Aquaporin-4 deletion ameliorates enterovirus 71 infection in mice

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Abstract. Aquaporin-4 (AQP4) is a major water channel of the central nervous system. The present study was designed to determine whether AQP4 deletion could ameliorate enterovirus (EV) 71 infection-induced hand, foot and mouth disease (HFMD) by inhibiting inflammation and apoptosis in mice. EV 71 strains were injected into neonatal BALB/c mice to induce HFMD. Western blotting and ELISA were used to measure the protein expression and cytokine levels. The levels of AQP4 mRNA and protein in the brain were increased in EV 71-infected mice, while the survival rate and health score were improved in AQP4-knockout (KO) mice with EV 71 infection. The EV 71 infection-induced increases of tumor necrosis factor-α, interleukin (IL)-1β, IL-6, monocyte chemotactic protein-1, interferon (IFN)-α and IFN-γ in plasma and brain were inhibited in AQP4-KO mice. AQP4 deletion reversed the decreased levels of Bcl2 and Bcl2/Bax, and the increased levels of Bax induced by EV 71 infection in the brain. These results demonstrated that AQP4 deletion ameliorated EV 71 induced-HFMD via inhibiting inflammation and apoptosis in mice.

Introduction

Hand, foot and mouth disease (HFMD) is an infectious illness, which predominantly affects infants and young children (1). The HFMD epidemic is a serious public health issue that could lead to an enormous economic and social burden (2). One of the causative agents of HFMD is enterovirus 71 (EV 71), a human virus species of the *Enterovirus* genus in the *Picornaviridae* family (3,4). EV 71-associated HFMD can be complicated by neurological manifestations, including cardiopulmonary failure, myoclonus, ataxia, polio-like paralysis, tremor and encephalomyelitis (5-7).

The aquaporin (AQP) family of water channels is a group of small membrane-spanning proteins that are vital for rapid transport of water across the plasma membrane. AQP4, as a major water channel of the central nervous system, is highly concentrated on astrocyte endfeet (8,9). Previous studies demonstrated that altered AQP4 expression serves a critical role in neuroinflammation during brain injury (10-12). In cultured rat articular chondrocytes, AQP4 downregulation attenuates interleukin (IL)-1β-induced chondrocyte apoptosis by regulating the expression of apoptosis-related genes and by inhibiting p38 MAPK (13). The levels of IL-6, IL-8, IL-1β, interferon (IFN)-γ and monocyte chemotactic protein (MCP)-1 are altered in the serum and cerebrospinal fluid of patients with AQP4-antibody seropositive neuromyelitis optica spectrum disorder (14-16). In astrocytes, AQP4 expression is altered through the release of tumor necrosis factor (TNF)-α and IL-6 by microglia during hypoxia-induced inflammatory responses (17).

Proinflammatory cytokines are demonstrated to be upregulated in the brains of EV 71-infected mice (18). EV 71 infection also directly impacts the mitochondrial apoptotic pathway by modulating the recruitment and activation of Bax (19). In addition, caspase-3 inhibition protects host cells from the cytopathic effect of EV 71 infection and prevents cell cycle arrest, leading to a decrease in EV 71 viral protein expression and viral production (20). The present study aimed to determine whether AQP4 deletion could ameliorate EV 71 infection by inhibiting inflammation and apoptosis in mice.

Materials and methods

In vivo experiments. All animal experiments were carried out in accordance with The Guidelines of the Xuzhou Medical University Institutional Committee for the Care and Use of Laboratory Animals and approved by The Xuzhou Medical University Laboratory Animal Management Ethics Committee. A total of 19 maternal mice (age, 8-10 weeks; weight, 210-240 g) were kept in a temperature (22±1˚C) and humidity (30-60%)-controlled room under a 12 h light-dark cycle with free access to standard chow and tap water. To minimize animal suffering and distress, the housing conditions, animal welfare, and experimental procedures were in accordance with The Guide for The Care and Use of

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Laboratory Animals (21). In addition, ≤8 newborn mice at a time were fed by a maternal mouse. A total of 78 one-day-old mice were used in the experiments. The sex of the pups was not determined, due to their young age.

