Original Research Article

Sevoflurane improves renal ischemia-reperfusion injury in rats through RISK signaling pathway

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Abstract

Purpose: To investigate the effect of sevoflurane on renal ischemia-reperfusion injury (IRI) in rats and its regulatory effect on reperfusion injury salvage kinase (RISK) signaling pathway.

Methods: A total of thirty (30) Sprague-Dawley rats were randomly divided into sham, model and sevoflurane groups with 10 animals per group. Renal IRI models were created in model and sevoflurane groups, while sham group had no ligation. Renal injury was determined using hematoxylin and eosin (HE) staining. Blood urea nitrogen (BUN) and serum creatinine (Scr) levels were assayed while apoptosis was determined via terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining. Enzyme-linked immunosorbent assay (ELISA) was used to assess malonaldehyde (MDA) content and inflammatory factors in kidney tissues and peripheral blood, respectively. Reactive oxygen species (ROS) level was determined using 2,7-dichlorodi-hydro fluorescein diacetate (DCFH-DA) while Western blotting was used to determine the expression of apoptosis- and RISK signaling pathway-related proteins in kidney tissues.

Results: Compared to model group, renal injury in sevoflurane group rats was significantly alleviated ($p < 0.01$). The levels of BUN and Scr in peripheral blood, apoptosis level in kidney tissues, MDA content and ROS level in kidney tissues, interleukin-1β (IL-1β), tumor necrosis factor-alpha (TNF-α) and IL-6 content, and content of caspase-3 protein in kidney tissues were significantly reduced ($p < 0.01$), whereas IL-10 content, Bcl-2/Bax ratio and expression levels of p-ERK1/2, p-Akt and phosphorylated glycogen synthase kinase 3β (p-GSK-3β) were significantly increased ($p < 0.01$) in the sevoflurane group.

Conclusion: Sevoflurane represses the release of inflammatory factors, lowers ROS level and apoptosis of renal cells and improves renal function through activation of RISK signaling pathway in kidney tissues of rats with renal IRI. Thus, sevoflurane is a potential agent for the treatment of IRI.

Keywords: Sevoflurane, Renal IRI, Apoptosis, RISK signaling pathway

INTRODUCTION

Ischemia-reperfusion injury (IRI) occurs in various organs including heart, brain, liver and kidneys, and it refers to certain factors that contribute to ischemia of organs or tissues, restoration of blood supply exacerbates organ damage and may even result in irreversible

effects [1,2]. During renal ischemia-reperfusion (IR), metabolites produced combine with oxygen free radicals, leading to cell membrane damage. This affects the intracellular oxidative phosphorylation process, eventually inducing inflammatory response, apoptosis and aggravated tissue damage [3]. Renal IRI frequently occurs during organ transplant and it is an inflammatory response induced by revascularization and anastomosis of blood vessels during transplantation. This results in renal cell apoptosis as well as renal fibrosis and organ rejection at specified future dates. It is an important factor affecting efficacy of organ transplantation [4]. For kidney transplantation, the urgent challenge to be surmounted is to alleviate renal IRI by drug intervention. Reperfusion injury salvage kinase (RISK) signaling pathway consists of extracellular signal-regulated kinase (ERK), protein kinase B (Akt), and glycogen synthase kinase-3β (GSK-3β) [5].

A study conducted by Zhang *et al* [6] showed that myocardial IR decreases levels of phosphorylated (p)-Akt and p-GSK-3β. Ischemia preconditioning increases levels of p-ERK1/2, p-Akt and p-GSK-3β, thus effectively protecting myocardial cells. Sevoflurane, the latest generation of inhaled anesthesia, is a commonly used anesthesia in many operations in clinical practice. It has the characteristics of stable action and fast induction [7]. Wang *et al* [8] studied and found that sevoflurane is capable of activating the phosphatidylinositol 3-kinase (PI3K)/Akt and ERK1/2 signaling pathways to inhibit damage of myocardial cells in diabetic rats. However, studies on the protective effect of sevoflurane on renal IRI in rats and whether the RISK signaling pathway participates therein are rare.

This study aims to investigate the protective effect of sevoflurane on renal IRI in rats and further ascertain its effect on RISK signaling pathway by establishing rat models of renal IRI.

