Journal of Inflammation Research

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ORIGINAL RESEARCH

Effects of Cooling Interventions with Different Target Temperatures on Heat Stroke Rats

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Purpose: To investigate the optimal target temperature of cooling intervention in heat stroke (HS) rats and explore the potential mechanisms of cooling intervention in alleviating heat stroke-induced damage.

Materials and Methods: A total of 32 Sprague-Dawley rats were randomly divided into 4 groups (n=8/group), including control, HS [core body temperature (Tc)], HS(Tc-1°C) and HS(Tc+1°C) group. Heat stroke model was established in rats of HS(Tc), HS(Tc-1°C) and HS(Tc+1°C) group. Rats in HS(Tc) group were cooled to baseline core body temperature after establishing heat stroke model, HS (Tc-1°C) group to baseline core body temperature minus 1°C and HS(Tc+1°C) group to baseline core body temperature plus 1°C. We compared the histopathological changes of lung, liver and renal tissue, as well as cell apoptosis and expression of critical proteins in phosphatidylinositol 3′-kinase (PI3K)/Akt signaling pathway.

Results: Heat stroke caused the histopathological damage and cell apoptosis of lung, liver and renal tissue, which could be alleviated by cooling intervention to a certain extent. Notably, HS(Tc+1°C) group demonstrated a better effect on alleviating cell apoptosis although the differences were not significant. Heat stroke lead to the elevated expression of p-Akt, which subsequently induced the elevated expression of Caspase-3 and Bax, as well as the decreased expression of Bcl-2. Cooling intervention could reverse this trend. Notably, the expression level of Bax in lung tissue of HS(Tc+1°C) group was significantly lower than that of HS(Tc) and HS(Tc-1°C) group.

Conclusion: The mechanisms of cooling intervention in alleviating heat stroke-induced damage were associated with the expression changes of p-Akt, Caspase-3, Bax and Bcl-2. The better effect of Tc+1°C might be associated with low expression of Bax.

Keywords: heat stroke, cooling intervention, target temperature, histopathological damage, cell apoptosis, PI3K/Akt signaling pathway

Introduction

Heat stroke (HS), characterized by an increased core body temperature (Hc)≥40°C, is a life-threatening illness.[1,2] Hyperthermia is the first stage of heat stroke development, which can lead to irreversible changes at the cellular level.[3] HS is associated with central nervous system (CNS) dysfunction including unconsciousness, coma, convulsion, etc.[4] Most of heat stroke patients can progress to multiple organ dysfunction syndrome (MODS),[5] and severe heat stroke can lead to multiple organ failure and death.[2] The incidence of heat stroke has been increasing as the intensity and duration of unprecedented heatwaves rose in the last few years, and it has become a serious public health problem.[6–9] Lowering core body temperature is the primary goal of treatment for heat stroke. However, the optimal target temperature still remains controversial. Most studies on evaporative cooling have utilized a target temperature near 38°C, while studies on immersion cooling instead have utilized a target temperature just below 39°C.[10]

At present, it is believed that apoptosis has an important role in cell death and tissue injury induced by high heat.[11] Furthermore, endothelial cell damage induced by acute heat stress can lead to apoptosis.[12,13] These results imply that apoptotic death of endothelial cells is involved in the pathological mechanism of heat stroke. The phosphatidylinositol 3′-kinase (PI3K)/Akt signaling pathway is a classical pathway to inhibit apoptosis, delivering important antiapoptotic signals in the survival of

Received: 16 February 2023
Accepted: 17 May 2023
Published: 31 May 2023

cells. It has been reported that Akt has an inhibitory effect on apoptosis pathways mediated by receptors and mitochondria.[14] In this study, different target temperatures were used to intervene the mouse heat stroke model. The aim was to investigate the optimal target temperature through comparing the changes in cell apoptosis and tissue damage and explore the potential mechanisms.

Materials and Methods

Animals

Male and specific pathogen free (SPF) Sprague-Dawley rats were selected as laboratory animals in this study. A total of 32 rats were included with an age of 12–14 weeks and weight of 225.97±13.24 g. All procedures received the approval of the Xinjiang Medical University Animal Protection and Use Committee [No.: SCXK(Xin)2016-0003] and were performed following Laboratory animal-Guideline for ethical review of animal welfare (GB/T 35892-2018). All the rats were allowed to acclimate for 7 days in the SPF facilities at 23±1°C, 40±5% humidity and 12:12 h light–dark cycle.

Grouping

All 32 rats were randomly divided into 4 groups (n=8/group), including control group, HS[core body temperature (Tc)] group, HS(Tc-1°C) group and HS(Tc+1°C) group. The control group was kept at 23±1°C and 40±5% humidity. Heat stroke model was established in rats of HS(Tc) group, HS(Tc-1°C) group and HS(Tc+1°C) group. Rats in HS(Tc) group were cooled to baseline core body temperature after being removed from the thermal simulation cabin, HS(Tc-1°C) group to core body temperature minus 1°C and HS(Tc+1°C) group to core body temperature plus 1°C.

Heat Stroke Model

The rats were fasted for 12 hours without water deprivation before the experiment. After intraperitoneal anesthesia with 10% chloral hydrate, the rats were placed in the thermal simulation cabin with a temperature of 37.5±0.5°C and humidity of 65+5% in the prone position, and heat stroke model was established when their core body temperature (anal temperature) reached to 42.0°C. The XR200 Animal Thermostatic Apparatus (Shanghai XinRuan Information Technology Co., Ltd, China) was used to measure anal temperature. This device has a stainless steel anal temperature sensor with 2 mm round head, non-irritating to animals, which can be used to dynamically monitor anal temperature. The core body temperature of each rat was monitored every 15 min when it was ≤41°C and was monitored every 10 min when it was >41°C until it reached to 42°C. The rat was then removed from the thermal simulation cabin and cooled immediately by a room temperature water bath to the target temperature. The target temperature should be maintained for 3 hours with a cooling blanket.