Mouse in vivo treatment. A total of 31 AQP4-knockout (KO) C57BL6/J mice (Cyagen Biosciences, Inc.) were used as the experimental group, and 47 wild-type (WT; Cyagen Biosciences, Inc.) mice were treated as a control group. Newborn mice were bred in-house. EV 71 was isolated from a HFMD clinical specimen (male; age, 1 year) in 2018 in Xuzhou Children's Hospital of Xuzhou Medical University, and written consent was provided by the parents of the donor. The present study was approved by The Ethics Committee of Xuzhou Children's Hospital.

One-day-old mice were challenged intraperitoneally with 50 μl EV 71 at a dose of 10^7 plaque-forming units per mouse. The mice in the control group were treated with 50 μl PBS (BioChannel; Nanjing Shenghang Biotechnology Co., Ltd.) and kept in a separate cage from the infected mice. All the mice received one EV 71 or PBS injection. Health was monitored and scored as follows: i) 0, healthy; ii) 1, lethargic and inactive; iii) 2, wasting; iv) 3, weak in limb; v) 4, paralyzed hind limb; and vi) 5, moribund or dead. In the survival analysis and health score experiments, WT-Control (n=8), KO-Control (n=8), WT-EV 71 (n=15) and KO-EV 71 (n=15) groups were used. For all other experiments, there were eight animals in each group.

Determination of inflammatory factor levels. The mice were sacrificed via 1.5% isoflurane inhalation for 2 min. The brain and serum samples of the mice were obtained 4 days after EV 71 injection. The brain tissues were homogenized in lysis buffer (Thermo Fisher Scientific, Inc.). The total protein in the homogenate was extracted and measured using a BCA protein assay kit (BioChannel Biotechnology Co., Ltd.). The levels of TNF-α, IL-1β, IL-6, MCP-1, IFN-α and IFN-γ (cat. nos. SE A133Mu, SEA076Mu, SEA079Mu, SEA087Mu, SEA033Mu and SCA049Mu, respectively) in the brain or serum were determined using ELISA kits (all Wuhan USCN Business Co., Ltd.) following the manufacturer's instructions.

Western blotting. The brain samples were sonicated in RIPA lysis buffer (BioChannel; Nanjing Shenghang Biotechnology Co., Ltd.) and homogenized. The debris was removed by centrifugation at 12,000 x g for 10 min at 4˚C and the supernatant was collected. Subsequently, ~30-40 μg protein (BCA protein assay kit) was separated by 8% gel electrophoresis, transferred to PVDF membrane The membrane was blocked with 5% skimmed milk powder at room temperature for 1 h and probed with primary antibodies overnight at 4˚C against AQP4, Bcl2 or Bax (all 1:1,000; cat. nos. 59678, 3498 and 14796, respectively; all Cell Signaling Technology, Inc.). Then, horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:10,000; cat. no. ab7090; Abcam) was added and incubated at room temperature for 1 h and GAPDH (1:10,000; cat. no. ab181602; Abcam) was used as an internal control. The bands were visualized via ECL (Beyotime, Shanghai, China). Images were analyzed using Image-Pro Plus software (version 6.0; XRayScan; CAD/CAM Services, Inc.).

Measurement of AQP4 mRNA using reverse transcription-quantitative PCR (RT-qPCR). AQP4 mRNA levels in the brain were measured using an RT-qPCR system (Roche Diagnostics). In brief, total RNA was extracted using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) cDNA was extracted from RNA via reverse transcription using random primers in a total volume of 10 μl, according to the instructions of the PrimeScript™ RT Master Mix (37˚C, 15 min; 85˚C, 5 sec; Takara Biotechnology Co., Ltd.). mRNA levels were determined via Power SYBR-Green PCR Master Mix (Thermo Fisher Scientific, Inc.). All samples were amplified in triplicates for 45 cycles in a 96-well plate (95˚C, 15 sec; 60˚C, 1 min). The relative gene expression was determined using the $2^{-\Delta\Delta Cq}$ method (22). Primer sequences were as follows: AQP4 forward, 5'-CGGCATCCTCTACCTGGTCACA-3' and reverse, 5'-GCCAGCGGTGAGGTTTCCAT-3'; and GAPDH forward, 5'-AGGTCGGTGTGAACGGATTTG-3' and reverse, 5'-TGTAGACCATGTAGTTGAGGTCA-3'.