EXPERIMENTAL

Animals

Thirty (30) healthy male Sprague-Dawley rats weighing 280 - 320 g, purchased from the Experimental Animal Center of Tongji Medical College, Huazhong University of Science and Technology, were fed in Specific Pathogen Free (SPF) environment (21 - 23 °C, humidity: 45 - 50 %) under circadian rhythm for 7 days of adaptive training, with free access to food and water. They were divided into sham operation group (n = 10), model group (n = 10) and sevoflurane group (n = 10) using a random number table.

Protocol in this study was reviewed and approved by the Laboratory Animal Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology (approval no. CN-WH-IACUC-2022-236794). All experimental operations involving animals were carried out in accordance with NIH Guide for Care and Use of Laboratory Animals [9].

Model establishment

Rats in each group were anesthetized with 5 % sodium pentobarbital (1.0 mL/kg) *via* intraperitoneal injection, fixed in the supine position and tracheally intubated for mechanical ventilation. Then, abdominal hair was removed and the abdomen was subsequently disinfected and draped. Abdominal skin, rectus abdominis and peritoneum were cut open along the midline of abdomen to expose abdominal cavity, the left and right kidneys separated, and intestine pulled out and covered with sterile gauze moistened with normal saline. Thereafter, left and right kidneys were exposed, right renal pedicle was isolated and ligated, while the left renal pedicle was exposed by gently puncturing the posterior peritoneum with tweezers and clamped with bulldog clamp [10]. Left kidney subsequently turned dark purple. After 45 min, the bulldog clamp was released and dark purple kidney colour turned bright red. Thereafter, abdominal tissues were sutured layer by layer and rats were placed in an electric thermal blanket and put back in rat cages after waking up. Rats in sham operation group only underwent exposure of the kidneys, without clamping the renal pedicle with bulldog clamps, while those in model and sevoflurane groups were prepared into models of renal IRI as described above. In addition, rats in sevoflurane group inhaled 2.2 % sevoflurane (Maruishi Pharmaceutical Co. Ltd) for 15 min immediately at reperfusion. Rats were sacrificed at 6 h after reperfusion to collect blood and kidney tissues for subsequent analyses. All operations were performed as per the previous publication guide [10].

Evaluation of serological indicators

Peripheral blood collected from rats at 6 h after reperfusion was centrifuged at 1000 g and 4 °C for 10 min and supernatant (serum) was collected and sent to the clinical laboratory of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology for determination of serum creatinine (Scr) and blood urea nitrogen (BUN) [11].

Determination of inflammatory response in the infarct zone

After 6 h of reperfusion, the kidneys of all rats were isolated, fixed with 4 % paraformaldehyde at 4 °C overnight, dehydrated in ethanol at different concentrations, permeabilized with xylene for 40 min, embedded in paraffin and cut into 3 µm-thick sections using a microtome. Afterward, sections were placed on clean glass slide, mounted in water to make the tissues fit the slide better, dried on a heating plate and stored for later use. The paraffin sections were deparaffinized, hydrated, stained with Hematoxylin and Eosin (H&E) (Wuhan Boster Biological Technology Co., Ltd., Wuhan, China) successively, decolorized in gradient ethanol, permeabilized with xylene and mounted with an appropriate amount of neutral resin. Lastly, morphological characteristics of each section were observed using an optical microscope (Olympus, Tokyo, Japan).

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) staining

At 6 h after reperfusion, TUNEL staining assay was conducted to determine apoptosis of cells in kidney tissues of rats in each group as follows: Paraffin sections of kidney tissues in each group were deparaffinized, dehydrated, treated with hydrogen peroxide (3 %) for 10 min, washed with phosphate-buffered saline (PBS) three times, digested with an appropriate volume of Proteinase K solution (added dropwise) at 37 °C for 10 min and washed three times in PBS. Next, sections were labeled with 40 µL of mixed solution of TdT and DIG-d-UTP added in drops at 4 °C for 2 h, washed 3 times in PBS, blocked with 40 µL of blocking solution added dropwise at room temperature for 30 min, incubated with biotinylated anti-digoxigenin antibody (1: 100) added in drops at 37 °C for 40 min, washed 3 times with PBS, incubated with SABC-FITC secondary antibody (1: 100) added dropwise at 37 °C for 40 min, washed with PBS for 3 times, mounted with anti-fluorescence quenching solution (added in drops) and neutral resin and photographed using a confocal fluorescence microscope. The apoptosis level of cells in kidney tissues was calculated. TUNEL-positive cells, i.e., apoptotic cells, are yellowish-green, while TUNEL-negative cells, i.e., normal cells, are non-fluorescent.

