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Original Article

The effects of cold-heat liver ischemia-reperfusion injury on HSP60 expression and ATPase activity in rats

Xidong Wang, Lianjie Liu, Zhixiong Wu

Department of Hepatobiliary, Pancreatic and Splenic Surgery, People's Hospital of Inner Mongolia Autonomous Region, Inner Mongolia Autonomous Region, China

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Abstract: Objective: To investigate the effects of cold-heat ischemia-reperfusion injury (IRI) on HSP60 and Na^+/K^+-ATPase in rat liver cells. Methods: 40 Sprague-Dawley rats were enrolled in the model of cold-heat IRI, consisting of the cold IRI group (group A, n = 10) and the heat IRI group (group B, n = 10) of liver transplantation, the control group (group C, n = 10) and the donor group (n = 10). The expression of HSP60 mRNA in the liver tissue was detected by RT-PCR, 3 h after the modeling. The relative expression level of HSP60 protein was detected by western blotting. The Na^+/K^+-ATPase activity in rat liver tissue was detected by measuring phosphorus levels 3 h after the modeling and the apoptosis of rat hepatocytes was detected by TUNEL apoptosis detection kit. The correlation between the levels of HSP60 mRNA, HSP60 protein and Na^+/K^+-ATPase activity in rat liver tissue was measured by the Pearson's test. Results: The activity of Na^+/K^+-ATPase in group A and group B was lower than that in group C ($P < 0.01$), and the activity of Na^+/K^+-ATPase in group A was higher than that in group B ($P < 0.01$). The expression of HSP60 mRNA in group A and group B were significantly higher than that in group C ($P < 0.01$). The expression of HSP60 protein in group A and group B was significantly higher than that in group C ($P < 0.01$). HSP60 mRNA and HSP60 protein levels in groups A and B were positively correlated with their Na^+/K^+-ATPase activity levels. Conclusions: Cold-heat IRI can promote the expression of HSP60 and inhibit the activity of Na^+/K^+-ATPase, and the expression of HSP60 is positively correlated with the Na^+/K^+-ATPase activity.

Keywords: HSP60, cold-heat ischemia-reperfusion, Na^+/K^+-ATPase, apoptosis

Introduction

Ischemia-reperfusion injury (IRI) is a non-surgical injury commonly occurring during liver transplantation and liver tumor resection [1]. When IRI occurs, it causes irreversible damage to the liver cells and affects the liver microcirculation. It can also lead to liver function-related metabolic disorder in patients, which has a serious impact on the postoperative recovery and prognosis [2]. According to the nature of ischemia, it can be divided into heat ischemia injury and cold ischemia injury. The liver heat IRI is more common in the microcirculation during shock, while the cold IRI is more common in liver transplantation [3]. These two forms of ischemic injury are mainly caused by ischemia-induced cell hypoxia and disruption in the energy transport system, which accelerates the consumption of stored substrates, and rapidly reduces ATP content in the cells, and eventually leads to cell apoptosis [4].

Heat shock proteins (HSP) are a kind of heat stress proteins widely found in bacteria as well as mammals [5]. When the body is exposed to high temperatures or other stresses, it induces the production of a set of defensive proteins that are highly conserved and help the cells to withstand environmental stress [6]. Heat shock protein 60 (HSP60), a member of the HSP family, is expressed in all eukaryotes and prokaryotes. Studies have shown that HPS60 is underexpressed in cells under normal conditions. When the cell is affected by external factors, the expression of HSP60 is rapidly increased [7]. ATP is a type of high-energy phosphate compound widely occurring in living cells. Na^+/K^+-ATPase, also known as sodium potassium pump, has been found to be an universal signal transductant [8]. Na^+/K^+-ATPase is a special type of protein present on the membrane of eukaryotic cells. Its main function is to obtain energy from ATP hydrolysis, and to realize the active transport of Na^+ and K^+ [9]. In recent

Table 1. Primer sequence

Gene	Upstream primer	Downstream primer
HSP60	5'-CTCGTGCGTGGGCGTGTTCC-3'	5'-TGGGCCTTGTAGTTCACCTG-3'
GAPDH	5'-GTTGGAACATCCGCAAAGAC-3'	5'-AAAGGGTGTAACGCAACTA-3'

years, there have been several studies reported on HSP60 and Na^+/K^+-ATPase in acute liver injury [10, 11], but there are relatively few reports on them in liver cold-heat IRI. The relationship between them is unclear.