Statistical analysis. Data are presented as the mean ± standard error of the mean. All data were analyzed using GraphPad Prism 7.0 (GraphPad Software, Inc.). Survival rates were evaluated by the Mantel-Cox log-rank test. Other data were analyzed using an unpaired t-test for pairwise comparison, or one-way ANOVA, followed by Bonferroni's correction when multiple comparisons were made. A two-tailed $P<0.05$ was considered to indicate a statistically significant difference.

Results

AQP4 levels in the brain. AQP4 mRNA level in brain was significantly higher in EV 71-infected mice compared with in control mice (2.04-fold-change). Furthermore, AQP4 protein levels were significantly higher in the brains of EV 71-infected mice compared with the control (Fig. 1).

Effects of AQP4 deletion on survival rate and clinical score in EV 71-infected mice. The survival rates and clinical scores of one-day-old mice intraperitoneally injected with 10^7 PFU EV 71 strain were recorded. In both the WT and KO groups, EV 71-inoculated mice exhibited shorter survival times compared with the controls. In addition, survival time was improved in EV 71-inoculated AQP4-KO mice compared with EV 71-infected WT mice (Fig. 2A). Furthermore, the mean heath score was reduced in infected AQP4-KO mice in the first 14 days of infection compared with infected WT-controls. Thus, AQP4 deficiency can delay disease development in EV 71-infected mice (Fig. 2B).

Effects of AQP4 deletion on inflammatory factors in the serum and brain of EV 71-infected mice. The levels of TNF-α, IL-1β, IL-6, MCP-1, IFN-α and IFN-γ in the serum of EV 71-infected mice were significantly higher compared with the control mice, both in WT and KO animals. However, the increase in levels of these inflammatory factors following EV 71 infection were reduced in AQP4-KO mice compared with WT mice (Fig. 3). These results also held true in the brain (Fig. 4).

Effects of AQP4 deletion on apoptosis in brain of EV 71-infected mice. The levels of Bcl2 in the brain were significantly reduced

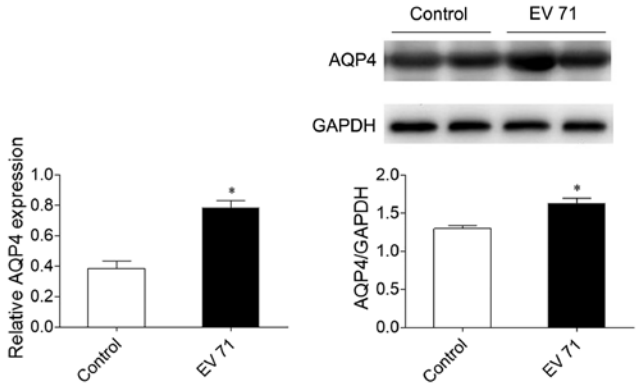

Figure 1. AQP4 levels in the brain. AQP4 mRNA and protein levels in the brain were significantly higher in EV 71-infected mice compared with in control mice. Data are presented as the mean ± SEM. n=8 in each group. *P<0.05 vs. Control. AQP4, aquaporin-4; EV 71, enterovirus 71.

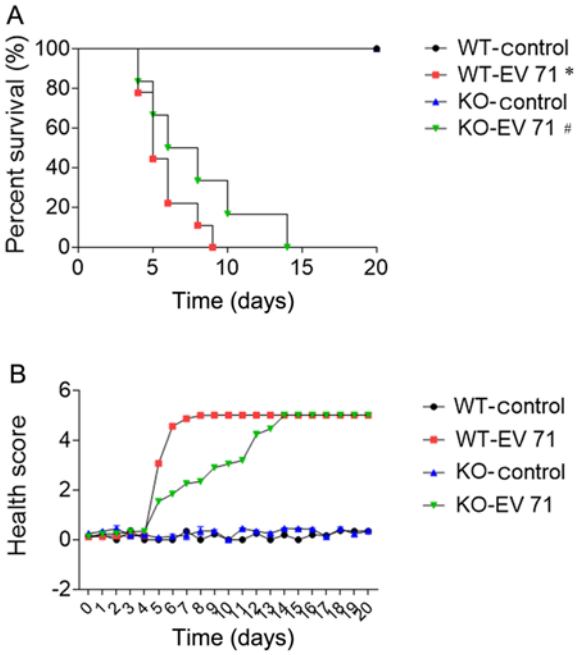

Figure 2. AQP4 deletion affects (A) survival rates and (B) health scores in EV 71-infected mice. AQP4-KO mice exhibited improved survival times and mean clinical scores compared with WT mice with EV 71 infection. Data are presented as the mean ± SEM. *P<0.05 vs. WT-Control; #P<0.05 vs. WT-EV 71. n=8 for WT-Control and KO-Control groups; n=15 in WT-EV 71 and KO-EV 71 groups. AQP4, aquaporin-4; EV 71, enterovirus 71; WT, wild-type; KO, knockout.