Evaluation of serum inflammatory factors

Peripheral blood of rats in each group was collected after reperfusion for 6 h, and levels of inflammatory factors such as interleukin-1β (IL-1β), IL-6, IL-10 and tumor necrosis factor-alpha (TNF-α) in rat serum were determined using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) in strict accordance with instructions of kits. Microplate reader was adopted to read absorbance of each group of samples and content of IL-1β, IL-6, IL-10 and TNF-α in peripheral blood was calculated.

Determination of malondialdehyde (MDA) content in kidney tissues

Paraffin sections of rats in each group were deparaffinized and an appropriate volume of lysis buffer was added to it. This was homogenized in an ultrasonic homogenizer and centrifuged at 12000 g and 4 °C for 10 min, followed by collection of supernatants. The MDA content in kidney tissue of each group of rats was determined using an MDA content determination kit (Wuhan Boster Biological Technology Co., Ltd., Wuhan, China). Standard curves and tissue samples were prepared strictly as per the instructions of the kit. Absorbance was read at 532 nm using the microplate reader and MDA content of each group was calculated based on standard curves.

Assessment of reactive oxygen species (ROS) level

Paraffin sections in each group were deparaffinized and appropriate amount of lysis buffer was added followed by homogenization in ultrasonic homogenizer and centrifugation at 12,000*g* and 4 °C for 10 min. Thereafter, supernatant was collected, and level of reactive oxygen species (ROS) was determined by 2,7-Dichlorodi-hydrofluorescein diacetate (DCFH-DA) method (Wuhan Boster Biological Technology Co., Ltd., Wuhan, China) according to instructions of relevant kit. Samples were processed and then photographed with a laser confocal microscope (excitation wavelength was 488 nm while emission wavelength was 525 nm). ROS level in rat kidney tissues in each group was calculated.

Measurement of expression levels of related proteins

After 6 h of IR, kidneys of rats in each group were immediately isolated and appropriate volumes of radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China), 1 % phosphatase inhibitor and 1 % protease inhibitor were added. Thereafter, it was homogenized using the ultrasonic homogenizer

and centrifuged at 12,000 *g* and 4 °C for 10 min. Next, supernatant was collected and protein concentration of samples determined by bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). A loading buffer system with equal solubility was prepared and samples subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein was transferred to a polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA) and subsequently incubated in 5 % skim milk at room temperature for 2 h to block non-specific binding. Target bands were cut, incubated with ERK1/2, phosphorylated (p)-ERK1/2, Akt, p-Akt, GSK-3β, p-GSK-3β antibodies (1:1000, Cell Signaling Technology, Danvers, MA, USA) at 4 °C overnight, washed with Tris-buffered saline with Tween®20 (TBST) 3 times, incubated with horseradish peroxidase (HRP)-labeled goat anti-rabbit secondary antibody (1: 5000, Cell Signaling Technology, Danvers, MA, USA) at room temperature for 2 h and finally washed with TBST 3 times. Subsequently, electrochemiluminescence (ECL) solution was prepared and added to target bands for development and exposure in a dark room. Finally, bands were scanned and expression levels of proteins were analyzed using LabWork 4.6 image analysis software.

Statistical analysis

Data are expressed as mean ± standard deviation (SD), and were analyzed and processed by Statistical Package for the Social Sciences (SPSS) 22.0 software (SPSS Inc, Chicago, IL, USA). Analysis of variance was employed for comparison among groups, Welch test was adopted for data with heterogeneity of variance while Bonferroni test was utilized for pairwise comparison of data with homogeneity of variance. $P < 0.05$ indicate that difference was statistically significant.