Therefore, in this study, we explored the effects of cold-heat IRI on HSP60 and ATPase in rat liver cells, providing a reference for clinical use.

Materials and methods

Experimental animals

In this study, 40 12-week-old Sprague-Dawley clean grade rats (purchased from Shanghai Kaixue Biotechnology Co., Ltd.) were used. The rats weighed 250-350 g and included 20 females and 20 males. They were routinely fed for one week after purchasing. They had free access to water and food and were kept at room temperature, 23°C ± 3°C, with humidity maintained at 40 ± 10%.

Main reagents and instruments

RNA reverse transcription kit (TransGen Biotech, AT101-03, China), real-time PCR kit (TransGen Biotech, China, AQ111-03), Trizol extraction reagent (Invitrogen, USA 15596018), Na^+/K^+-ATPase kit (Shanghai Suobao Biotechnology Co., Ltd., F104), TNF-α ELISA kit (Shanghai Biyuntian Biotechnology Co., Ltd., PT512), IL-1β ELISA kit (Shanghai Biyuntian Biotechnology Co., Ltd., PI301), RIPA lysis buffer (Shanghai Biyuntian Biotechnology Co., Ltd., P0013B), BCA protein quantification kit (Shanghai Biyuntian Biotechnology Co., Ltd., P0009), mouse anti-human HSP60 protein monoclonal antibody (BD Biosciences, USA 611562), horseradish peroxidase labeling GaMIgG (BD Biosciences, USA 554002), and Annexin V-FITC kit (Shanghai Fushen Biotechnology Co., Ltd. BD 556547) were purchased from Nanjing Shengxing Biotechnology Co., Ltd., MB16-414. Microplate reader was purchased from Shanghai Guansen Biotech Co., Ltd. ABI 7900 PCR amplifier was purchased from USA ABI. Beckman-CytoFLEX flow cytometer was purchased from the USA Beckman, and the primer (**Table 1**) was designed and synthesized by Shanghai Shenggong Biological Co., Ltd.

Animal grouping

The rats were randomly divided into four groups of ten rats each by computer. Among them, group A (cold IRI model) had four males and six females, group B (heat IRI model) had five males and five females, and group C (control group) had six males and four females. The remaining ten rats were five females and five males in the group D (donor group), which were the liver organ donor to establish cold IRI model in group A. Therefore, rats in group D did not participate in the following experiments of this study. There was no statistical difference in age, sex and body weight among the groups ($P > 0.05$).

Preoperative preparation and surgery

The rats in the donor group were not fasted before surgery, whereas the rats in recipient group were fasted 12 h before surgery but had free access to water. The donor group was anesthetized with atropine (0.05 mg/kg) 30 min before surgery, while the recipient group received 8U sodium-penicillin per rat by intramuscular injection. During the surgery, the operator performed a naked-eye operation, and inhalation anesthesia was administered using diethyl ether. The rats were placed in the supine position on the operating table, and the abdomen and the lower chest were prepared for skin preservation and disinfected with iodophor. The rats in group C were first subjected to laparotomy and then the liver was obtained and then the rats were sacrificed. The cold-heat IRI model was established according to the literature of Bao et al. [12]. The color of the liver from the modeled rats was observed, and a color change from red to white to red indicates that the rat liver experienced IRI, i.e., a successful modeling of IRI was achieved. A filament lamp was used to maintain the body temperature of the rats. When the rats were not restrained from movement, the lamp was removed. The rats were sacrificed 3 h after reperfusion, after collection of the venous blood. The liver tissues were obtained for subsequent experiments, and the rest were stored at -80°C.