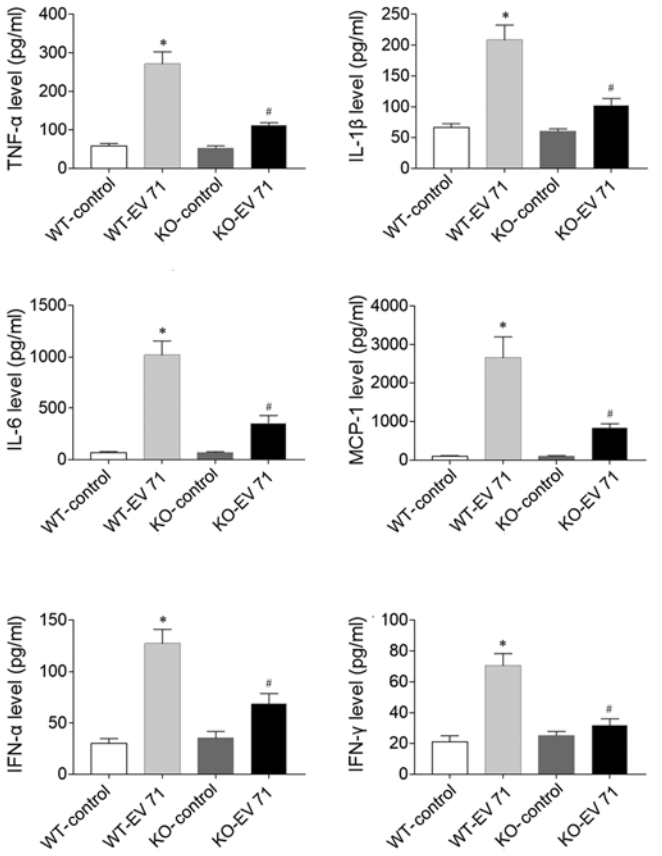

Figure 3. AQP4 deletion affects inflammatory factor levels in the serum of EV 71-infected mice. The EV 71-induced increases of TNF-α, IL-1β, IL-6, MCP-1, IFN-α and IFN-γ in serum were inhibited in AQP4-KO mice. Data are presented as the mean ± SEM. n=8 in each group. *P<0.05 vs. WT-Control; #P<0.05 vs. WT-EV 71. AQP4, aquaporin-4; EV 71, enterovirus 71; WT, wild-type; KO, knockout; IL, interleukin; IFN, interferon; TNF-α, tumor necrosis factor-α; MCP-1, monocyte chemotactic protein.

in EV 71-infected mice compared with the respective control group, while Bax levels were significantly increased. The Bcl2/Bax ratios were also reduced accordingly. However, AQP4 deletion limited the decrease in Bcl2 levels and the increase in Bax levels induced by EV 71-infection in the brain compared with WT. AQP4 deficiency also decreased the decrease Bcl2/Bax ratios seen after EV 71 infection (Fig. 5).

Discussion

The first case of EV 71 infection was reported more than half a century ago (23), yet specific therapy for this severe inflammatory disease is still lacking. Human EV 71, a member of the *Enterovirus* genus of the *Picornaviridae* family, is the main etiologic agent of HFMD (24,25). The AQP4 water channel is widely expressed in the brain and represents a potential drug target for neurological disorders (26). The present study demonstrated that, in addition to its potential for the treatment of neurological disease, AQP4 deletion ameliorated EV 71 induced-HFMD by inhibiting inflammation and apoptosis in mice.

