

# Rewarming rats at 37°C rescues hypothermia with significant changes in IL-1 $\beta$ levels in the intestinal tissue and blood

**Dan-Dan Li**

Sun Yat-Sen Memorial Hospital <https://orcid.org/0000-0002-5079-9442>

**Wei Ma**

Sixth Medical Center of PLA General Hospital

**Ming Xiong**

Sixth Medical Center of PLA General Hospital

**You-Xin Feng**

Sixth Medical Center of PLA General Hospital

**Dong-Dong Liu**

Sixth Medical Center of PLA General Hospital

**Yuan-Yuan Qiao** (✉ [qiaoyuan75@126.com](mailto:qiaoyuan75@126.com))

Sixth Medical Center of PLA General Hospital <https://orcid.org/0000-0002-9114-2746>

**Cheng-He Shi**

Sixth Medical Center of PLA General Hospital

---

## Research

**Keywords:** Hypothermia, Intestinal injury, Inflammatory factors, IL-1 $\beta$

**Posted Date:** April 7th, 2021

**DOI:** <https://doi.org/10.21203/rs.3.rs-396184/v1>

**License:**  This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

---

# Abstract

## Background

Hypothermia secondary to accidental exposure is becoming increasingly prevalent in the general population; however, the mechanisms and early treatments of hypothermia require additional study.

## Methods

A hypothermia-rewarming SD rat model was established by immersing rats in 15°C seawater for 5h and then rewarming at 37°C for 2, 6 and 12 h. The rats were randomly divided into a normal control group (group C), hypothermia group (group H) and rewarming group (group R). The changes in the levels of inflammatory factors and pathophysiology of the intestinal tissues of rats were assessed. The blood was collected in test tubes, and the levels of cytokines in the separated plasma were detected using ELISA. The intestinal tissue was ground and lysed, and protein expression profiles of 67 inflammatory factors were measured using a protein chip. These samples were further subjected to reverse transcription-quantitative (RT-q)PCR analysis and tissue section staining.

## Results

The temperature of the abdomen and the physiological state of the rats was significantly altered during immersion in the hypothermic seawater, and returned to normal after rewarming. The protein chip showed that inflammatory factors, including IL-1 $\beta$ , IL-10 and IL-6, were differentially expressed in the intestine. Using ELISA, it was shown that IL-1 $\beta$ , IL-6 and IL-10 levels were also upregulated in the plasma. Comparing the ratios of IL-1 $\beta$  to IL-6 and IL-1 $\beta$  to IL-10, IL-1 $\beta$  was found to be significantly more upregulated compared with IL-10 and IL-6 in the intestine during hypothermia. The immunohistochemical staining of IL-1 $\beta$  showed that IL-1 $\beta$  expression first increased then decreased during the rewarming period, and similar results were obtained based on RT-qPCR analysis.

## Conclusion

Rewarming at 37°C may be a suitable method for early treatment of hypothermia, and IL-1 $\beta$  may serve as a potential biomarker for assessing the severity of hypothermia.

## Background

Hypothermia is defined as a central body temperature < 35°C, and it is a type of clinical syndrome and a major cause of death in individuals who die in the sea [1, 2]. Stay in low temperature environment for a long time could result in hypothermia. Shipwrecks and naval battles could cause to people falling into the sea. Since the temperature of seawater is low and the thermal conductivity coefficient of seawater is ~ 23

times that of air, hypothermia after falling into the sea is considerably more likely to occur than on the land [3, 4]. With the prevalence of activities such as marine shipping, marine operations and diving increasing, the incidence of shipwrecks and drowning is increasing [5]. Existing research has shown that ~ 2/3 of the people who fall into the sea will develop hypothermia. With the activities in the ocean and expeditions to colder regions of the Earth, such as the north pole, are becoming increasingly popular, the incidence of hypothermia is increasing on a yearly basis [6]. Moreover, the mortality rate of hypothermia is very high. Hundreds of people die of hypothermia in America every year [7]. In hospitals, the mortality rate of hypothermia is 12%, while the mortality rate of moderate to severe cases may be as high as 40% [8].

The tolerance period of the body when immersed at different temperatures of seawater is distinguishable. The lower the seawater temperature is, the shorter the survival time is, and thus the higher the risk of mortality [9, 10]. When the central body temperature is  $< 32^{\circ}\text{C}$ , cardiac dysfunction occurs. When the central body temperature is  $< 28^{\circ}\text{C}$ , dysfunction of the respiratory system, circulatory system, nervous system and coagulation system is observed, which may progress to multiple organ dysfunction syndrome (MODS) [11, 12]. The longer the period of immersion in seawater, the greater the extent of deterioration of organs. Under severe hypothermic conditions, the physiology of the body is altered notably, and the so-called 'death triad' including hypothermia, coagulation disorders, and acidosis may occur. If the central body temperature is  $< 32^{\circ}\text{C}$  and suffers trauma at the same time, it could be fatal [13].

Researches showed that hypothermia was associated with alterations in IL-6 and the other pro-inflammatory cytokines, including IL-1 and TNF- $\alpha$ , which stimulates the release of acute phase proteins [14]. Inflammation is correlated with NF- $\kappa$ B activation. Wang et al [15] showed that adenosine 5'-monophosphate-induced hypothermia inhibited the activation of NF- $\kappa$ B in endotoxemic rats. Thus, the pro or anti-inflammatory effects of hypothermia may be associated with the activation or inhibition of NF- $\kappa$ B activation.

At present, treatment measures for hypothermia primarily include rewarming, reversing acidosis and treating any dysregulated coagulation [16, 17]. Among these, rewarming is the most important treatment measure for hypothermia patients [18]. It has been shown that hypothermia patients may still suffer from 'rewarming shock' after their body temperature returns to the normal levels, that is, improper rewarming may lead to 'secondary damage' [19]. Additionally, it has been shown that hypothermia can also induce dysfunction of the digestive system, including a reduction in intestinal motility. Ulcers of the ileus and pancreatitis may occur when the central body temperature is  $< 32^{\circ}\text{C}$  [13]. The intestinal tract is one of the most likely affected and severely injured internal organs following a traumatic shock, burn or infection. Intestinal mucosa is vulnerable to ischemia due to the special clamped structure of its blood vessels. Therefore, ischemia-reperfusion injury of intestinal mucosa following trauma may lead to structural and functional changes of the intestinal mucosa [20]. Furthermore, it may also lead to translocation of enteric bacteria, intestinal-derived release of media, cascade reactions of the reticuloendothelial system, release of a large quantities of inflammatory media and cytokines, and thus, systemic inflammatory response syndrome (SIRS), sepsis and even MODS [16]. However, currently, there are only a few studies examining

the changes in the intestine following hypothermia. The rewarming methods for hypothermia also require improvement.

In the present study, an improved hypothermia-rewarming model for Sprague-Dawley (SD) rats was established based on a hypothermia model established in our previous study [17, 21]. A focus was placed on exploring the changes in the physiological state, structure and the profile of inflammatory cytokines in the intestinal tissue under hypothermic and rewarming conditions. Additionally, the damage caused by hypothermia to the intestinal tract was assessed.

## Methods

### Ethics statement

The animal experimentation protocol was reviewed and approved by the Ethical Committee of The Sixth Medical Center of Chinese PLA General Hospital (Approval No.MDKN-2020-158), and all rats were handled in accordance with the guidelines described in the Declaration and the National Institutes of Health Guide for Care and Use of Laboratory animals [22].