Detection of serum ALT and AST

Rat venous blood (1 ml) was allowed to stand at room temperature for 6 h, centrifuged at 3000

rpm for 15 min, and then the supernatant was collected and the expression of ALT and AST was detected by Hitachi 7600 automatic biochemical analyzer.

Detection of serum TNF-α and IL-1β

50 μl of different concentrations of standard solution was added to the plate microwells; 50 μl of distilled water and 50 μl of antibody was added to the blank control wells; to the remaining microwells 40 ul of sample was added initially, followed by 10 μl of biotinylated antibody. The next series of operations were performed following the kit instructions. The microplate reader was used for detection within 15 min to determine the maximum absorption at 450 nm.

Detection of Na^+/K^+-ATPase activity

The activity of Na^+/K^+-ATPase in rat liver tissue was detected by phosphorus measurement as follows: 0.1 g of collected tissue was added to 1 mL extract for homogenization in ice bath, followed by centrifugation at 8,000 rpm, 4°C for 10 min. The supernatant was extracted and placed on ice for testing. The buffer was separately added at 50 μL/well in a 96-well plate, and then the supernatant was added at 50 μL/well. A standard well and a negative control well were prepared with 50 μL volume. Then, 50 μL of the reaction solution was added to each well, the microtiter plates were gently shaken and incubated in a 37°C water bath for 10 min. Finally, 50 μL of the substrate solution was added to each well and mixed, followed by absorbance measurement at 660 nm to detect the Na^+/K^+-ATPase activity.

Detection of relative expression of HSP60

The total RNA was extracted from the collected liver tissue using Trizol extraction reagent and was analyzed by ultraviolet spectrophotometry and agarose gel electrophoresis to determine the purity, concentration and integrity. The total RNA was reverse transcribed using a reverse transcription kit. The procedure was carried out in strict accordance with the manufacturer's instruction, and the reverse transcribed cDNA was collected and a part of it was utilized in subsequent experiments. The following PCR reagents were used: 2 X TransStart Top Green qPCR SuperMix 10 μL, 0.4 μL of upstream and downstream primers, 2 μL of cDNA, ROX Dye II 50 × 0.4 μL, and nuclease-free water to add up to 20 μL. PCR conditions were as follows: predenaturation at 95°C for 30 s, 95°C for 5 s, 60°C for 30 s, for a total of 40 cycles. Three replicate wells were set for each sample and the experiment was performed three times independently. In this study, GAPDH was used as an internal reference, and the data was analyzed with $2^{-\Delta\Delta ct}$.

Detection of HSP60 protein

Total protein from rat liver tissue was extracted at -80°C by RIPA lysis method and quantified by BCA method. 5 x SDS buffer solution was added to the protein samples and electrophoretically separated by SDS-PAGE. For 8% spacer gel, 80 V constant pressure was used, and for 5% separation gel, it was changed to 120 V. The protein bands were transferred to a film of difluoroethylene and detected after dyeing in Lichunhong working solution. The cells were immersed in PBST for 5 min, and 5% skim milk powder was added for overnight incubation at 4°C. Each antibody was diluted with PBST containing 1% skim milk powder, and a mouse anti-human HSP60 protein monoclonal antibody (1:500) was added and incubated at 4°C overnight. The primary antibody was removed, and the membrane was washed with TBST, and horseradish peroxidase labeling reagent GaMIgG (1:5000) was added, incubated at 37°C for 1-2 h, and rinsed 5 times with TBST for 5 min each. The image was developed in a darkroom, and the liquid on the membrane was dried using a filter paper, and the ECL illuminant was added thereon, and exposed after 5 min. The protein bands were scanned, and the gray values were analyzed using the Quantity One software, where the relative expression level of the protein = the gray value of the target protein band/the gray value of the GAPDH protein band.