Histopathological Examination

The rats were sacrificed by cervical dislocation after anesthesia, and the lung, liver and renal tissues were collected and fixed with 10% formalin. The paraffin embedded sections of rat tissues were prepared according to the procedure of histopathological section preparation, and the sections were then stained by hematoxylin and eosin (HE). The histopathological changes were observed under the microscope and the images were collected.

Detection of Cell Apoptosis

The cell apoptosis of lung, liver and renal tissues was detected by terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) staining. TUNEL staining was performed with In Situ Cell Death Detection Kit, POD (Boster Biological Technology Co. Ltd, China) strictly following the manufacturer's instruction. Apoptotic index (AI) was calculated by counting apoptotic cells and total cells in 10 visual fields (100× magnification) selected randomly.

Measurement of Protein Expression

Western blotting was performed to measure the expression levels of critical proteins in PI3K/Akt signaling pathway in lung, liver and renal tissues. The total protein was extracted from the above tissues with radio immunoprecipitation assay (RIPA) lysis buffer (Boster Biological Technology Co. Ltd, China) and protein concentration was measured using BCA Protein Assay Kit (TransGen Biotech, China). The total protein was firstly isolated through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto Immobilon-P membranes (Millipore co, USA). Blocking was conducted in Tris-buffered saline containing 0.1% Tween-20 and 5% skimmed-milk powder. Anti-mouse Bax (1:400), Bcl-2 (1:400), Caspase-3 (1:400), PI3K (1:400), AKT (1:500) and p-AKT (1:300) were purchased from Boster Biological Technology Co. Ltd and utilized as primary antibodies, and anti-mouse antibodies conjugated to HRP were utilized as secondary antibodies. The intrinsic quality control selected β-actin (Abmart, Shanghai, China, 1:1000). The bands were detected with the Chemiluminescence Imaging System (Chemiscope 3000, Clinx, Shanghai, China). The software of Image J was employed to evaluate the relative expression levels of proteins.

Statistical Analysis

Statistical significance of all data was analyzed with the SPSS version 20.0 (SPSS Inc., USA), and a two-sided P value <0.05 was considered to be statistically significant. The normality of continuous data was assessed with the Shapiro–Wilk test. Normally distributed data were expressed as mean ± SD and compared with One-Way Analysis of Variance (ANOVA) followed by post hoc multiple comparisons.

Results

General Data

There were no significant differences in body weight, baseline core body temperature and preparation time of heat stroke model between control, HS(Tc) group, HS(Tc-1°C) group and HS(Tc+1°C) group. The required times of cooling to the target temperature were statistically different between HS(Tc) group, HS(Tc-1°C) group and HS(Tc+1°C) group (Table 1).

Histopathological results

The lung histopathological results are shown in Figure 1. In the control group, the lung tissue was structurally intact, a small number of alveolar walls were thickened, and inflammatory cell infiltration could be observed under some bronchial mucosae. In the HS(Tc) group, the lung tissue structure was incomplete, some alveolar walls were ruptured and dilated or thickened significantly, interstitial congestion accompanied by intravascular thrombosis could be observed, and a large number of inflammatory cells were infiltrated in the submucosa and interstitium of the bronchus. Compared with HS(Tc) group, alveolar wall thickening and rupture were significantly alleviated in HS(Tc-1°C) group and HS(Tc+1°C) group, as well as interstitial inflammatory cell infiltration, congestion and intravascular thrombosis.

The liver histopathological results are shown in Figure 2. In control group, the structure of liver tissue was clear with polygonal liver cells, pink cytoplasm, round nuclei and visible nucleoli. In the HS(Tc) group, the structure of hepatic lobules was disordered, the structure of hepatic cords was unclear, some hepatocytes were of edema and vacuolar degeneration, some areas showed more nuclear shrinkage, necrotic foci and hepatic sinus congestion, and some central veins were dilated accompanied with congestion, and some portal areas had more inflammatory cell infiltration.

Table 1 General Data of Blank Control, HS(Tc) Group, HS(Tc-1°C) Group and HS(Tc+1°C) Group

Groups	Body Temperature (g)	Baseline Core Body Temperature (°C)	Preparation Time of Heat Stroke Model (Min)	Required Times of Cooling to Target Temperatures (Min)
Blank control group	229.650±16.404			
HS(Tc) group	220.800±11.420	38.138±0.699	27.38±3.543	11.75±2.121$^{▲}$
HS(Tc-1°C) group	230.475±13.538	38.438±0.297	28.00±5.345	15.62±2.669^{Δ}
HS(Tc+1°C) group	228.578±13.884	38.537±0.293	27.00±3.665	7.75±1.488$^{▲Δ}$

Notes: $^{Δ}P < 0.05$, vs HS(Tc) group; $^{▲}P < 0.05$, vs HS(Tc-1°C) group.