### Preparation of artificial seawater

The experimental seawater was prepared according to the standard of the Third Institute of Oceanography of the State Ocean Bureau: Osmotic pressure  $1,250.00 \pm 11.52$  mOsm/l, pH 8.20,  $[Na^+] 630.00 \pm 5.33$  mmol/l,  $[K^+] 10.88 \pm 0.68$  mmol/l, room temperature ( $25 \pm 1$ ) °C. The seawater was contained in a chromatography cabinet at a temperature of  $15^\circ C \pm 0.2^\circ C$ .

### Implantation of the temperature recorder into the abdominal cavity of rats

SD male adult rats (weight, 280-300g; age, 2-3 months) were obtained from the Institute of Zoology, Academy of Military Medical Sciences. All animals were kept under specific pathogen-free conditions with controlled light/dark cycles and free access to water and standard chow and were fasted for 24 hours before surgical procedures. Rats were anaesthetized with an intraperitoneal injection of 1.5% pentobarbital sodium (40mg/kg body weight), and then fixed in the supine position on a plate. A ventral midline incision of 2cm on the right side of the xiphoid process was made, and a temperature recorder wrapped in paraffin was placed under the abdominal cavity of the rat. Subsequently, the long incision was sutured. The wound was smeared with erythromycin ointment and rats were intraperitoneally injected with penicillin sodium (200,000 u), which lasted for 3 days after operation. After 10 days, the rats which grew regularly and had normal bowel movements were used for further experiments.

### Animal preparation and hypothermia-rewarming model

We built a hypothermia-rewarming rats model based on our previous study [17]. A total of 50 SD male rats implanted with a temperature recorder were randomly assigned to three groups: Normal control group (C group, n=10), hypothermia group (H group, n=10) and a hypothermia followed by rewarming

group (R group, n=30). The R group included three time-point subgroups: 2, 6 and 12h (R2, R6 and R12, respectively). There were 10 rats in each subgroup. Rats in the C group were fixed into the cylindrical upright retainer and placed at room temperature for 5 h. Then, they were anesthetized, and samples of blood and various tissues were obtained. The other rats were fixed into the cylindrical upright retainer and then immersed into the 15°C seawater for 5 h, such that the seawater reached the armpits of the rats. The abdominal temperature and other vital indicators were observed for 5 h, unless the rat died as judged by respiratory and cardiac arrest (Tables SI and SII). After immersion for 5 h, the survived rats in the groups H were anesthetized, and samples of blood and various tissues were obtained. The rats which survived in the 3 R groups were transferred to a 37°C constant temperature water bath for 2, 6 and 12 h, respectively. After each detection time point, the rats were anaesthetized with an intraperitoneal injection of 1.5% pentobarbital sodium (40mg/kg body weight) to obtain the samples of blood and tissues including the intestinal tissue. The blood was collected via the left ventricle of the rats into test tubes containing citrate solution (1:9 citrate to blood). The blood samples were centrifuged at 1,000 × g for 15min, and the plasma was separated within 1 h and was stored at -80°C for subsequent use. The small intestinal tissue from the Treitz ligament to the ileocecal junction was rapidly excised from the mesentery and rinsed gently with 5ml ice-cold PBS. A 5cm segment of the ileum, 15cm above the ileocecal junction, was removed for histopathological analysis. The lung, kidney, heart were stored in liquid nitrogen immediately until required for tissue analysis.

### **Assessment of the expression of inflammatory factors in the intestinal tissues using the protein chip GSR-CAA-67**

The frozen small intestinal tissues (~1cm) were fixed in 3-fold phosphate-buffer (0.1M, pH7.2), homogenized and centrifugalized (10,000rpm/min for 30 min) for estimation of the levels of inflammatory factors. The protein chip GSR-CAA-67 was used to simultaneously examine the protein levels of 67 different inflammatory factors in the supernatants of the intestinal tissues. Each well of the chip was filled with 100µl tissue lysis supernatant (500µg/ml) and then incubated overnight at 4°C on a shaker. Well wash Versa(Thermo Fisher Scientific, Inc.) was used to wash the slides. Subsequently, 80µl detection antibody was added to each well. After washing the slide again, 80µl Cy3-streptavidin was added to each well. The slide was covered with aluminum foil to protect against light and incubated at 37°C for 1 h on a shaker. Fluorescence signals were detected and observed using a microscope.

### **Determination of plasma cytokine levels using ELISA**

Based on the differential analysis, three inflammatory factors (IL-6, IL-10 and IL-1β) were selected for further analysis. To detect the levels of cytokines in the plasma, IL-6(cat.BMS603-2; eBioscience; Thermo Fisher Scientific, Inc.), IL-10 (cat.BMS629; eBioscience; Thermo Fisher Scientific, Inc.) and IL-1β (cat.BMS6002; eBioscience; Thermo Fisher Scientific, Inc.) were measured using a double-antibody sandwich ELISA according to the manufacturer's protocol. The assays were repeated three times for each group and subgroup.

Briefly, 100µl plasma and 50µl enzyme conjugate was added to the antibody precoated 96-well ELISA plate and incubated for 1h at 37°C. The ELISA plate was washed five times with PBS and then incubated for 15 min with a TMB substrate. The absorbance was measured using an ELISA reader.

### **Reverse transcription-quantitative (RT-q)PCR**

Small intestinal tissue was harvested and homogenized in 1ml TRIzol® Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) per 50-100mg tissue. Total RNA was isolated according to the manufacturer's protocol and was treated with RNeasyMinElute™ Cleanup kit (Qiagen, Inc.). RNA quantity and quality were measured using a NanoDrop 8000 (Thermo Fisher Scientific, Inc.). RNA (1 µg) was reverse transcribed using an RT kit (Takara Bio, Inc.) according to the manufacturer's protocol. qPCR was performed using SYBR green technology (Promega Corporation) using an iCycler real-time detection system (Bio-Rad Laboratories, Inc.). Relative mRNA levels were determined following normalization to the housekeeping gene, GAPDH, and the data were analyzed using the  $2^{-\Delta\Delta C_q}$  method. Assays were repeated three times for each group and subgroup.

### **Histopathological (HE) examination**

The paraformaldehyde fixed small intestinal tissue was embedded in paraffin, sectioned (5µm thick) using a microtome, and stained with hematoxylin and eosin (H&E) for histological examination by the pathologists.

### **Immunohistochemical staining of IL-1β**

Slides were deparaffinized in xylene and rehydrated by immersion using a series of ethanol concentrations (100, 95, 90, 80 and 70%). The sections were then incubated in 3% H<sub>2</sub>O<sub>2</sub> for 30 min to block the endogenous peroxidase activity, and antigen retrieval was performed using citrate buffer. Slides were blocked (37°C, 2.5h) with 10% FBS and then incubated the anti-IL-1β primary antibody (cat.EPR16805-15; no.ab234437; Abcam; 1:100) for 12 h at 4°C. IgG-purified normal rabbit serum (2µg/ml) (cat.MFCD00165673; no.I5006; Sigma-Aldrich; Merck KGaA) was used as the control. After washing with PBS, sections were incubated with polyperoxidase-anti-rabbit IgG for 30 min at 37°C. Signals were visualized using DAB and observed using a microscope. The intensity of staining was scored on a scale of 0-3: 0, negative staining; 1, weakly positive staining; 2, moderately positive staining; and 3, strongly positive staining. The extent of positivity (extent of distribution of positive cells) was estimated on a scale of 0-4: 0, negative; 1, positive staining in 1-25% of cells; 2, positive staining in 26-50%; 3, positive staining in 51-75%; and 4, positive staining in 76-100%. A combined staining score (extension +intensity) ≥3 was considered as positive staining [23].

