RESEARCH ARTICLE

Attenuation of Cardiac Ischaemia-reperfusion Injury by Treatment with Hydrogen-rich Water

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Abstract: Background: Hydrogen has been shown to exert a bioactive effect on the myocardium. This study examined the signalling pathways for hydrogen attenuating ischaemia-reperfusion injury.

Methods: In total, 20 male Wistar rats were evaluated for the effects of hydrogen-rich water on ischaemia-reperfusion in hearts. Left ventricular tissue was taken for screening and analysis of active protein factors by protein chip technology. The enrichment of the KEGG pathway was obtained by using the Gene Ontology (GO) enrichment principle. The expression of JAK2, STAT1, STAT3, p-STAT1, p-JAK2, p-STAT3 in rat myocardium was detected by Western blot analysis and immunohistochemistry. The apoptosis rates of the control and hydrogen-rich water groups were detected by TUNEL staining.

Results: The expression levels of 25 proteins, including five transduction pathways, were downregulated in the hydrogen-rich water group. The expression levels of p-JAK2/JAK2, p-STAT3/STAT3 were upregulated in the hydrogen-rich water group compared with the control group, and p-STAT1/STAT1 was downregulated in the hydrogen-rich water group compared with the control group. Furthermore, the apoptosis rate was significantly decreased in the hydrogen-rich water group, as well.

Conclusion: Hydrogen-rich water may inhibit the apoptosis of cardiomyocytes after ischaemia-reperfusion by upregulating the expression of the JAK2-STAT3 signalling pathway, which reduces ischaemia-reperfusion injury.

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1. INTRODUCTION

Ischaemia-reperfusion injury (IRI) occurs in tissues or organs that receive oxygen-rich blood reflow after a given time of ischaemia. Increasing oxygen radicals, mitochondrial dysfunction, autophagy, calcium overload and energy metabolism disorders may contribute to inflammation and apoptosis during IRI [1-4]. The heart undergoes a process of ischaemia-reperfusion during open heart surgery under cardiopulmonary bypass. The restoration of blood perfusion after myocardial ischaemia can cause ventricular systolic dysfunction, impaired microvasculature, and lethal reperfusion

arrhythmias. On the cellular level, IRI triggers cardiovascular cell injury and necrosis, resulting in scar tissue formation.

Hydrogen is a reductive small gaseous molecule that is demonstrated to exhibit "antioxidant", "anti-inflammatory" and "anti-apoptotic" effects in vivo [5]. Hydrogen reportedly reduces ischaemia-reperfusion by inhibiting apoptosis and inflammation [6-8]. Our previous study has shown that hydrogen can protect the cardiac function of isolated rat heart and reduce mitochondrial damage in cardiac myocytes [9]. To define the molecular pathways involved in the protective impact of hydrogen-rich water (HRW), in the current study, we used a high-throughput, miniaturized, and automated protein chip technology to investigate the subcellular localization of proteins, and protein-protein interactions and to undertake a biochemical

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analysis of the protein functions [10]. Next, we applied the chip technology to study the mechanism of cardiac ischaemia-reperfusion injury in rats treated with or without HRW.

2. MATERIALS AND METHODS

2.1. Animals and Materials

A total of 20 male Wistar rats that weighed 290-320 g were provided by Beijing Vital River Laboratory Technology Co., Ltd. (Beijing, China), Certificate of Conformity: SCXK (Beijing) 2016-001. Experimental hydrogen-rich water (HRW) was provided by Dr. Zhilin Li from the College of Chemistry and Environmental Science of Hebei University of China (China's National Patent, patent number ZL102557227B). A Raybiotech kit (G-Series Rat Cytokine Array 67, GSR-CAA-67) was purchased from RayBiotech, Inc. JAK2, STAT1 and STAT3 antibodies were purchased from Proteintech (Chicago, USA). P-STAT1, P-JAK2 and P-STST3 were purchased from Abcam (Cambridge, MA). A TUNEL kit was purchased from Vazyme (Jiangsu, China). An InnoScan 300 Microarray Scanner Fluorescent scanner (Equipment model: InnoScan 300 Microarray Scanner) was purchased from Innopsys.