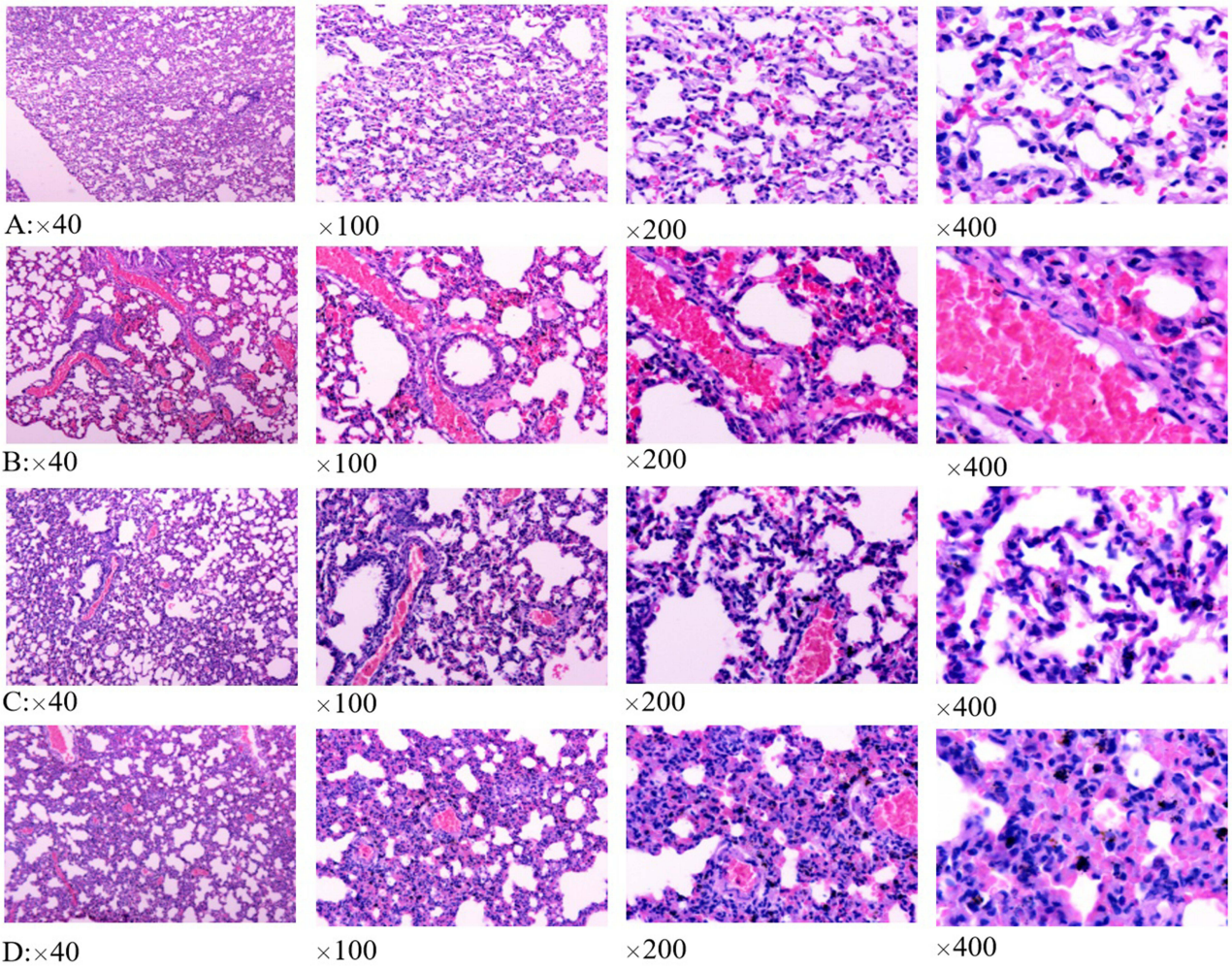

Figure 1 Lung histopathological results of different groups. (**A**–**D**) were the results of control group, HS(Tc) group, HS(Tc-1°C) group and HS(Tc+1°C) group, respectively.
Abbreviations: HS, heat stroke; Tc, core body temperature.

Compared with HS(Tc) group, hepatocyte edema and inflammatory cell infiltration were significantly alleviated in HS (Tc-1°C) group, as well as congestion of hepatic sinusoids, portal areas and central veins. The histopathological results of HS(Tc+1°C) group were not significantly different from those of HS(Tc) group.

The Renal histopathological results are shown in Figure 3. In control group, the structure of the renal tissue was clear with normal glomeruli and renal tubules. In HS(Tc) group, the structure of renal tissue showed focal disorder, some tubular epithelial cells were of edema and vacuolar degeneration with tubular formation, and there were more bleeding foci and inflammatory cell infiltration in the interstitium. The histopathological results of HS(Tc-1°C) group were not significantly different from those of HS(Tc) group, only demonstrating slight alleviation of edema of tubular epithelial cells. Compared with HS(Tc) group, tubular epithelial cell edema and damage were alleviated in HS(Tc+1°C) group, as well as tubular formation, interstitial bleeding and inflammatory cell infiltration.

Cell Apoptosis

The TUNEL staining results of lung, liver and renal tissue are demonstrated in Figure 4. As shown in Figure 5, the AIs of HS groups were significantly higher than those of control group in lung, liver and renal tissue ($P < 0.05$), the AIs of HS (Tc-1°C) group and HS(Tc+1°C) group were significantly lower than that of HS(Tc) group in lung and liver tissue ($P < 0.05$), and the AI of HS(Tc+1°C) group was significantly lower than that of HS(Tc) group in renal tissue ($P < 0.05$). Meanwhile, the AIs of HS(Tc+1°C) group were slightly lower than those of HS(Tc-1°C) group in lung, liver and renal