### **Statistical analysis**

Data were analyzed using SPSS version 20.0 (IBM Corp.) and R software version 3.6.2. All data are presented as the mean ± standard deviation. One-way ANOVA was used under one factor condition while

two-way ANOVA was used under two factors condition. Bonferroni method was used to adjust multiple test p-value. A  $\chi^2$  test was used to compare categorical variables.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

### Changes in the abdominal temperature of the rats

After immersion in 15°C seawater for 2 h, compared with the C group, the abdominal temperature of the rats in group H and R dropped significantly at all time points ( $P < 0.05$ ). The abdominal temperature decreased sharply in the first 30 min, and then decreased slowly. After 2 h, the temperature dropped to ~15.6°C, and then fluctuated at this temperature until the 5<sup>th</sup>h. During this time, some rats died. There were 30 rats in R group (three sub-groups) and 10 rats in H group. During immersing in 15°C seawater, 2 rats and 8 rats were dead in H group and R group respectively. Fisher exact test shows that there no difference between the two groups ( $P = 1$ , odds ratio = 0.75). The number of rats which remained alive at each time period for each group is summarized in Tables SI and SII. After rewarming in a 37°C water bath for 1 h, the abdominal temperature of the rats in group R increased rapidly to ~38°C, and then dropped to ~35°C. After rewarming for 2 h, the temperature returned to normal until the 12<sup>th</sup>h. During the rewarming procedure, no rat died (Table SII). Thus, rewarming in 37°C water helped the body to warm up quickly and safety. Compared with the 37<sub>0m</sub> (Rewarming in 37°C water for 0 minute) time point, the abdominal temperature of the rats was significantly increased at all time points ( $P < 0.05$ ; Fig. 1A). Since the temperature was almost constant after immersion in 15°C seawater for 2 h, the remaining 3 h are not shown in Fig. 1.

### Changes in the physiological state of the rats

In the early stages of immersion in the low-temperature seawater, the stress caused a significant increase in heart rate, respiration and amyostasia of rats in the H and R groups. With the core temperature decreasing consistently, the stress exhibited decreased eventually. After immersing in 15°C temperature seawater for 10 min, compared with the C group, the respiratory rate and heart rate of rats in the H and R group increased, although the difference was not statistically significant. After immersion in 15°C temperature seawater for 10-120 min, compared with the C group, the respiratory rate and heart rate of rats in groups R and H decreased significantly ( $P < 0.05$ ), where it remained stable. The respiratory and heart rates of rats in group R increased significantly after rewarming for 1 h and returned to normal after 2 h. Compared with 37<sub>0m</sub>, the respiratory rate and heart rate of rats was significantly higher at all time points ( $P < 0.05$ ; Fig. 1B and C).

When immersed in 15°C seawater for 10 min, compared with the C group, the rats in groups H and R showed notable amyostasia. After 30 min of immersion, the muscle tremor in the R and H groups peaked. With the core temperature decreasing, the amyostasia of R and H groups also decreased rapidly and was absent after 90 min. However, after rewarming for 30 min, the amyostasia of rats in the R group increased

significantly, and peaked again after rewarming for 60 min. As the core temperature increased, amyostasia gradually decreased and after 3 h of rewarming, the amyostasia was absent (Fig. 1D).

Based on the above results, rewarming in a 37°C water bath may recover the vital signs quickly and safely in hypothermic rats.

### **Expression of intestinal inflammatory factors**

The expression levels of 67 inflammatory factors in the small intestine were detected using a protein chip. Based on the expression of the inflammatory factors, the C group was clustered into a class via hierarchical clustering, which indicated that hypothermia resulted in notable changes to the body (Fig. S1). The H, R2 and R6 groups could not be clustered into one class; however, the R12 group could almost be clustered into a class, which indicated that rewarming for 12 h could be distinguishable from the H group. Expression of inflammatory cytokines in rats rewarmed for short periods (2 or 6 h) could not be clustered. This suggested that a rewarming period of 12 h was considered sufficient to stabilize the physiology in the rats (Fig. S1).

Next, the differentially expressed proteins in the H and R groups (R2, R6 and R12) was compared with the N group (Fig. S2). Compared with the C group, 11 inflammatory factors were differentially expressed, including 7 upregulated and 4 downregulated inflammatory factors, (Fig.S2A). There were 15 and 14 significantly differentially expressed inflammatory factors in the R2 and R6 groups, respectively (Fig. S2B and C). The R12 group exhibited the highest number of differentially expressed inflammatory factors; 15 upregulated and 5 downregulated (Fig. S2D). These changes in inflammatory factors may participate in the development of hypothermia. We selected IL-6, IL-10 and IL-1 $\beta$  for further analysis based on our lab condition (our lab had sufficient conditions to study IL-10 because we had studied IL-10 before) and the differential analysis (IL-1 $\beta$  and IL-6 were the most significant proteins when compared the H group with the C group (Fig. S2A). These two proteins were also significant in the other three comparisons (Fig. S2B, C, D).

### **Plasma levels of IL-6, IL-10 and IL-1 $\beta$**

The expression levels of certain inflammatory factors were altered in the intestine following hypothermia. Thus, whether these changes were also observed in the blood was next assessed. For analysis, three inflammatory factors (IL-6, IL-10 and IL-1 $\beta$ ) were selected for further analysis in the blood. Using ELISA, it was shown that following immersion in 15°C seawater for 5 h, the levels of the three cytokines in the plasma from the H group was significantly higher than that of the C group ( $P < 0.05$ ). Compared with the H group, the levels of the three cytokines in the R group was gradually reduced following rewarming in a warm water bath ( $P < 0.05$ ; Fig.2A-C). The ratio of IL-1 $\beta$ :IL-10 and IL-1 $\beta$ :IL-6 ratios were used to measure the balance between pro- and anti-inflammatory cytokines. The ratio of IL-1 $\beta$ :IL-6 and IL-1 $\beta$ :IL-10 in the plasma of the H group rats were increased, and they gradually decreased in the R group with time. However, the increase in IL-1 $\beta$  expression was greater than that of IL-10 and IL-6 in the H and R groups (Fig. 2D). Thus, the levels of inflammatory factors in the blood were also altered. The changes in these

three inflammatory factors was similar to that observed in the intestine. Thus, rewarming both recovered the vital status and also restored the levels of inflammatory factors in the blood.