RESULTS

Renal function

There were no noticeable abnormalities in rat kidney tissues in sham operation group. There was necrosis of partial epithelial cells, edema and infiltration of inflammatory cells in substantial quantities in rat kidney tissues of model group, while mild edema, necrosis of few cells and infiltration of few inflammatory cells in rat kidney tissues of sevoflurane group was observed. Rats which were sacrificed immediately at 6 h after IR (Figure 1) showed that content of BUN and Scr were significantly higher ($p < 0.01$) in model group than sham group, while a significant

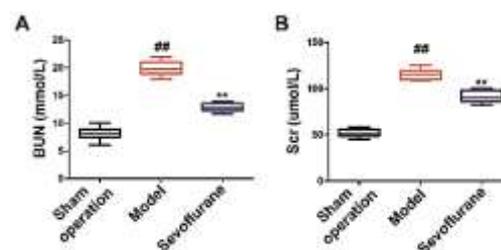decrease was observed in sevoflurane group ($p < 0.01$).

Figure 1: Myocardial infarct size measured in rats. (A) TTC staining (B) Statistical map of infarct size. The myocardial infarct size was significantly larger in the model group than in sham operation and sevoflurane groups. **$P < 0.01$ *vs.* model group, ##$p < 0.01$ *vs.* sham operation group

Apoptosis level

It was found that apoptosis level of cells in kidney tissues of rats was significantly higher ($p < 0.01$) in model group compared to sham operation group, whereas a significant decrease was observed in the sevoflurane group ($p < 0.01$; Figure 2).
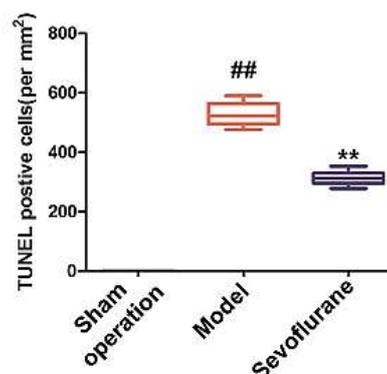

Figure 2: Apoptosis level of cells in kidney tissues determined through TUNEL staining. **$P < 0.01$ *vs.* model group, ##$p < 0.01$ *vs.* sham group

MDA content and ROS level

The results (Figure 3) demonstrate that MDA content and ROS level in kidney tissues of rats were distinctly higher in the model group than those in the sham operation group ($p < 0.01$), but they were significantly lower in sevoflurane group ($p < 0.01$).

Content of inflammatory factors in rat peripheral blood

After peripheral blood was collected from rats sacrificed immediately at 6 h after IR, levels of inflammatory factors were determined. Results revealed that in comparison with sham operation

group, model group had significantly increased levels of IL-6, TNF-α and IL-1β ($p < 0.01$) and an obviously significantly reduced level of IL-10 in peripheral blood. This trend was completely reversed in sevoflurane group ($p < 0.01$; Figure 4).
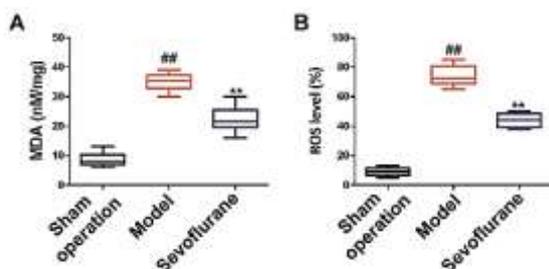

Figure 3: MDA content and ROS level in rat kidney tissues. (A) MDA content (B) ROS level. ***P < 0.01 vs.* model group, ##$p < 0.01$ *vs.* sham group
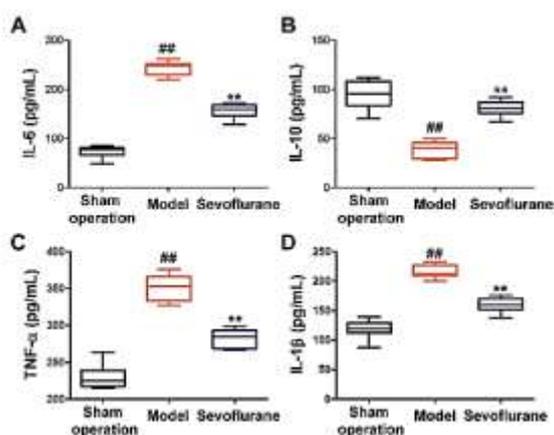