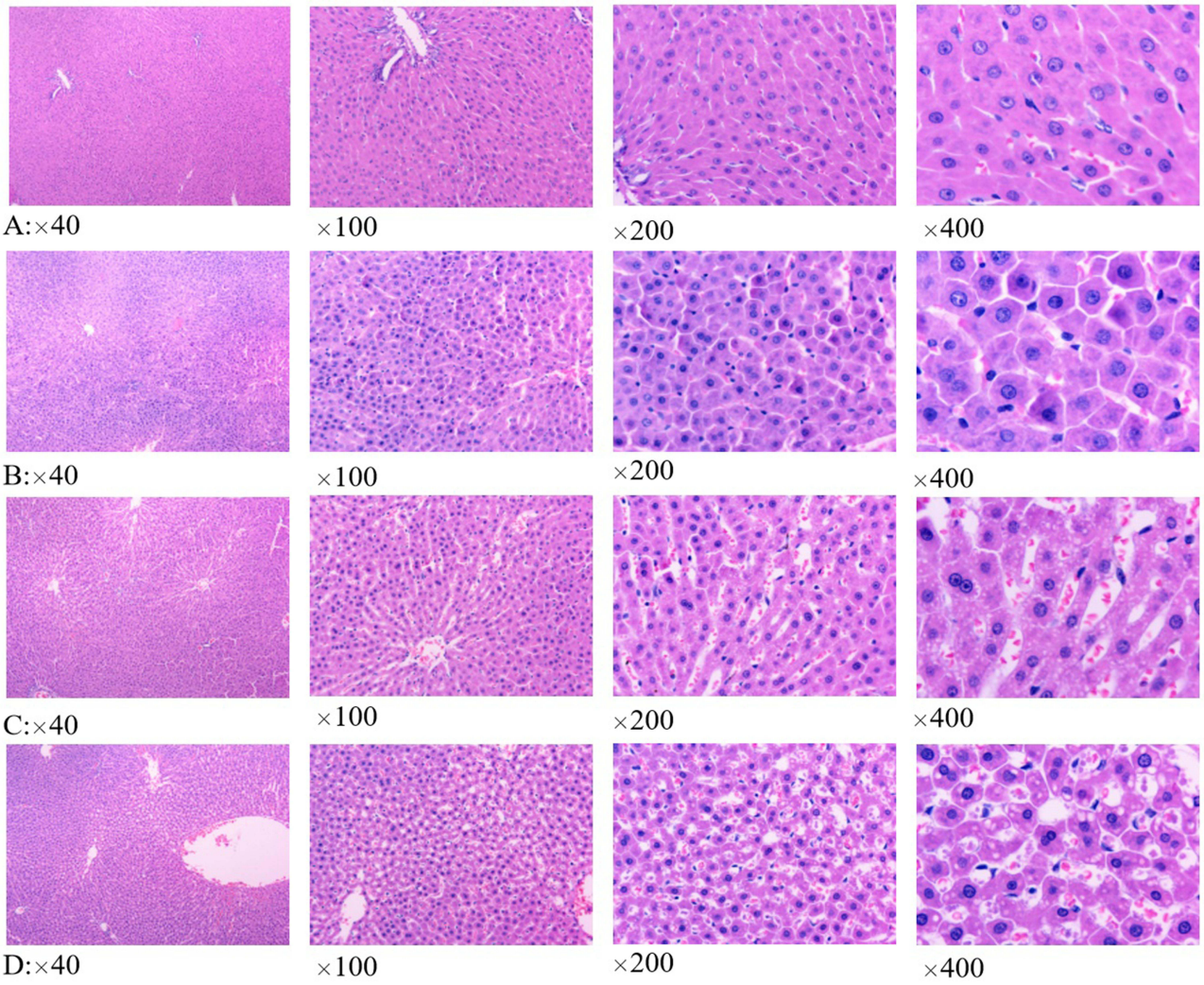

Figure 2 Liver histopathological results of different groups. (**A–D**) were the results of control group, HS(Tc) group, HS(Tc-1°C) group and HS(Tc+1°C) group, respectively.
Abbreviations: HS, heat stroke; Tc, core body temperature.

tissue although the differences were not significant ($P > 0.05$). These results suggested that heat stroke resulted in cell apoptosis in the lung, liver and renal tissue, and cooling intervention could alleviate the cell apoptosis. Notably, the target temperature set at core body temperature plus 1°C might have a better effect on alleviating cell apoptosis.

Protein Expression

Figures 6–8 demonstrated the expression levels of p-AKT, AKT, Caspase-3, Bcl-2, Bax and PI3K in lung, liver and renal tissue in different groups. According to the results, the expression levels of p-AKT, Caspase-3 and Bax elevated significantly following establishment of heat stroke model, and cooling intervention could reverse this trend. The expression levels of Bcl-2 decreased significantly following the establishment of heat stroke model, and cooling intervention could reverse this trend. The expression levels of AKT and PI3K were not statistically different between control group, HS(Tc) group, HS(Tc-1°C) group and HS(Tc+1°C) group. Notably, the expression level of Bax in lung tissue of HS(Tc+1°C) group was significantly lower than that of HS(Tc) group and HS(Tc-1°C) group. These results suggested that heat stroke leads to elevated expression of p-AKT, which subsequently induced the elevated expression of Caspase-3 and Bax, as well as the decreased expression of Bcl-2. Cooling intervention could reverse this trend, which was associated with the mechanism of alleviating cell apoptosis induced by heat stroke. In addition, the better effect on alleviating cell apoptosis might be associated with lower expression of Bax in HS(Tc+1°C) group.