### **Relative gene expression levels of the cytokines in the gut**

Differentially expressed inflammatory factors were found in the hypothermic rats using a protein chip. To verify the change in these factors, IL-1 $\beta$ , IL-10 and IL-6 were selected for qPCR analysis. The results showed that the expression levels of IL-1 $\beta$  in the H group was significantly increased ~4.5-fold compared with the C group ( $P < 0.05$ ; Fig. 3A). When rewarming for 2 h, the levels of IL-1 $\beta$  mRNA decreased to normal. However, after rewarming for 6 h, the levels of IL-1 $\beta$  were increased significantly, ~3.5-fold higher than that of the C group. As the rewarming time increased, the expression levels of IL-1 $\beta$  gradually decreased (Fig. 3A). Compared with group C, the mRNA levels of IL-10 in group R2, R6 and R12 increased ( $P < 0.05$ ; Fig. 3B). The mRNA expression levels of IL-6 in the R2 group increased, whereas it decreased in the H, R6 and R12 groups, although the differences were not statistically significant (Fig. 3C). The ratio of IL-1 $\beta$ :IL-6 and IL-1 $\beta$ :IL-10 were analyzed. Compared with the C group, IL-1 $\beta$  expression was higher than that of IL-10 in the H group and all the R groups ( $P < 0.05$ ; Fig. 3D).

### **Pathological changes in the intestinal tissues**

The histological analysis showed that the morphology of intestinal mucosa in the C group was normal (Fig. 4). The villi of the intestinal mucosa and the epithelial cells were neatly arranged. They were free of edema in the interstitial tissue and the villi structure was intact. However, a mass of necrosis was observed in the epithelium of intestinal mucosa villi in the H group, with a large degree of neutrophil and inflammatory cell infiltration, and the shed villi interstitial structure was lost. In the R group, the tight junction structure of the intestinal tissue and the permeability was altered. At the top of the villi, there was increased neutrophil infiltration. These findings suggest that severe inflammation occurred in the intestine following hypothermia.

### **IL-1 $\beta$ expression in intestinal tissues based on immunohistochemistry (IHC) analysis**

To investigate the expression and distribution of IL-1 $\beta$  in intestinal tissues, IHC was performed. As shown in Fig. 5, positive IHC staining, which was considered as brownish yellow or brown particles that could be distinguished from the background cells, was primarily localized in the cytoplasm of the intestinal tissue cells. Compared with the C group, the proportion of positive cells was higher in the H group. In the R2 group, the staining of IL-1 $\beta$  was decreased compared with the H group. However, the number of positive cells was increased in the intestinal tissue of the R6 group. With a longer rewarming period, the intensity of IL-1 $\beta$  expression gradually decreased. This phenomenon was observed with regard to the amyostasia. When hypothermic or rewarming, amyostasia was first increased and then subsequently decreased (Fig. 1D). However, for IL-1 $\beta$ , there was only one time-point and the end of immersion. A possible explanation for this is that when the temperature is decreasing, the attempts to correct this to maintain the normal body temperatures. However, as the temperature decreases further, the body is unable to maintain a physiological temperature. Thus, perhaps the lower temperature suppresses certain physiological

functions. Additionally, this observation may be the result of evolution. When the temperature is too low, the body stops trying to increase the core temperature, instead reserving energy, thus resulting in damage to the body from the colder temperatures.

## Discussion

Hypothermia is becoming increasingly prevalent, and when caused by seawater, it has a high rate of mortality. Longer periods of immersion may result in worse outcomes [24]. Appropriate early treatment is very important for reversing and limiting hypothermia [25]. In the present study, a hypothermia-rewarming model was established via immersion of rats in 15°C seawater for 5h followed by rewarming in a 37°C water bath. Physiological changes, changes in the inflammatory response and damage of the intestinal tissue structure was observed. When rats were immersed in 15°C seawater, the heart rate, respiratory frequency, and amyostasia were initially increased and subsequently decreased. This may be an early response to cold stimulation. As the time the rats were immersed in the cold water increased, the damage to the thermoregulatory center increased, and the more the body's aerobic metabolism was reduced. The rat's consciousness deteriorated as the respiratory rate and heart rate reduced. In addition, muscle tremor disappeared. During immersion in 15°C seawater, some rats died. No rewarming shock was observed during the rewarming period and there was no death during this period either. Thus, rewarming at 37°C is a good choice for early treatment of hypothermia. After rewarming, the temperature of the abdomen in the rats first increased to 38°C and then decreased to 35°C, and the temperature increased to normal body temperatures.

It was found that the intestine could initiate a 'second attack' when the level of stress experienced increased [26], resulting in a release of inflammatory factors, platelet-activation factor and tumor necrosis factor [27]. Hypothermia is associated with white blood cell activation, increased levels of cytokines and SIRS [10, 28].

In the present study, the expression of certain inflammatory factors, including IL-1 $\beta$ , IL-6 and IL-10 was altered in the intestinal tissue and blood, particularly for IL-1 $\beta$ . IL-1 $\beta$  is a pro-inflammatory cytokine, whereas IL-6 may serve as a pro- or anti-inflammatory cytokine [11, 29]. In addition, according the ratios of IL-1 $\beta$  to IL-10 and IL-1 $\beta$  to IL-6 ratios, IL-1 $\beta$  expression was significantly higher than that of IL-10 and IL-6 in the intestine following hypothermia, and it returned to around normal expression levels following rewarming for 2 h. Thus, it is speculated that IL-1 $\beta$  is a hypothermia specific inflammatory factor that could be used to assess the severity of hypothermia.

IL-1 $\beta$  is a common inflammatory factor that is produced by monocytes, endothelial cells, fibroblasts and other types of cells in response to infection [30, 31]. IL-1 $\beta$  could stimulate the production of colony-stimulating factor, and platelet-derived growth factor and also could induce T cells to produce IL-2, which serves an important role in immune response and tissue repair. As a pre-inflammatory regulatory factor, IL-1 $\beta$  may be used to reflect the severity of intestinal damage during the early phase, and activate NF- $\kappa$ B to increase the expression of IL-6 and TNF- $\alpha$  in an autocrine manner [32, 33]. It has been reported that the

expression of inflammatory factors is upregulated in the plasma of hypothermia patients [34]. The high ratios of IL-1 $\beta$  to IL-10 and IL-1 $\beta$  to IL-6 in the present study supported the hypothesis that IL-1 $\beta$  was the predominant pro-inflammatory factor that mediated the initial inflammatory response following ischemia reperfusion mediated by hypothermia [23]. The inflammatory response following immersion in low-temperature seawater and rewarming is complex. According to Cryer *et al* [35], the exaggerated pro-inflammatory response possibly coexists with an exaggerated counter-inflammatory response. According to Moldawer *et al* [36] the situation is more complicated than a pro-inflammatory response followed by an anti-inflammatory response. After rewarming for 2 h, the ratios returned to around normal levels, and IHC analysis of the intestinal tissue indicated that IL-1 $\beta$  expression was higher in the intestinal tissue of the hypothermic rats compared with the control group. This may be related to the increase in the levels of pro-inflammatory factors stimulated by the stress response in the intestine under hypothermic conditions. After rewarming for 2h, IL-1 $\beta$  expression was significantly downregulated, which indicated that rewarming may be a useful method of reversing hypothermia. The above results together show that IL-1 $\beta$  is a specific inflammatory factor in intestinal tissues expressed following immersion at low-temperatures followed by rewarming.