2.2. Methods

Rats (10 per group) were randomized into control and HRW groups and were provided with adaptive feeding for one week. The animals were anaesthetized with sodium pentobarbital (50 mg/kg), heparin (250 U / kg) by intraperitoneal injection. After the onset of anaesthesia, a sternotomy was performed, and the heart was removed and was immediately placed in ice-cold water at 4 °C. Aortic cannulation was performed, and the myocardium was fixed through an ex vivo perfusion Langendorff apparatus. In the control group, cardiac perfusion was performed at 37 °C in Krebs-Ringer perfusate with oxygen balance (95% O₂ + 5% CO₂). The hydrogen-rich water group was treated with 37 °C Krebs-Ringer solution + hydrogen-rich water perfusion (0.6 mmol / L, pH 7.3) with oxygen balance (95% O₂ + 5% CO₂); the perfusion pressure of the two groups was 7.85 kPa. After undergoing reverse perfusion for 10 min at room temperature, the hearts were set aside for 20 min and then were reperfused for 20 min, and the left ventricular myocardia were reserved.

2.3. Protein Arrays

1. The glass slides were removed from the box and were allowed to equilibrate to room temperature inside the sealed plastic bag for 20-30 min. The slides were removed from the plastic bag, the cover film was removed, and the slides were allowed to air dry for another 1-2 hours. Incomplete drying of the slides before use may cause the formation of "comet tails," thin directional smearing of antibody spots. 2. Sample Diluent (100 µl) was added into each well and was incubated at room temperature for 30 min to block the slides. 3. The buffer was decanted from each well, and

100 µl of sample was added to each well. The arrays were incubated at room temperature for 1-2 hours. Longer incubation time is preferable for higher signals. This step may be undertaken overnight at 4 °C. We recommend using 50 to 100 µl of original or diluted serum, plasma, conditioned media, or other body fluid, or 50-500 µg/ml of protein for cell and tissue lysates. The incubation chamber was covered with the adhesive film during incubation, especially if less than 70 µl of sample or reagent was used. 4. Wash: The samples were decanted from each well, and the wells were washed 5 times (5 min each) with 150 µl of 1x Wash Buffer I at room temperature with gentle shaking. The wash buffer was completely removed in each wash step. Wash Buffer I was diluted 20x with H₂O (Optional for Cell and Tissue Lysates). The glass slides with their frames were placed into a box with 1x Wash Buffer I (the whole glass slide and its frame were covered with Wash Buffer I), and the slides were washed at room temperature with gentle shaking for 20 min. The 1x Wash Buffer I was decanted from each well and the wells were washed 2 times (5 min each) with 150 µl of 1x Wash Buffer II at room temperature with gentle shaking. The wash buffer was completely removed in each wash step. Then, 20x Wash Buffer II was diluted with H₂O. Incomplete removal of the wash buffer in each wash step may cause "dark spots," which are background signals higher than the spots. 5. The detection antibody was reconstituted by adding 1.4 ml of Sample Diluent to the tube, and the tubes were spun briefly. 6. The detection antibody cocktail (80 µl) was added to each well, and the wells were incubated at room temperature for 1-2 hours. Longer incubation time is preferable for higher signals. 7. The samples were decanted from each well, and the wells were washed 5 times (5 mins each) with 150 µl of 1x Wash Buffer I and then 2 times with 150 µl of 1x Wash Buffer II at room temperature with gentle shaking. The wash buffer was completely removed in each wash step. 8. After the samples were briefly spun down, 1.4 ml of Sample Diluent was added to the Cy3 equivalent dye-conjugated streptavidin tube, and the contents were mixed gently. 9. Then, 80 µl of Cy3 equivalent dye-conjugated streptavidin was added to each well. The device was covered with aluminium foil to avoid exposure to light and was incubated in a dark room at room temperature for 1 hour. 10. The samples were decanted from each well and were washed 5 times (5 mins each) with 150 µl of 1x Wash Buffer I at room temperature with gentle shaking. The wash buffer was completely removed in each wash step. 11. The device was disassembled by pushing the clips outward from the side of the slide. The slide was carefully removed from the gasket without touching the surface of the array side. 12. The slide was placed in the Slide Washer/Dryer (a 4-slide holder/centrifuge tube), and enough 1x Wash Buffer I (approximately 30 ml) was added to cover the whole slide; then, the slide was gently shaken at room temperature for 15 min. Next, Wash Buffer I was decanted, and the slides were washed with 1x Wash Buffer II (approximately 30 ml) and gently shaken at room temperature for 5 min. 13.