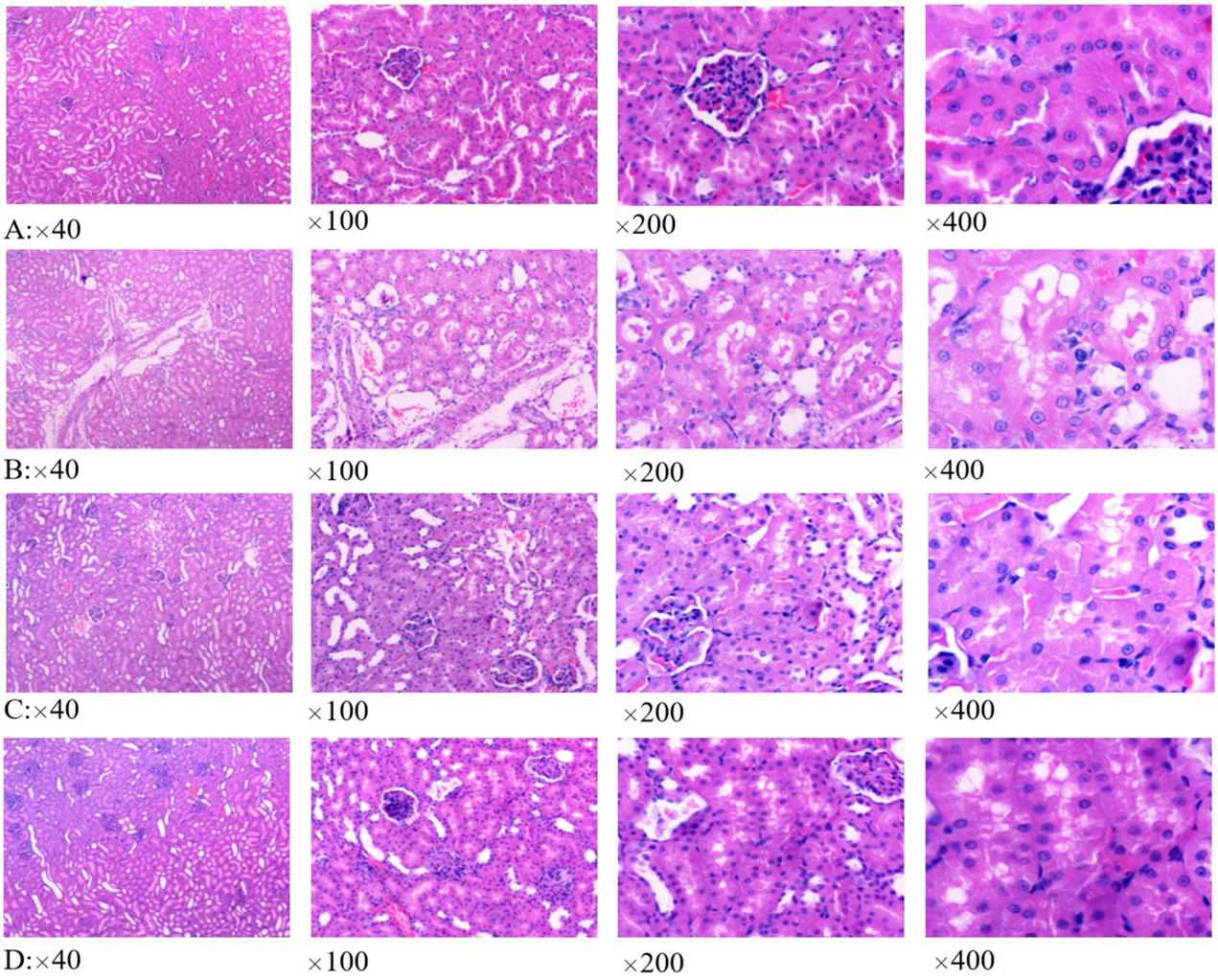

Figure 3 Renal histopathological results of different groups. (**A**–**D**) were the results of control group, HS(Tc) group, HS(Tc-1°C) group and HS(Tc+1°C) group, respectively.
Abbreviations: HS, heat stroke; Tc, core body temperature.

Discussion

Thermal injury can lead to cell toxicity,[12] which is continually intensified as the core body temperature continues to increase. This process can induce extensive tissue and organ damages, and even result in multi-organ failure. The mortality of heat stroke will be significantly increased if effective treatment is not taken in the early stage.[15] It has been demonstrated that endothelial cells may be an early target in tissue injury associated with heat stress.[16] Heat stress may induce apoptosis of multiple kinds of cells.[16–22] Our study demonstrated that heat stroke caused the histopathological damage of lung, liver and renal tissue, and cooling intervention could alleviate the histopathological damage to a certain extent. Furthermore, heat stroke resulted in cell apoptosis in the lung, liver and renal tissue, and cooling intervention could alleviate the cell apoptosis. Notably, the target temperature set at core body temperature plus 1°C demonstrated a better effect on alleviating cell apoptosis although the differences were not significant.

As a natural process, apoptosis is associated with tissue remodeling, normal development and removal of mutated or damaged cells. There is a delicate balance between anti- and pro-apoptotic signals in cells, and abnormal apoptosis contributes to plenty of diseases and angiogenesis. Apoptosis has an important role in cell death and tissue injury induced by high heat[11] and meanwhile endothelial cell damage induced by acute heat stress can lead to apoptosis,[12,13] leading to a vicious circle. Apoptosis can be mediated by multiple signaling pathways. The PI3K/Akt signaling pathway is a classical pathway to inhibit apoptosis, delivering important antiapoptotic signals in the survival of cells. Akt has been demonstrated involvement in a wide range of cellular functions including cell survival, metabolism, proliferation,