The present study has some limitations. First, only 67 inflammatory associated genes were detected in the present study. Some inflammatory modulators, such as IL-8 and cyclooxygenase-2, were not included. Other genes may also serve important roles in response to hypothermia, and these may have been missed. Additionally, a larger range of rewarming temperatures should be assessed to determine the optimal temperature. Third, knockdown and overexpression of IL-1 $\beta$  should be performed to better determine its role in hypothermia. Forth, more time-points of immersion at 15°C seawater is required to observe the extent of changes over time, particularly with regard to IL-1 $\beta$ . Finally, it will be more rigorous if the immersion of rats in water at room temperature (for five hours) as blank control, and the rats which died during immersion in 15°C seawater should also have been analyzed. In the future, the mechanism of IL-1 $\beta$  in hypothermia will be further studied.

## Conclusions

The present study established a hypothermia-rewarming rat model. The results showed that: i) Rewarming in 37°C may serve as a candidate method for early treatment of hypothermia. ii) Expression of certain inflammatory factors, including IL-1 $\beta$ , IL-6 and IL-10 is altered notably under hypothermic conditions. iii) IL-1 $\beta$  may serve as a biomarker and/or therapeutic target for assessing the severity and management of hypothermia, respectively. The other inflammatory factors assessed, such as IL-10 and IL-6 may not be of particular relevance with regard to hypothermia.

## Abbreviations

IHC, immunohistochemistry; MODS, multiple organ dysfunction syndrome; SIRS, systemic inflammatory response syndrome

# Declarations

## Acknowledgments

We are very grateful to professor Yuan-yuan Qiao and Cheng-He Shi for providing assistance in the experimental design stage and Ph.D Wei MA for analyzing the data.

## Authors' contributions

DDL, WM and MX contributed equally to this study. DDL and MX did the experiments. WM and DDL performed the analysis and manuscript. YYQ and CHS advised and supervised the study. YXF and DDL helped DDL do the experiments. . All authors read and approved the final manuscript.

## Funding

This study was supported by military logistics scientific research and military innovation and cultivation project (Project no. 16QNP023 and BHJ14C009 ).

## Availability of data and materials

The datasets used and/or analyzed in the current study are available from the corresponding author upon reasonable request.

## Ethical Approval

The animal experimentation protocol was reviewed and approved by the Ethical Committee of the Sixth Medical Center of the General Hospital of the Chinese People's Liberation Army (Approval No. MDKN-2020-158).

## Consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

## Author details

<sup>1</sup> Basic Medical Research Center, the Sixth Medical Center of the General Hospital of the Chinese People's Liberation Army, Beijing 100048, China

<sup>2</sup> Department of Blood Transfusion, Sun Yat-sen Memorial Hospital of Sun Yat-Sen University, Guangzhou 510515, China

## References

1. Douglas J. A. Brown M D, Hermann Brugger M D, Jeff Boyd M B B S, et al. Accidental Hypothermia. *N Engl J Med.*2012;367(20):1930-1938.
2. Su Y J. Hypothermic lung edema after accidental hypothermia with out of hospital cardiac arrest.*Heart Lung Vessel.* 2015;7(4):328-329.
3. Petrone P, Asensio JA, Marini CP. In brief: hypothermia.*CurrProbl Surg.* 2014;51(10):414-415.
4. Kempainen RR, Brunette DD. The evaluation and management of accidental hypothermia.*Respir Care.*2004;49(2):192-205.
5. Brown DJ, Brugger H, Boyd J, et al. Accidental hypothermia. *N Engl J Med.*2012;367(20):1930-1938.
6. K. Zafren, G. G. Giesbrecht, D. F. Danzl et al. Wilderness medical society practice guidelines for the out-of-hospital evaluation and treatment of accidental hypothermia: 2014 update. *Wilderness and Environmental Medicine.*2014;25(4):66-85.
7. Kempainen RR, Brunette DD. The evaluation and management of accidental hypothermia.*Respir Care.*2004;49(2):192-205.
8. Zhang J, Yu XC, and Wang M. Current situation and countermeasures of hypothermia treatment for sea drowning person. *Chinese Medical Equipment Journal.* 2016;37(5):121-124.(Chinese).
9. Lim C, Duflou J. Hypothermia fatalities in a temperate climate: Sydney, Australia. *Pathology.* 2008;40:46-51.
10. McCullough L, Arora S. Diagnosis and treatment of hypothermia. *Am Fam Physician.*2004;70:2325-2332.
11. Paal P, Gordon L, Strapazzon G, et al. Accidental hypothermia-an update : The content of this review is endorsed by the International Commission for Mountain Emergency Medicine (ICAR MEDCOM). *Scand J Trauma Resusc Emerg Med.*2016;24(1):111.
12. Li S, Qiu C, Shi W, et al. A survey of accidental hypothermia knowledge among navy members in china and the implication for training. *Intern J Envir Res Pub Health.*2016;13(3):315.
13. Vardon F, Mrozek S, Geeraerts T, et al. Accidental hypothermia in severe trauma. *Anaesthesia Critical Care & Pain Medicine.* 2016;35(5):355-361.
14. McInerney J J ,Breakell A, Madira W et al. Accidental hypothermia and active rewarming: the metabolic and inflammatory changes observed above and below 32 degrees C. *Emerg Med J.* 2002;3(19):219-223.
15. P.O. Scumpia, L.L. Moldawer, et al. Biology of interleukin-10 and its regulatory roles in sepsis syndromes. *Grit. Care Med.* 2005;33(12):468-471.
16. B. J. Tsuei and P. A. Kearney. Hypothermia in the trauma patient. *Injury.*2004;35(1):7-15.
17. Zhang D, Qu J, Xiong M, et al. Role of Vascular Endothelial Cells in Disseminated Intravascular Coagulation Induced by Seawater Immersion in a Rat Trauma Model. *BioMed Research International.* 2017; 5147532-5147532.

18. A. Hope, L. Aanderud, and A. Aakvaag. Dehydration and body fluid-regulating hormones during sweating in warm (38°C) fresh—and seawater immersion. *Journal of Applied Physiology*. 2001;91(4):1529-1534.
19. Jarosz A, Darocha T, Kosinski S, et al. Profound Accidental Hypothermia: Systematic Approach to Active Recognition and Treatment. *ASAIO J*. 2017;63(3):26-30.
20. van der Ploeg G, Goslings J C, Walpoth B H, et al. Accidental hypothermia: Rewarming treatments, complications and outcomes from one university medical centre. *Resuscitation*. 2010;81(11):1550-1555.
21. Liqun Shang, Wei Wang, Jiyao Yu, et al. Effect of hypothermia on the survival of seawater-immersed rabbits with open abdominal wound and effect of early rewarming. *Medical Journal of Chinese People's Liberation Army*. 2010;35(12):1496-1498 (Chinese).
22. National Research Council. Guide for the care and use of laboratory animals. NIH Publication. 1996;85-23.
23. Md. AtiqurRahman, Dipok Kumar Dhar, Emi Yamaguchi, et al. Coexpression of Inducible Nitric Oxide Synthase and COX-2 in Hepatocellular Carcinoma and Surrounding Liver: Possible Involvement of COX-2 in the Angiogenesis of Hepatitis C Virus-positive Cases. *Clinical Cancer Research*. 2001;7:1325-1332.
24. Roggla M, Frossard M, Wagner A, et al. Severe accidental hypothermia with or without hemodynamic instability: rewarming without the use of extracorporeal circulation," *Wien KlinWochenschr*, vol.114, no.8-9, pp.315-320, 2002.
25. Polderman K H. "Mechanisms of action, physiological effects, and complications of hypothermia," *Crit Care Med*, vol.37, no.7 Suppl, pp.S186-S202, 2009.
26. G. M. Swank and E. A. Deitch, "Role of the gut in multiple organ failure: bacterial translocation and permeability changes," *World Journal of Surgery*, vol. 20, no. 4, pp. 411–417, 1996.
27. Z. W. Wang, Y. A. Wu, X. T. Liu et al., "Changes of gut mucosal barrier in rats with open celiac seawater immersion wounds," *Journal Of Nautical Medicine and Hyperbaric Medicine*, vol. 10, no. 3, pp. 129–132, 2003 (Chinese).
28. Z. H. Han, J. Y. Yu, M. Hu et al., "Effect of seawater immersion on the NF-κB, IκB and TLR4 expression in small intestinal tissues in rats with abdominal open injury," *African Journal of Pharmacy and Pharmacology*, vol. 7, no. 17, pp. 893–897, 2013.
29. O, Collange, A.-L, T. Lavaux et al. "Compartmentalization of Inflammatory Response Following Gut Ischemia Reperfusion," *European Journal of Vascular & Endovascular Surgery*, vol.1, no.49, pp.60–65, 2015.
30. Yeonsil Yu, SaeMiYoo, Hwan Hee Park, et al. "Preconditioning with interleukin-1 beta and interferon-gamma enhances the efficacy of human umbilical cord blood-derived mesenchymal stem cells-based therapy via enhancing prostaglandin E2 secretion and indoleamine 2,3-dioxygenase activity in dextran sulfate sodium-induced colitis," *J Tissue Eng Regen M*, vol.13, no.10, pp.1792-1804, 2019.