A previous study suggested that serum AQP4 levels significantly differed according to disease severity before and after treatment in EV 71-associated HFMD (27). Similarly, in the present study, the levels of AQP4 mRNA and protein increased in the brain of EV 71-induced HFMD mice. Indeed, AQP4 mRNA levels were doubled in EV 71-infected mice compared with control mice. Compared with EV 71-infected WT mice, AQP4-KO mice exhibited increased survival time. In addition, the mean health scores were higher in EV 71-infected mice compared with the controls. However, in AQP4-KO mice, the increase in health scores induced by EV 71 infection was reduced. These results indicated that AQP4 may be a therapeutic target for HFMD. A previous study suggested that AQP4-overexpressing mice exhibit intracranial pressure elevation, leading to brain herniation and death (28). Altogether, these results demonstrated that AQP4 deficiency improved survival in diseases such as HFMD.

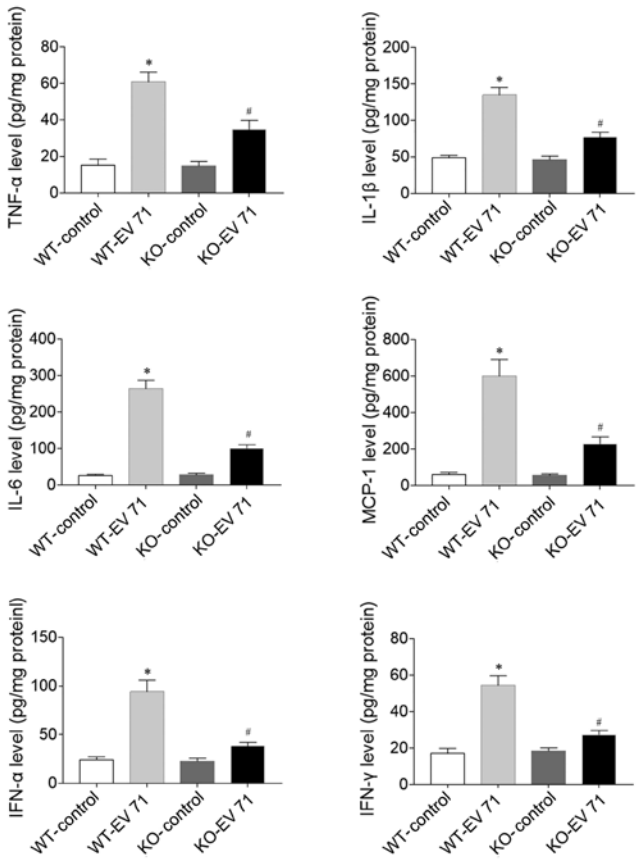

Figure 4. AQP4 deletion affects inflammatory factor levels in the brains of EV 71-infected mice. The EV 71-induced increases of TNF-α, IL-1β, IL-6, MCP-1, IFN-α and IFN-γ in serum were inhibited in AQP4-KO mice. Data are presented as the mean ± SEM. n=8 in each group. *P<0.05 vs. WT-Control; #P<0.05 vs. WT-EV 71. AQP4, aquaporin-4; EV 71, enterovirus 71; WT, wil-type; KO, knockout; IL, interleukin; IFN, interferon; TNF-α, tumor necrosis factor-α; MCP-1, monocyte chemotactic protein.

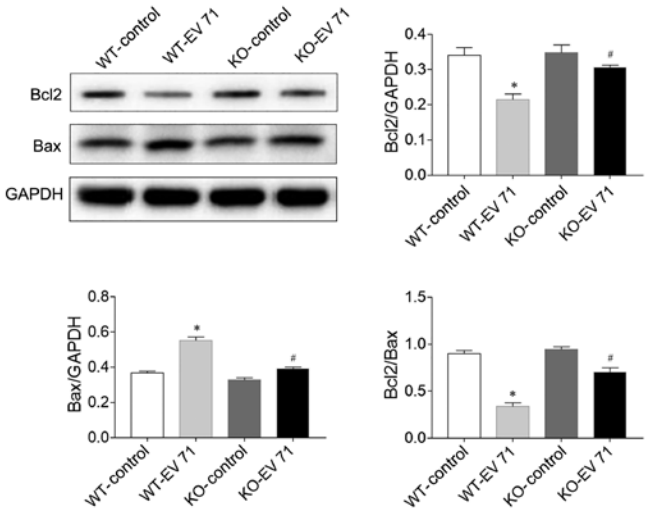

Figure 5. AQP4 deletion affects apoptosis in the brains of EV 71-infected mice. AQP4 deletion reversed the decreases of Bcl2 and Bcl2/Bax levels, and the increase of Bax level induced by EV 71 infection in the brain. Data are presented as the mean ± SEM. n=8 in each group. *P<0.05 vs. WT-Control; #P<0.05 vs. WT-EV 71. AQP4, aquaporin-4; EV 71, enterovirus 71; WT, wild-type; KO, knockout.