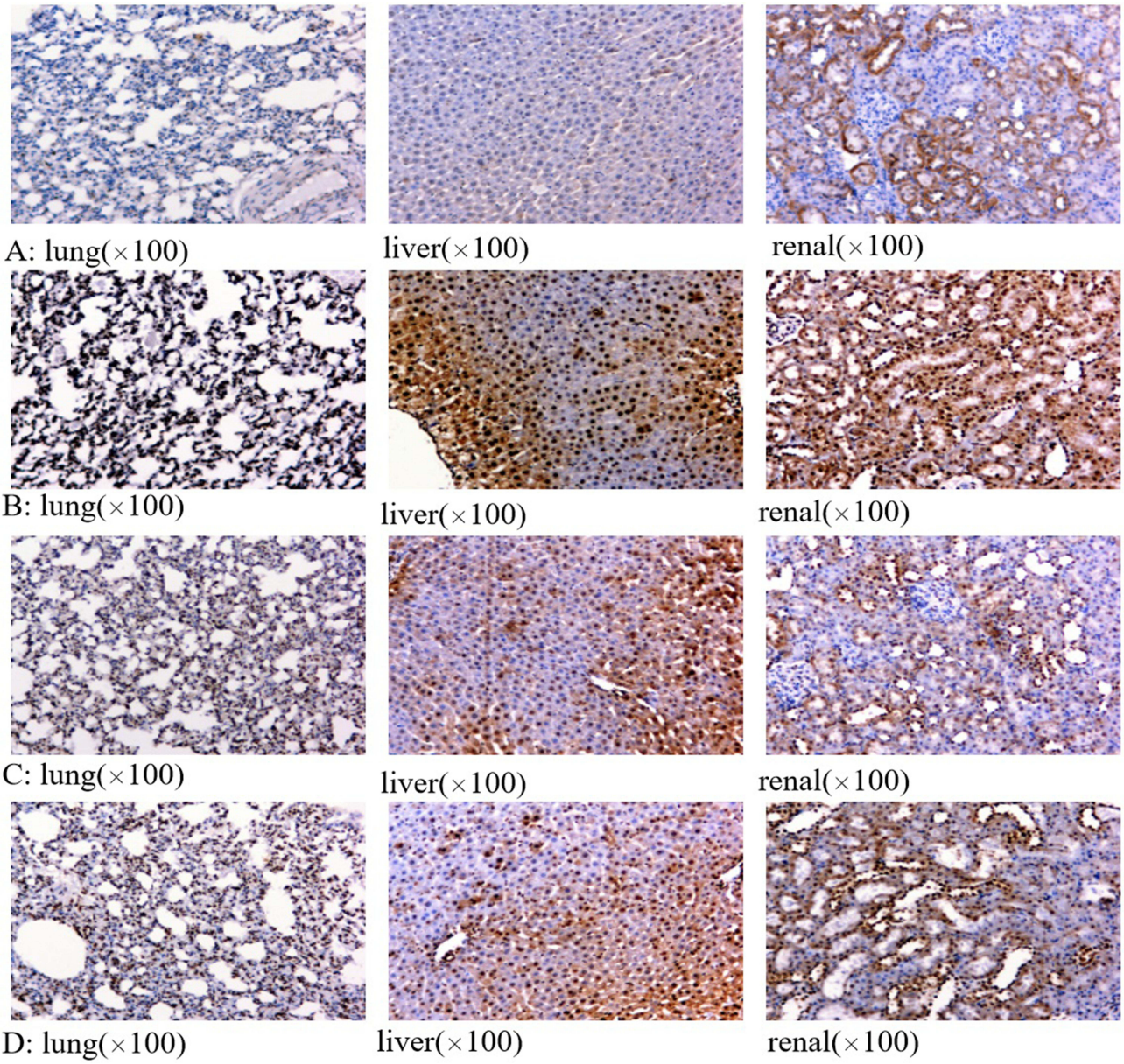

Figure 4 TUNEL staining results of lung, liver and renal tissue in different groups. (**A**–**D**) were the results of control group, HS(Tc) group, HS(Tc-1°C) group and HS(Tc+1°C) group, respectively.

Abbreviations: TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick end labeling; HS, heat stroke; Tc, core body temperature.

migration and angiogenesis. Akt may mediate cell survival via phosphorylation of apoptosis-associated proteins, including the Bcl-2-family members.[23] Bcl-2 family members, having a critical role in cell apoptosis, are divided into proapoptotic members such as Bax and Bad and antiapoptotic members such as Bcl-2 and Bcl-xL.[24,25] It has been demonstrated that antiapoptotic members (Bcl-2 and Bcl-xL) are able to inhibit the release of cytochrome c from the mitochondria to cytosol and subsequent caspase activation through binding to proapoptotic members (Bax and Bad).[26] Thus, the expression levels of Bcl-2 and Bax appear to be associated with cell apoptosis.

Bcl-2 is a critical modulatory component in the mitochondrial death pathway,[27,28] and its expression is regulated by multiple mechanisms such as phosphorylation, transcription, post-translational modification and degradation.[29,30] Akt activation can result in an increased expression of Bcl-2 in BMSCs.[31] In other words, Akt phosphorylation can inhibit the expression of Bcl-2. Bcl-2 can inhibit the release of cytochrome c from mitochondria through inhibition of mitochondrial

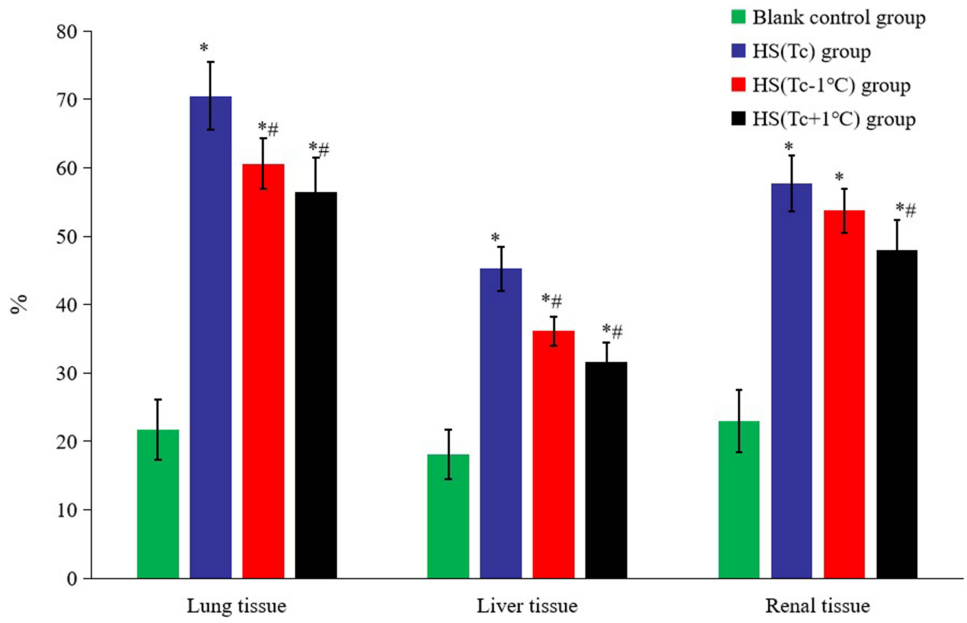

Figure 5 AIs of lung, liver and renal tissue in different groups. *P < 0.05, vs control group, #P < 0.05, vs HS(Tc) group.
Abbreviations: AI, apoptotic index; HS, heat stroke; Tc, core body temperature.