31. Stewart C R, Landseadel J P, Gurka M J, et al. "Hypothermia increases interleukin-6 and interleukin-10 in juvenile endotoxemic mice," *Pediatric Critical Care Medicine*, vol.11, no.1, pp.109-116, 2010.
32. Tang M, Zhao X, He Y, et al. "Aggressive re-warming at 38.5°C following deep hypothermia at 21°C increases neutrophil membrane bound elastase activity and pro-inflammatory factor release," *Springer Plus*, vol.5, no.1, pp.1-7, 2016.
33. Aibiki M, Maekawa S, Nishiyama T, et al. "Activated cytokine production in patients with accidental hypothermia. *Resuscitation*,1999;41(3):263-268.
34. Stewart C R, Landseadel J P, Gurka M J, et al. Hypothermia increases interleukin-6 and interleukin-10 in juvenile endotoxemic mice. *Pediatric Critical Care Medicine*.2010;11(1):109-116.
35. Cryer HG. Ischemia and reperfusion as a cause of multiple organ failure. *Multiple organ failure*. 2000. pp.112-113.
36. Moldawer LL, Minter RM, Rectenwald III J. Emerging evidence of a more complex role for proinflammatory and antiin-flammatory cytokines in the sepsis syndrome. *Multiple organ failure*. 2000;145-154.

## Figures

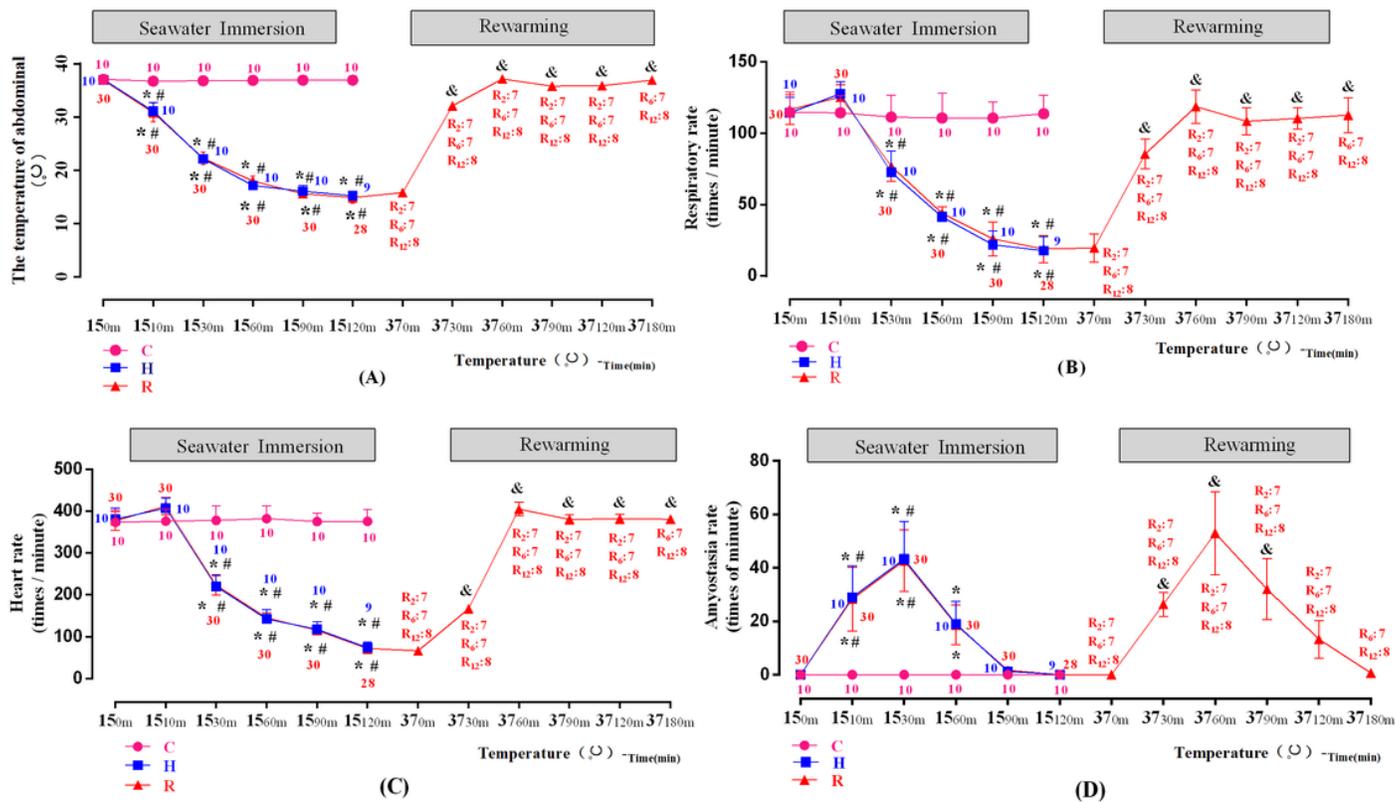
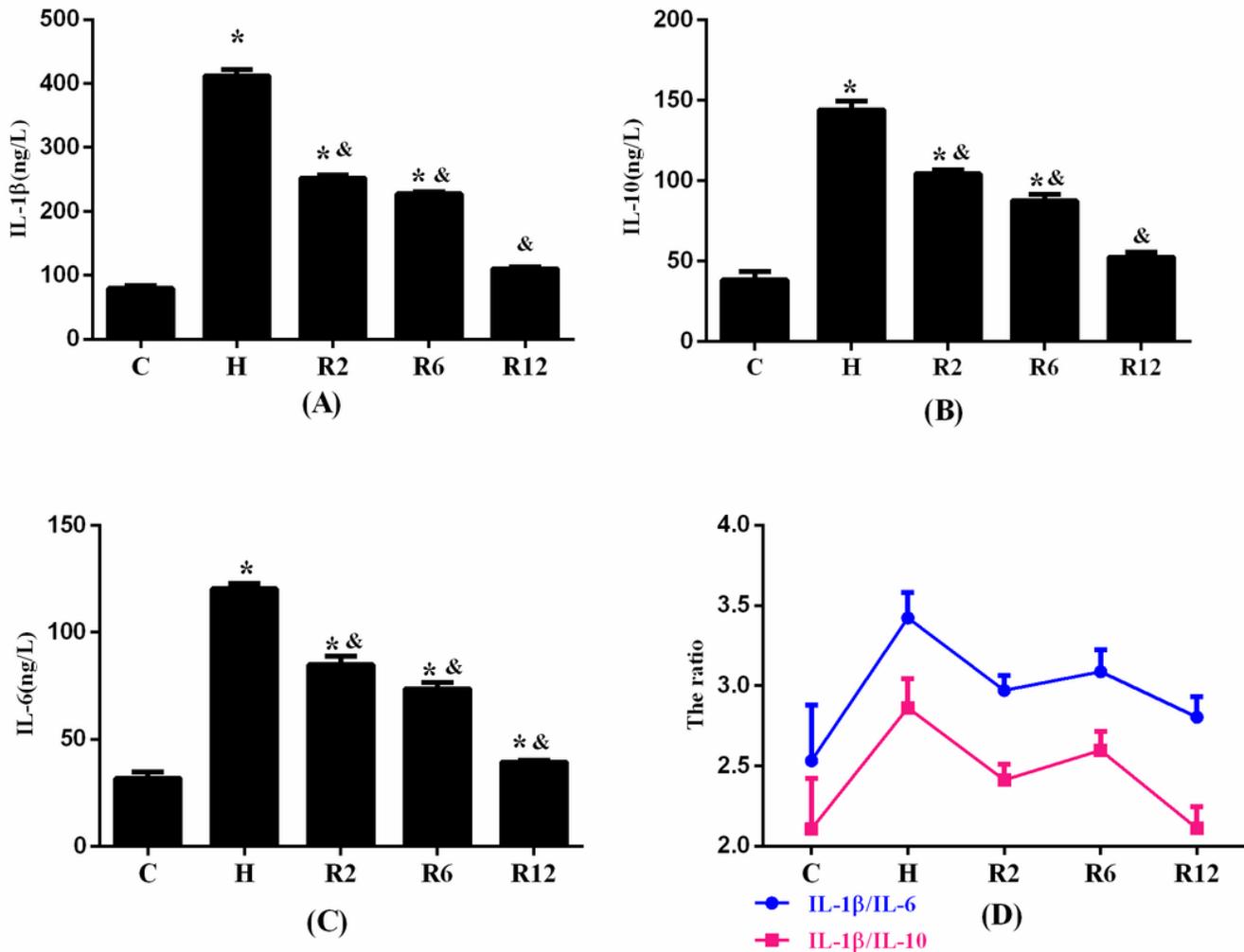