The water droplets were completely removed by gently applying suction with a pipette, being careful not to touch the array, only the slides. The glass slides were also dried by a compressed N₂ stream. 14. Imaging: The signals were visualized through the use of a laser scanner equipped with a Cy3 wavelength (green channel) such as Axon GenePix. When the signal intensity for different cytokine varies greatly in the same array, multiple scans were performed with a higher PMT for low signal cytokines and with a low PMT for high signal cytokines. 15. Data extraction was performed using the GAL file that was specific for this array along with the microarray analysis software (e.g., GenePix, ScanArray Express, ArrayVision, and MicroVigene).

2.4. Detection of Immunohistochemistry and Western Blotting

Detection of p-STAT1, p-JAK2, p-STAT3 and other proteins was pursued by immunohistochemistry. Myocardial tissue was fixed with 10% formalin, dehydrated with gradient ethanol dehydration, paraffin embedded and sectioned into 4- μ m-thick sections. The immunohistochemical detection was performed according to the kit instructions. Images were taken at $\times 200$ with a light microscope; each slice was randomly taken for 5 different fields of vision using the Image-pro plus (IPP) image analysis system to analyse images. The expression of JAK2, STAT1, STAT3, p-STAT1, p-JAK2, and p-STAT3 proteins was detected by Western blotting. After the tissue was homogenized, the protein was extracted, and 60 μ g of total protein was denatured. If not used immediately, the samples were stored at -20 °C. Proteins were separated by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and the PVDF membranes were wet-transferred. Skim milk powder and PBST was used to dilute the primary antibodies. For the primary antibody incubation, a good amount of primary antibody diluted in PBST was added to PVDF membrane, which was placed on 4 °C shaker overnight. The membrane was washed and was incubated with the secondary antibody at 37 °C, and then the membrane was washed again. A fluoroskan to tablet was used to develop the chemiluminescent signal, and then an Image-pro plus (IPP) image analysis system was used to analyse the results.

2.5. Detect Apoptosis by TUNEL

In strict accordance with the kit instructions, after sample pretreatment, labelling and detection and the completion of the photograph under a fluorescence microscope, 5 positive fields were randomly taken from each section, the average was calculated, and the apoptosis rate was analysed.

2.6. Data Analysis

2.6.1. The Normalization of the Original Data

The scanning background reading of the original data obtained by the chip was removed by Raybiotech

software, and the background removal between the chips was normalized.

2.6.2. Differential Gene Screening

After the original data was normalized by software, the data were selected for analysis. Differential proteins were screened by t-test, and the Fold-change (expression of fold difference) was calculated. The selection criteria was Foldchange ≤ 0.83 or Foldchange ≥ 1.2 , fluorescence signal > 150 (p-value < 0.05).

2.6.3. Differentiation Gene GO Enrichment Analysis

Gene Ontology (GO) is an international standard classification system of gene function. GO can be divided into a molecular function, biological processes and cellular components. By GO enrichment analysis, it is possible to find important functions that lead to changes in traits and to find the gene to which this function corresponds. Using Fisher's exact test, the data packet was clusterProfiler from R / bioconductor; the selection criteria was the number of proteins that fell on a term / GO ≥ 2 , $p < 0.05$.

2.6.4. Cluster Analysis

The 2-function heatmap approach was used, and the datagram was gplots, from R / Bioconductor. The distance between two samples was calculated with Euclidean geometry; the distance between two clusters was calculated as the nearest neighbour; and the distance between two classes was defined as the maximum distance between two classes of data.

2.6.5. Enrichment Analysis of Differential Protein KEGG Pathway

Fisher's exact test was adopted. The data packet was clusterProfiler from R / bioconductor. The criteria for selection was the number of genes that fall in one term/pathways is greater than or equal to 5, $p < 0.05$.

2.7. Statistical Analysis

Results of WB, IHC-P and TUNEL have been indicated in terms of mean $\pm$ SD. The difference between groups was analysed by ANOVA test. The minimum level of significance was fixed at $p < 0.05$.