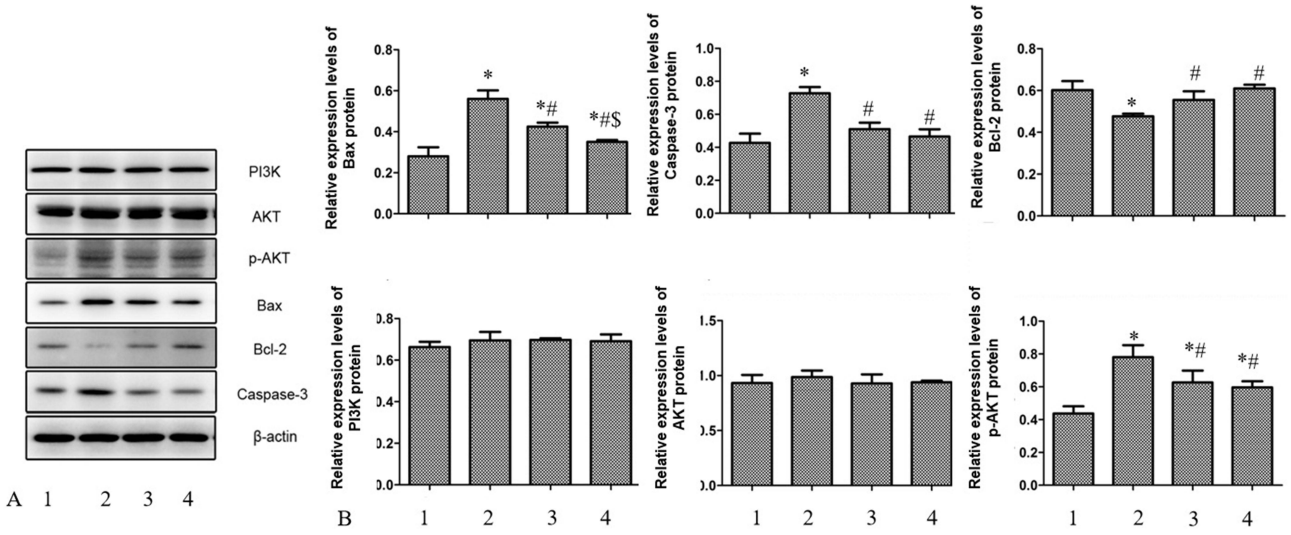

Figure 6 Protein expression results of lung tissue in different groups. (**A**) Protein levels were determined by Western blotting; (**B**) Relative expression levels of Bax, Caspase-3, Bcl-2, PI3K, AKT and p-AKT. 1–4 were the results of control group, HS(Tc) group, HS(Tc-1°C) group and HS(Tc+1°C) group, respectively. *P < 0.05, vs control group, #P < 0.05, vs HS(Tc) group, $$P$ < 0.05, vs HS(Tc-1°C) group.
Abbreviations: HS, heat stroke; Tc, core body temperature.

pore formation induced by apoptosis. The downregulation of Bcl-2 can promote apoptosis through upregulating caspase-3 due to significant association of translocation of cytochrome c from the mitochondria to the cytoplasm with activation of caspase-3 and induction of apoptosis.[32] Bax plays an important role in controlling apoptosis of cells, especially for hematopoietic cells. Cells overexpressing Bax demonstrate increased apoptosis,[33] while Bax-null cells demonstrate resistance to both induced and spontaneous apoptosis. It has been shown that activation of Akt can directly phosphorylate Bax, which subsequently inactivates its proapoptotic function.[34] Therefore, enhancement of Akt phosphorylation can result in decrease of Bax phosphorylation that activates its proapoptotic function.