Figure 4: Content of inflammatory factors in rat peripheral blood measured *via* ELISA. (A) Level of IL-6 (B) Level of IL-10 (C) Level of TNF-α (D) Level of IL-1β. The levels of IL-6, TNF-α and IL-1β in peripheral blood are remarkably higher in model group than sham and sevoflurane groups, while level of IL-10 is lower in model group than sham and sevoflurane groups. ***P < 0.01 vs.* model group, ##$p < 0.01$ *vs.* sham group

Expression levels of apoptosis-related proteins in rat kidney tissues

Rats were sacrificed immediately after 6 h of IR to collect kidney tissues to detect the expression level of apoptosis-related proteins therein. It was discovered that compared with sham operation group, expression level of caspase-3 was significantly increased ($p < 0.01$) and B-cell lymphoma 2 (Bcl-2)/Bcl-2-associated X protein (Bax) ratio was significantly reduced in kidney tissues in model group ($p < 0.01$). However, sevoflurane group exhibited reduced expression of caspase-3 ($p < 0.01$) but significantly increased Bcl-2/Bax ratio in kidney tissues compared with model group ($p < 0.01$; Figure 5).
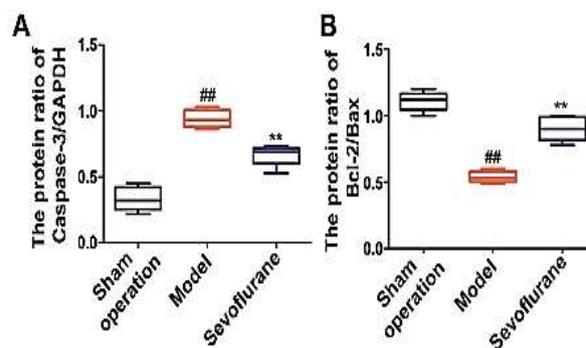

Figure 5: Expression levels of apoptosis-related proteins in rat kidney tissues. (A) Expression level of Caspase-3 (B) Bcl-2/Bax ratio. Expression level of Caspase-3 in kidney tissues is distinctly higher in model group than sham operation group and sevoflurane group, while an opposite trend is seen in Bcl-2/Bax ratio. ***P < 0.01 vs.* model group, ##$p < 0.01$ *vs.* sham operation group

Expression levels of RISK signaling pathway-related proteins

Results presented in Figure 6 show that expression levels of p-ERK1/2, p-Akt and p-GSK-3β in rat kidney tissues were significantly reduced in model group than sham operation group ($p < 0.01$), and significantly higher in sevoflurane group than model group ($p < 0.01$).
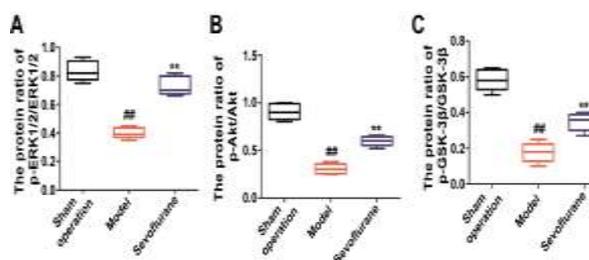

Figure 6: Expression levels of RISK signaling pathway-related proteins in rat kidneys. (A) Expression level of p-ERK1/2 (B) Expression level of p-Akt (C) Expression level of p-GSK-3β. Expression levels of p-ERK1/2, p-Akt and p-GSK-3β in rat kidneys are significantly lower in model group than sham operation sevoflurane groups. ***P < 0.01 vs.* model group, ##$p < 0.01$ *vs.* sham group