3. RESULTS

3.1. Microarray Show Differential Protein Expression in the Hearts with IRI

In isolated ischaemia-reperfusion injury in the rat heart, left ventricular tissue was taken for screening and analysis of active protein factors by protein chip technology. Using the GSR-CAA-67 data analysis software to analyse data, we found that compared to the control group, there were 25 proteins that were downregulated ($p < 0.05$) (Table I).

3.2. Differential Expression of the Plasma Membrane in the Hearts with IRI

The results of GO analysis showed differential expression of proteins mainly distributed in the lateral

Table I. The differentially expressed proteins between case group and control group (N=10/group).

Protein name	Gene ID	Foldchange (HRW group/ control group)	P
Gas 1	683470	0.67366	0.000045
Fit-3L	103691134	0.66336	0.000088
IL-17F	301291	0.65264	0.000154
Galectin-1	56646	0.72155	0.000775
JAM-A	116479	0.70737	0.000943
TIM-1	286934	0.64563	0.028159
Adiponectin	246253	0.65508	0.002857
IL-3	24495	0.81244	0.003768
Erythropoietin	24335	0.75290	0.003970
4-1BB	500590	0.67767	0.005473
MIP-1a	25542	0.82481	0.006305
B7-1	25408	0.81611	0.006446
GFR alpha-1	25454	0.80775	0.009870
IL-2 Ra	25704	0.68152	0.011098
Notch-1	25496	0.77854	0.013427
Fractalkine	89808	0.68499	0.015341
IL-22	500836	0.59740	0.015728
P-Cadherin	116777	0.70260	0.017220
Decorin	29139	0.72011	0.020027
Nope	363081	0.76400	0.020463
CINC-3	114105	0.75123	0.024528
CD48	245962	0.82264	0.027651
TIMP-1	116510	0.64563	0.002634
Prolactin	24683	0.75411	0.036032
Prolactin R	24684	0.75411	0.045943

plasma membranes of the plasma membrane, particularly those proteins involved in binding and catalytic activities in the regulation of signalling pathways, in the inflammatory response, in apoptosis, in immune regulation and in cytokine secretion (Fig. 1).

3.3. Differential Expression of Proteins in the Hearts with IRI by KEGG Pathway Enrichment Analysis

In this experiment, a total of 5 pathways were screened. They were cytokine-cytokine receptor interactions, the JAK-STAT signalling pathway, Haematopoietic cell lineage, the PI3K-Akt signalling pathway and Th17 cell differentiation (Fig. 2).

3.4. JAK-STAT Signalling Pathway Expression in the Hearts with IRI

Compared to the control group, the proportion of p-JAK / JAK and p-STAT3 / STAT3 increased, while the proportion of p-STAT1 / STAT1 decreased in the hydrogen-enriched water group (all $p < 0.01$) (Fig. 3).

The results of IHC-P showed that the expression of p-JAK2 and p-STAT3 levels in the hydrogen-rich water group were higher than those in the control group. In contrast, the expression of p-STAT1 was significantly lower ($p < 0.01$). Details are shown in Fig. 4.

3.5. HRW Inhibits Apoptosis in the Hearts with IRI

TUNEL was used to detect the effect of hydrogen-rich water on myocardial cell apoptosis after myocardial ischaemia-reperfusion injury. The results showed that the apoptosis in the hydrogen-rich water group was significantly reduced compared to that in the controls ($p < 0.01$) (Fig. 5).

4. DISCUSSION

In this study, a protein chip technology was used for assessing myocardial proteins in IRI rats treated with or without HRW. Furthermore, GO analysis was performed on the biological function of differential proteins. The results showed that the expression of 25 proteins in the HRW group was downregulated

Fig. (1). The GO classification map of expression protein annotation, protein microarrays of 10 results were analysed. Three illustrations represents cellular component, molecular function and bioprocess respectively.

compared with those in the control group. Five proteins showed the most significant difference, including Gas 1, Flt-3L, IL-17F, Galectin-1 and JAM-A. Differential proteins are mainly involved in the regulation of signalling pathways, the inflammatory response, apoptosis, immune regulation, the regulation of cytokine secretion and other biological processes.

