visiting www.nrronline.org.

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