DISCUSSION

Renal revascularization after kidney transplantation results in renal IRI. Research evidence showed that renal solid and immune cells participate in renal IR. Activated vascular endothelial cells produce reactive oxygen species, enhance the permeability of renal cell membranes and promote infiltration of inflammatory factors into kidney tissues. Release of pro-inflammatory factors significantly

aggravates the inflammatory response in kidney tissues [12]. Renal tubular epithelial cells are important components of renal parenchymal cells and dysregulated energy metabolism of these cells due to IRI will lead to edema, degeneration and even apoptosis [13]. In this study, it was found that in rats undergoing renal IR, there was severe edema in renal tubular epithelial cells, infiltration of numerous inflammatory cells in renal tubular cells, increased levels of inflammatory factors like IL-6, IL-1β and TNF-α in peripheral blood. Increased MDA content, ROS level in kidney tissues, apoptosis level of renal cells and significantly reduced levels of anti-inflammatory factor IL-10 were also observed in such rats. Research by Xu *et al* [14] noted that ROS produced in cases of cerebral IRI activates inflammatory responses in brain tissues and elevates the level of neuronal apoptosis [14].

In this study, sevoflurane was administrated to rats undergoing renal IR and it was uncovered that sevoflurane significantly mitigated edema of renal tubular cells, reduced levels of inflammatory factors in peripheral blood, MDA, ROS and apoptosis level of cells in kidney tissues. A study by Lu *et al* [15] revealed that sevoflurane inhaled significantly reduces myocardial infarct size and improves cardiac function in rats with myocardial IRI [12]. Numerous studies have emerged from multiple perspectives including animal experiments and clinical trials that sevoflurane exerts an obvious protective effect against myocardial IRI, probably by reducing ROS level in myocardial cells and ameliorating inflammatory responses [16,17]. Hao *et al* [18] found that protective effect of sevoflurane against IRI is dose-dependent and inhalation of 2 – 4 % sevoflurane significantly attenuates IR-induced damage to myocardial cells. Research by Ye *et al* [19] demonstrated that pretreatment with sevoflurane can improve the peroxidation of liposomes in brain tissues of rats after cerebral IR, relieve inflammatory response, reduce expression of apoptosis proteins and alleviate apoptosis of nerve cells. A study by Zhang *et al* [20] noted that sevoflurane activates PI3K/Akt signaling pathway in myocardial cells of myocardial IRI rats to facilitate level of p-ERK1/2, leading to opening of mitochondrial ATP-sensitive potassium channels as well as reduced release of ROS, thereby protecting myocardial cells.

Zhou *et al* [21] found that activating RISK signaling pathway in isolated heart of rats and increasing level of p-GSK-3β ameliorates IR-induced damage to isolated heart. Results of this study revealed that renal IR inhibited the RISK signaling pathway and reduced the levels of p-

ERK1/2, p-Akt and p-GSK-3β in kidney tissues, while treatment with sevoflurane activated the RISK signaling pathway in kidney tissues and protected kidney cells. Xie *et al* [22] found that opioid receptor agonists are capable of increasing levels of p-ERK1/2 and p-Akt in myocardial cells of IR rats, activating RISK signaling pathway and reversing IR-induced damage to rat myocardial cells. GSK-3β is a signaling protein under the control of multiple signaling molecules. Research by Wang *et al* [23] noted that activating PI3K/Akt signaling pathway can markedly increase level of p-GSK-3β, prevent mitochondrial potassium channel from closing and prevent mitochondrial permeability transition pore from opening and protect cells from IRI. As a target molecule of GSK-3β, Bcl-2 is able to effectively modulate the expression of both apoptotic and anti-apoptotic proteins in mitochondria [24]. It was observed in this study that treatment with sevoflurane significantly reduced expression of Caspase-3 in renal tissue cells, increased expression of Bcl-2, decreased apoptosis level of renal cells and ultimately improved renal function of rats undergoing renal IR.

CONCLUSION

Sevoflurane lowers the release of inflammatory factors, ROS level and apoptosis of renal cell. Thus, improvement of renal function through activation of RISK signaling pathway in kidney tissues of rats undergoing renal IR has been observed with the use of sevoflurane. Therefore, sevoflurane can potentially be developed for the management of IRI.

DECLARATIONS

Ethical approval

This study was approved by the Laboratory Animal Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology (approval no. CN-WH-IACUC-2022-236794).

Availability of data and materials

The datasets used and/or analyzed during the

current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them. Bo Wang and Xin Yan contributed equally to this work.

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