Fig. (2). Changes in the KEGG pathways in the hearts with IRI enriched with the differentially expressed proteins.

The results showed that the expression of Gas 1, Flt-3L, IL-17F and IL-22 in the HRW group significantly decreased compared with the levels in the control group. Gas 1 is one of the tumour suppressors that is expressed when cell development blocked. Gas 1 can also regulate cell proliferation and induce the apoptosis of different cell lines [11]. Flt-3L can promote the proliferation and differentiation of DC, and DC can induce the proliferation and differentiation of Th17, which can then secrete IL-17F and IL-22 [12-15]. IL-17F is a member of the IL-17 cytokine family, similar to IL-17. IL-17 uses IL-17RA and IL-17RC as its receptors, and it uses Act1 and TRAF6 as its signal transduction factors to induce many different cell types to express proinflammatory cytokines and chemokines [16,17]. IL-22 is also a kind of proinflammatory factor that plays an important role in regulating inflammation. According to the experimental results, HRW may reduce inflammatory responses and apoptosis, and it may alleviate myocardial ischaemia-reperfusion injury by inhibiting the expression of Gas 1 and Flt-3L, leading to the inhibition of the expression of IL-17F and IL-22, which are induced by Flt-3L.

The experiment results show that compared with the control group, the expression of Galectin 1 and JAM-A decreased significantly in the HRW group. Galectin 1 is the first known factor in the galactose lectin family, which participates in inflammation, apoptosis, cell adhesion, and other biological processes [18]. However, the role of Galectin-1 in the modulation of inflammation is still controversial. It has been reported that Galectin 1 can induce inflammation [19]. However, Seropian IM found simulated hypoxia and proinflammatory cytokines selectively raised the expression of Gal-1 in mouse cardiomyocytes, while the anti-inflammatory cytokine inhibited the expression of the lectins. In this case, myocardial inflammation increased when Gal-1 was reduced; so, Galectin-1 had a protective effect on the myocardium [20].

JAM-A is a member of the immunoglobulin superfamily, which plays an important role in normal tissue structure maintenance, inflammation and immune response, wound repair and other processes.

Fig. (3). Changes in the hearts with IRI and treatment of HRW. (A) Representative blots. 1 represents the MI/R group, 2 represents the MI/R+H2 group. (B) p-JAK2/JAK2 ratio. (C) p-STAT1/STAT1 ratio. (D) p-STAT3/STAT3 ratio. (E) JAK2/ β -actin ratio. (F) STAT1/ β -actin ratio. (G) STAT3/ β -actin ratio. The results are expressed as the mean $\pm$ SD, n=10/group. #p<0.01 and *p>0.05 vs the MI/R+H2 group.

Fig. (4). Changes in the hearts with IRI and treatment of HRW. (A) p-JAK2 expression results of the MI/R group; (B) p-JAK2 expression results of the MI/R+H2 group; (C) p-STAT1 expression results of the MI/R group; (D) p-STAT1 expression results of the MI/R+H2 group; (E) p-STAT3 expression results of the MI/R group; (F) p-STAT3 expression results of the MI/R+H2 group; (G) p-JAK2 positive cell expression rate; (H) p-STAT1 positive cell expression rate; (I) p-STAT3 positive cell expression rate. The results were expressed as the mean $\pm$ SD, n=10/group. #p<0.01 vs the MI/R+H2 group.

In myocardial ischaemia-reperfusion injury, neutrophil infiltration can cause tissue damage; JAM-A is an essential protein for neutrophil infiltration. When the JAM-A gene was knockout, neutrophil infiltration was significantly reduced [21]. However, during ischaemia-reperfusion of the liver, it was found that although the lack of JAM-A gene reduced neutrophil infiltration, it

also increased hepatocyte apoptosis [22]. HRW may reduce myocardial ischaemia-reperfusion injury by inhibiting the expression of JAM-A and Galectin 1.