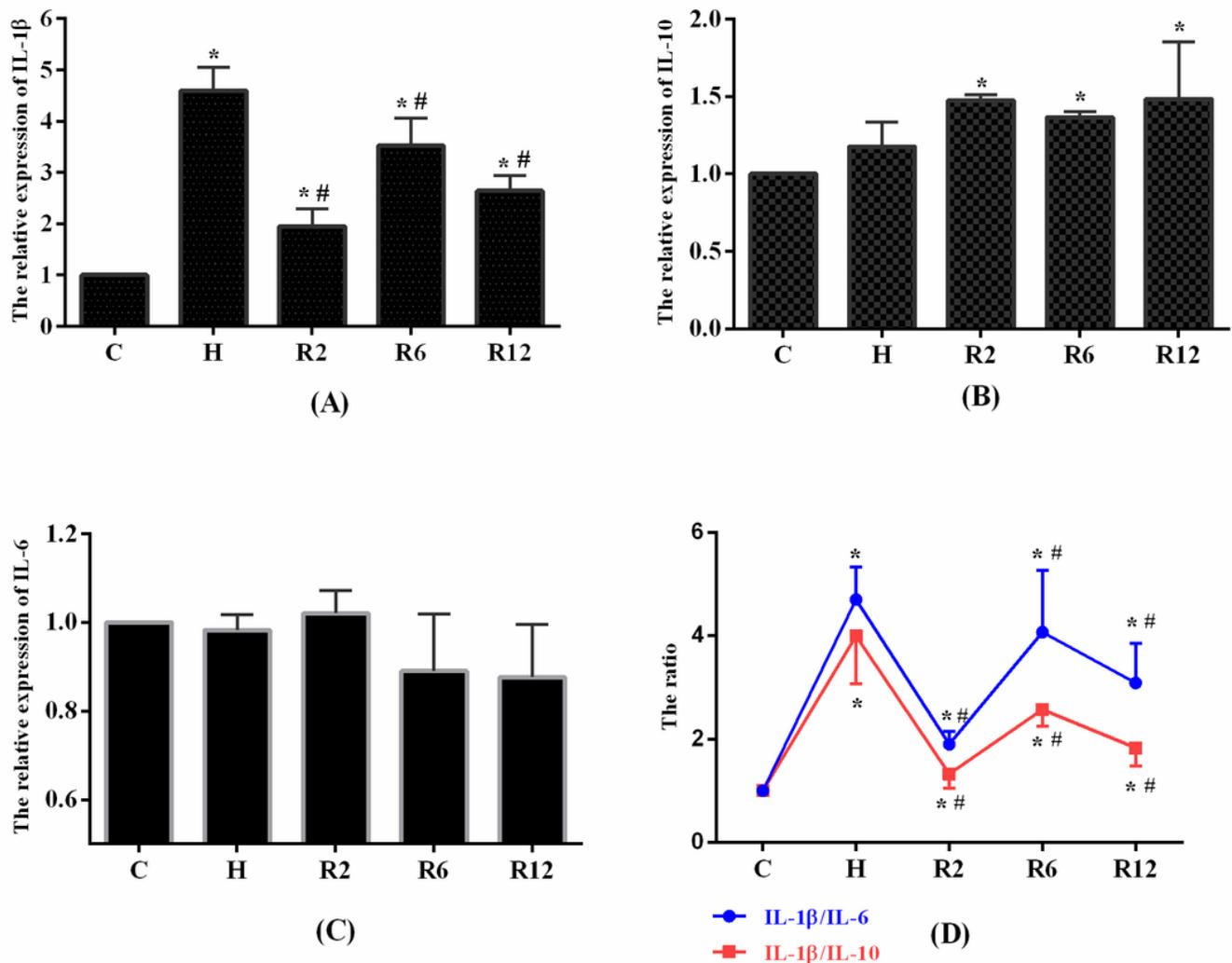
Figure 1

Changes in vital signs of rats under the overall process of hypothermia and rewarming(A,B,C,D). Time-matched hypothermia immersion (15°C) group for 120minutes and rewarming (37°C) group for 180minutes. (A) Changes in temperature of abdominal. (B) Changes in heart rate. (C) Changes in respiratory rate. (D) Changes in Amyostasia rate. N: normal control group; C: 15°C seawater immersion for 5 h; R: 15°C seawater immersion for 5 hand rewarming in 37°C water.150m:immersion in 15°C water for 0 minute. 370m:rewarming in 37°C water for 0 minute. Values are means  $\pm$  SD; \*P < 0.05 compared to C; #P < 0.05 compared with 150m in the hypothermia immersion group; &P < 0.05 compared with 370m in the rewarming group.