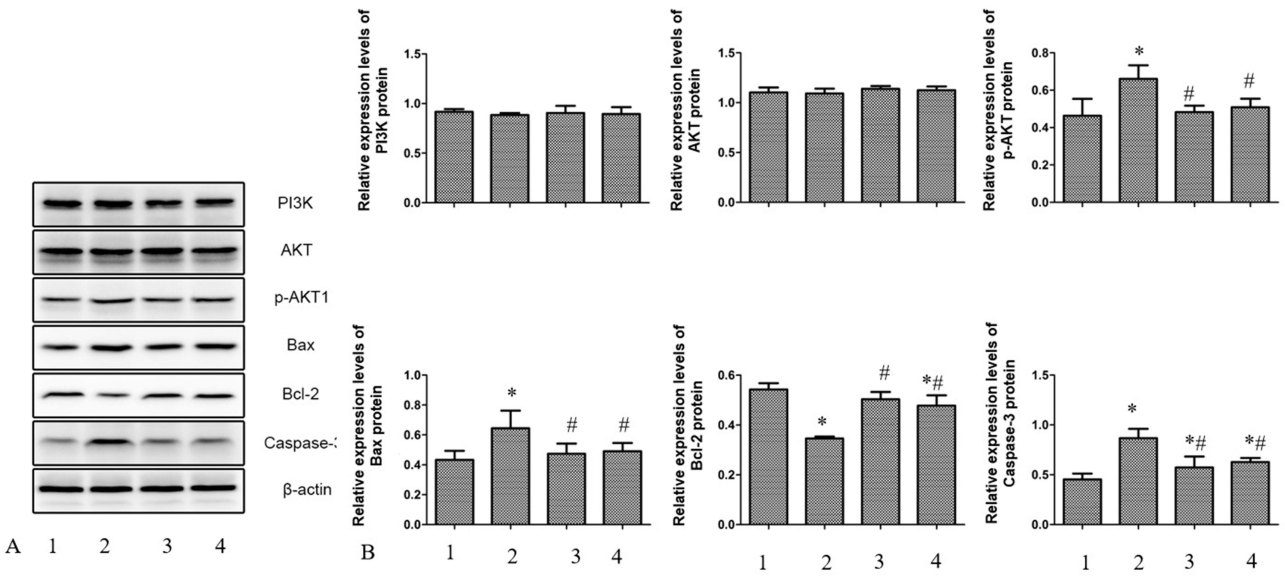

Figure 7 Protein expression results of liver tissue in different groups. (**A**) Protein levels were determined by Western blotting; (**B**) Relative expression levels of Bax, Caspase-3, Bcl-2, PI3K, AKT and p-AKT. 1–4 were the results of control group, HS(Tc) group, HS(Tc-1°C) group and HS(Tc+1°C) group, respectively. *$P < 0.05$, vs control group, #$P < 0.05$, vs HS(Tc) group.
Abbreviations: HS, heat stroke; Tc, core body temperature.

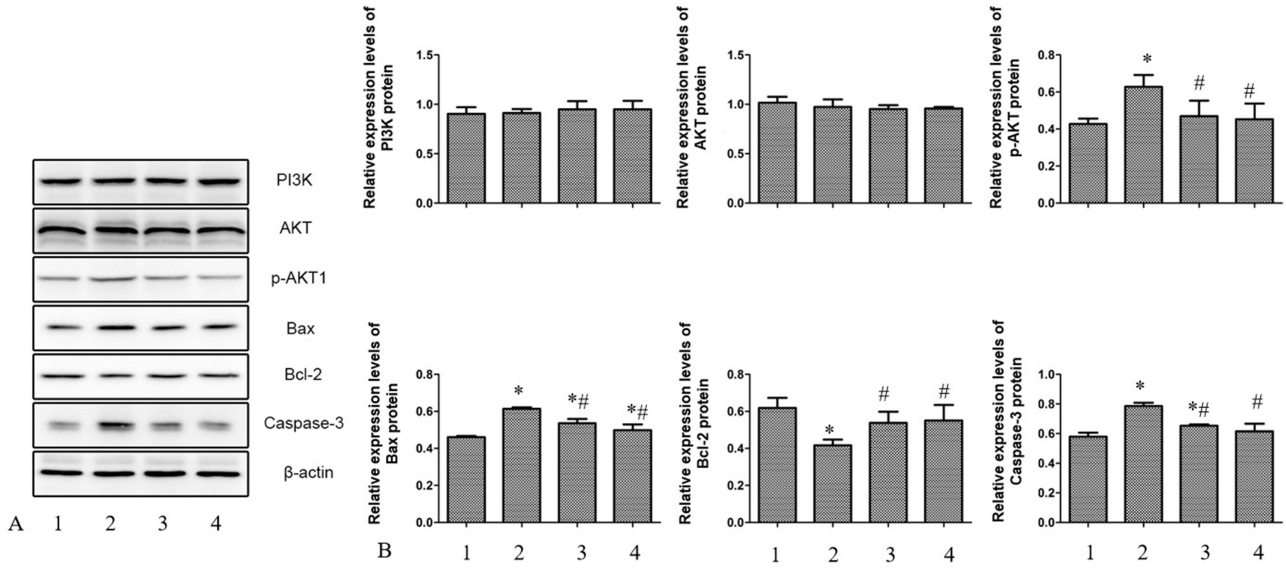

Figure 8 Protein expression results of renal tissue in different groups. (**A**) Protein levels were determined by Western blotting; (**B**) Relative expression levels of Bax, Caspase-3, Bcl-2, PI3K, AKT and p-AKT. 1–4 were the results of control group, HS(Tc) group, HS(Tc-1°C) group and HS(Tc+1°C) group, respectively. *$P < 0.05$, vs control group, #$P < 0.05$, vs HS(Tc) group.
Abbreviations: HS, heat stroke; Tc, core body temperature.

In this study, heat stroke led to the elevated expression of p-Akt (Akt phosphorylation), which subsequently induced the elevated expression of Caspase-3 and Bax, as well as the decreased expression of Bcl-2. Cooling intervention could reverse this trend, which was associated with the mechanism of alleviating cell apoptosis induced by heat stroke. In addition, the better effect on alleviating cell apoptosis might be associated with lower expression of Bax in HS(Tc+1°C) group. In conclusion, the mechanisms of cooling intervention in alleviating heat stroke-induced damage were associated with the expression changes of p-Akt, Caspase-3, Bax and Bcl-2. The better effect of Tc+1°C might be associated with low expression of Bax.

There were two main limitations in our study. The first was no negative controls, ie, heat stroke rats without cooling intervention; and the other was a small sample size. In the next step, we will perform a large sample study to further verify the conclusion and explore the potential mechanisms.

Conclusion

The mechanisms of cooling intervention in alleviating heat stroke-induced damage were associated with the expression changes of p-Akt, Caspase-3, Bax and Bcl-2. The better effect of Tc+1°C might be associated with low expression of Bax.

Acknowledgments

This study was supported by Natural Science Foundation of Xinjiang Uygur Autonomous Region (Contract No.: 2020D01C106) and Intrahospital Projects of People's Hospital of Xinjiang Uygur Autonomous Region (20210111). The funders had no role in design, data collection and analysis, decision to publish, or preparation of the manuscript.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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