In this experiment, a total of 5 pathways were screened. The pathways were cytokine-cytokine receptor interaction, the JAK-STAT signalling pathway,

Fig. (5). TUNEL of apoptotic cardiomyocytes after myocardial ischaemia-reperfusion injury with or without treatment of HRW. (A) Representative photomicrographs of TUNEL staining. The green fluorescence shows the TUNEL-positive nuclei; the red fluorescence shows the nuclei of all cardiomyocytes; original magnification $\times 200$. (B) Percentage of TUNEL-positive nuclei. The results are expressed as the mean $\pm$ SD, $n=10$ /group. $\# p<0.01$ vs. the MI/R+H₂ group.

the hematopoietic cell lineage, the PI3K-Akt signalling pathway and Th17 cell differentiation. The PI3K-AKT pathway is composed of PI3K and Akt, which are widely found in various organ tissues in the body. Its main functions include controlling energy metabolism, inhibiting cell apoptosis, and promoting cell proliferation [23,24]. It has been reported that activating the PI3K-AKT signalling pathway has a protective effect on myocardial ischaemia-reperfusion injury [25,26]. In addition, hydrogen can also protect cells from ischaemia-reperfusion injury through the PI3K-AKT signalling pathway [27]. The KEGG pathway results showed that HRW may protect cells from myocardial ischaemia-reperfusion injury through the PI3K-AKT signalling pathway.

The JAK-STAT signalling pathway was demonstrated to participate in various biological processes of cell proliferation, differentiation, apoptosis and inflammation [28-30]. JAK is a family of non-receptor tyrosine kinases, the members of which include JAK1, JAK2, JAK3 and TYK2. STAT is the substrate of JAK, which is the signal transduction and transcriptional activator. STAT has seven family members (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6) that are expressed in myocardial cells [31].

JAK-STAT signalling pathways proceed as follows: The cytokine binds to the receptor and causes the dimerization of the receptor→The activation of JAKs→The phosphorylation of STATs→STATs form a dimer and enter the nucleus→The STAT dimer binds to the target gene promoter to regulate gene expression [32].

In this study, the results showed that the JAK-STAT signalling pathway was closely related to myocardial ischaemia-reperfusion injury. Activation of the JAK-STAT signalling pathway can alleviate myocardial ischaemia-reperfusion injury [33,34]. In the JAK-STAT signalling pathway, STAT1 played a role in promoting the apoptosis of myocardial cells, while STAT3 inhibited apoptosis and protected the heart [35]. Based on the above theoretical basis, we established an ex vivo Langendorff model to further study the role of hydrogen-rich water in myocardial IRI and provide experimental data for the use of hydrogen for myocardial protection in open heart surgery with cardiopulmonary bypass.

The results showed that the P-JAK2/JAK2 and P-STAT3/STAT3 increased when compared to the control, while the P-STAT1/STAT1 decreased in the hydrogen-rich water group. In addition, the apoptosis

rate of the HRW group decreased significantly. These results will provide experimental data for the use of hydrogen in myocardial protection for open heart surgery with extracorporeal circulation. However, as the experiment is still at the level of basic research, further research is needed to clarify this method's clinical application.

CONCLUSION

HRW inhibits the apoptosis of myocardial cells after the ischaemia reperfusion and alleviates ischaemia-reperfusion injury by upregulating the expression of the JAK-STAT signalling pathway.

ABBREVIATIONS

GO	=	Gene Ontology
IRI	=	Ischaemia-reperfusion injury
HRW	=	Hydrogen-rich water
IPP	=	Image-pro plus
SDS-PAGE	=	SDS-polyacrylamide gel electrophoresis

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study is approved by the animal Welfare and ethics committee of Hebei University, China.

HUMAN AND ANIMAL RIGHTS

No human was used in this research. All animal research procedures followed were in accordance with the standards set forth in the eighth edition of Guide for the Care and Use of Laboratory Animals published by the National Academy of Sciences, The National Academies Press, Washington, D.C.).

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Availability of data and materials is real and effective.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Declared none.

AUTHORS' CONTRIBUTIONS

Fulin Liu and Yu YuJuan Zhou gave guidance on the design and writing of our experiments. Xiangzi Li and Liangtong Li did the main part of the experiment and wrote this article. Liu Xuanchen, Wu Jiawen, Sun Xiaoyu contributed to the manufacture of animal experimental models. Zhilin Li provided us with some of the reagents needed for the experiment. Yan Yongjian gave guidance on the writing of the article. All authors read and approved the final manuscript.

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