AQP4 deletion protects the blood-brain barrier from hypoglycemia by reducing inflammatory responses through the inhibition of proinflammatory cytokine release (29). In a previous study, AQP4 deficiency resulted in a significant increase in the production of IL-1β and TNF-α in a murine model of Parkinson's disease (30). Furthermore, mice treated with the AQP4 inhibitor TGN-020 displayed attenuated lipopolysaccharide-induced lung injury, improved survival rates and reduced proinflammatory cytokines release, including IL-1α, IL-1β, IL-6, TNF-α, IL-23 and IL-17A (31). Whether downregulation of AQP4 also attenuates inflammatory cytokine release in the brain of HFMD mice is not well-studied. The present study demonstrated that the levels of TNF-α, IL-1β, IL-6, MCP-1, IFN-α and IFN-γ in the serum and brain of EV 71-infected mice were higher compared with those of the control mice. In addition, the EV 71-induced increases in the levels of these inflammatory factors were limited in AQP4-KO mice. These findings indicated that AQP4 deletion could ameliorate inflammation in EV 71-induced HFMD.

A previous study demonstrated that gene expression levels of caspase-1 and active caspase-1 were significantly increased in the SH-SY5Y human neuroblastoma cell line following EV 71 infection (32). EV 71 protease 3C plays an important role in EV 71-induced apoptosis (33). AQP4 small interfering RNA knockdown markedly decreases Bax and caspase-3 and increases Bcl-2 mRNA levels in joint diseases, such as rheumatoid arthritis (13). Knockdown of AQP4 significantly decreases astrocyte apoptosis, and promotes viability of astrocytes in anoxic conditions (34). In the present study, the levels of Bcl2 and Bcl2/Bax ratios in the brain were reduced in EV 71-infected mice, while AQP4 deletion limited this EV 71-induced decrease. The levels of Bax were increased in the brains of EV 71-infected mice, and AQP4 deletion inhibited this EV 71-induced increase. These results indicated that AQP4 deletion could attenuate apoptosis in EV 71-induced HFMD in mice.

AQP4 expression can be regulated by inflammatory cytokines. High mobility group box-1, a mediator of inflammatory responses, upregulates AQP4 expression and promotes cell swelling in cultured spinal cord astrocytes after oxygen-glucose deprivation/reoxygenation, which is mediated by IL-6 (35). AQP4 expression levels are also increased in astrocytes following treatment with IL-1β, TNF-α and IFN-γ (36). AQP4 and inflammatory cytokines levels are changed in lipopolysaccharide-treated astrocytes (37). Altogether, previous studies and the present study indicate AQP4 regulates the levels of several inflammatory cytokines. Inflammatory cytokines also regulate AQP4 levels.

In conclusion, inflammation and apoptosis were increased in HFMD. AQP4 expression levels were increased in the brains of mice with of EV 71-induced HFMD. This increase in AQP4 levels may lead to the inflammation and apoptosis seen in EV 71 infection and HFMD. AQP4 deletion improved survival rates and health scores, and attenuated inflammation and apoptosis in EV 71-infected mice. Thus, the findings of the present study suggested that AQP4 deletion could ameliorate EV 71 infection and HFMD by inhibiting inflammation and apoptosis in mice.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

LZ and XWH conceptualized the study and performed the experiments; XLW and BXQ performed the experiments and analyzed data; LZ, TY and HJW wrote the manuscript. TY and HJW designed the study and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments in the present study were carried out in accordance with The Guidelines of The Xuzhou Medical University Institutional Committee for The Care and Use of Laboratory Animals and were approved by The Xuzhou Medical University Laboratory Animal Management Ethics Committee. The present study was also approved by The Ethics Committee of Xuzhou Children's Hospital. Written consent was obtained from the parents of the children involved in the present study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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