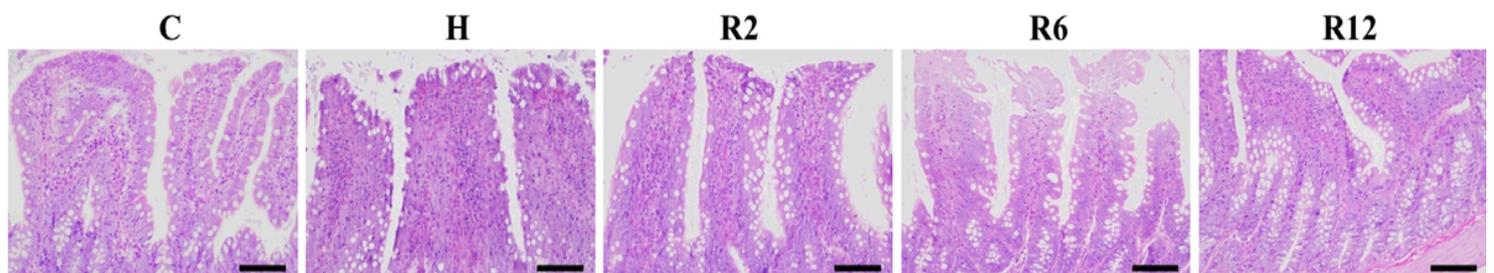
**Figure 2**

Cytokine expression levels in serum after hypothermia seawater immersion and rewarming at 37°C. (A) Changes of IL-1 $\beta$  in serum. (B) Changes of IL-10 in serum. (C) Changes of IL-6 in serum. (D) Changes of the ratio of IL-1 $\beta$ :IL-10 and IL-1 $\beta$ :IL-6. N: normal control group; C: 15°C seawater immersion for 5 h; R2, R6, R12: 15°C seawater immersion for 5 h, and rewarming in 37°C warm water bath for 2, 6, 12 h respectively. \*P < 0.05, compared with C; &P < 0.05, compared with H.



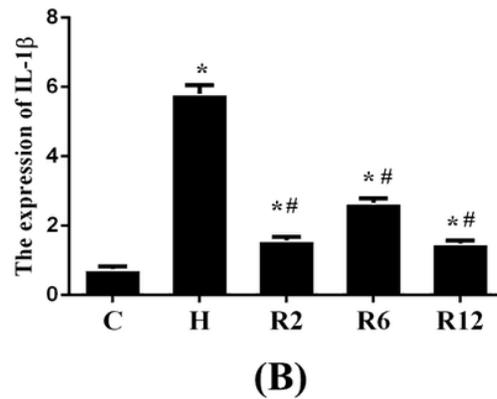
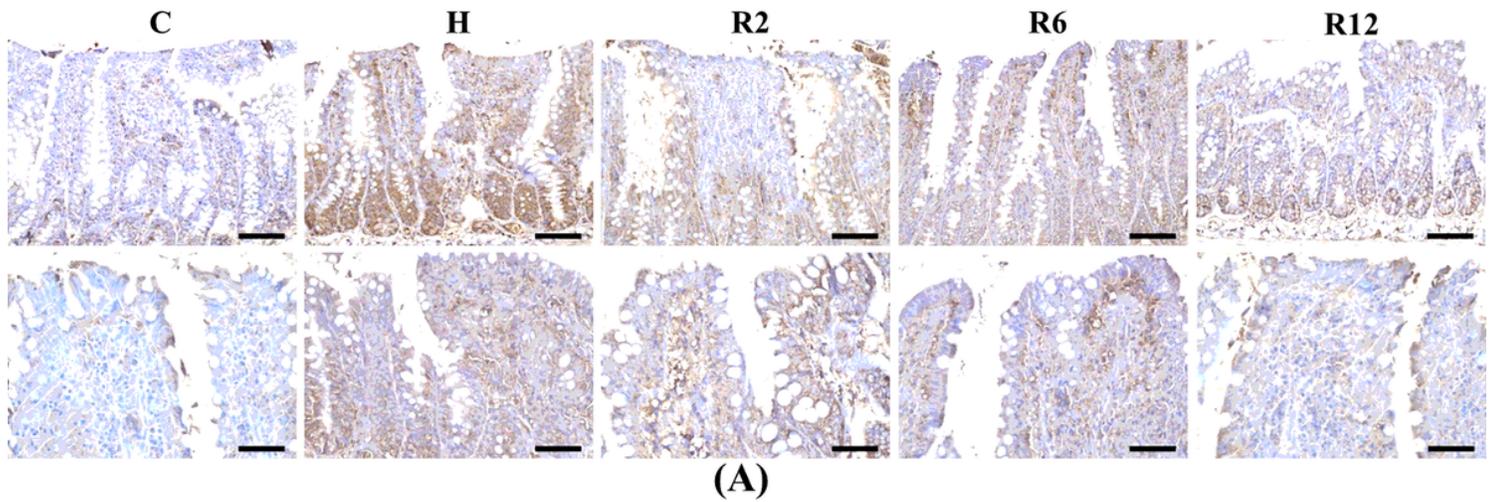
**Figure 3**

Cytokine gene expression in gut after hypothermia seawater immersion and rewarming at 37°C. (A) Changes of IL-1 $\beta$  in mRNA levels. (B) Changes of IL-10 in mRNA levels. (C) Changes of IL-6 in mRNA levels. (D) Changes of the ratio of IL-1 $\beta$ :IL-10 and IL-1 $\beta$ :IL-6. C: normal control group; H: 15°C seawater immersion for 5 h; R2, R6, R12: 15°C seawater immersion for 5 h, and rewarming in 37°C warm water bath for 2, 6, 12 h respectively. \* $P < 0.05$ , compared with C; # $P < 0.05$ , compared with H.



**Figure 4**

HE staining of the intestinal tissue in each group. C: normal control group; H: 15°C seawater immersion for 5 h; R2, R6, R12: 15°C seawater immersion for 5 h, and rewarming in 37°C warm water bath for 2, 6, 12 h respectively.



**Figure 5**

The expression level of IL-1 $\beta$  in the intestinal tissue in each group achieved by immunohistochemistry (IHC). (A) IHC of IL-1 $\beta$  in intestinal tissues of each group (pictures above: 200x; pictures below: 400x). (B): Comparison of IHC of IL-1 $\beta$  ("\*" indicated the comparison between group N and other groups with p-value < 0.05; "#" indicated the comparison between H and R2, R6, R12 group with p-value < 0.05). C: normal control group; H: 15°C seawater immersion for 5 h; R2, R6, R12: 15°C seawater immersion for 5h, and rewarming in 37°C water for 2, 6, 12 h respectively.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryMaterial1.docx](#)
- [SupplementaryMaterial2.docx](#)
- [SupplementaryMaterial3.xlsx](#)

- [SupplementaryMaterial4.xlsx](#)
- [OnlineS1.png](#)
- [OnlineS2.png](#)