Detection of apoptosis

1 gram of rat liver tissue was taken, fixed with 4% paraformaldehyde, dehydrated by gradient ethanol, embedded in paraffin, sectioned, and the apoptosis of rat flap cells was detected by TUNEL apoptosis detection kit. The experimental method was carried out in strict accordance with the manufacturer's instructions. After staining, the light microscopy was used to observe the nucleus staining of the flaps. The nucleus staining of the normal flaps was blue, and the nucleus staining of the apoptotic flaps

Table 2. ALT and AST expression in serum of three groups of rats

Group	A group (n = 9)	B group (n = 10)	C group (n = 9)	F value	*P* value
ALT (U/L)	1258.69 ± 155.42[a,b]	835.74 ± 82.14[a]	60.32 ± 3.85	327.749	0.000
AST (U/L)	1288.91 ± 160.58[a,b]	894.96 ± 108.55[a]	59.84 ± 5.39	284.361	0.000

Note: [a]indicates differences compared with group C. (P < 0.05). [b]indicates differences compared with group C. (P < 0.05).

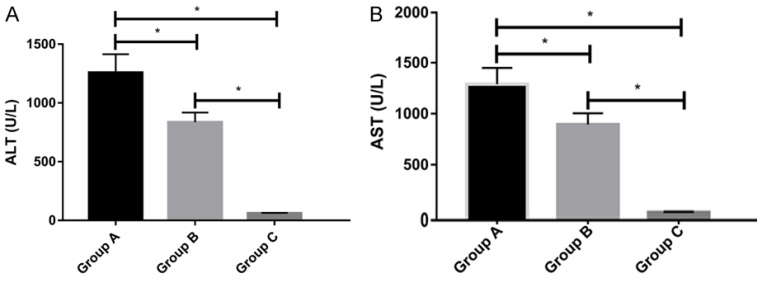

Figure 1. The expression of ALT and AST in the serum of three groups of rats.

was yellow or brownish yellow. Five area of each section were randomly observed. Apoptotic cells were counted (apoptosis rate = total apoptotic number/total number of cells * 100%).

Outcome measures

Main outcome measures: The relative expression levels of HSP60 mRNA and HSP60 protein in liver tissue was observed 3 h after the successful modeling of IRI in the rats. The activity of NA^+/K^+-ATPase in liver tissue was observed 3 h after successful modeling, and the apoptosis of hepatocytes, the relationship between HSP60 mRNA and HSP60 protein and Na^+/K^+-ATPase activity were examined. Secondary outcome measures: The expression of ALT, AST, TNF-α and IL-1β in serum was observed after the modeling in the rats.

Statistical analysis

In this study, the collected data was statistically analyzed using the SPSS20.0 software package (Guangzhou Bomai), and the data was plotted using GraphPad Prism 7 (Shanghai Beka), in which the enumeration data was expressed by rate (%), tested by chi-square test, and indicated as chi-square. The measurement data were expressed as mean ± standard deviation (mean ± SD). The comparison between groups was analyzed by ANOVA with post hoc Bonferroni tests. Pearson's correlation was used to analyze the relationship between HSP60 mRNA and HSP60 protein and NA^+/K^+-ATPase activity. P < 0.05 means a statistical difference.

Results

Rat model of liver IRI

In this experiment, one rat in group C died of anesthesia accident; one rat in group A died of superior and inferior vena cava anastomosis bleeding in the modeling process, and no death occurred in group B.

The expression of ALT and AST in the serum of rats

We detected the expression of ALT and AST in the serum of three groups of rats. The serum levels of ALT and AST in group A and group B were significantly higher than those in group C (P < 0.01). The expression of ALT and AST in group A was higher than that in group B (P < 0.01) (**Table 2** and **Figure 1A**, **1B**).

The expression of TNF-α and IL-1β in the rat serum

We detected the expression of TNF-α and IL-1β in the serum of three groups of rats. It was found that the expression of TNF-α and IL-1β in serum of group A and group B was higher than that of group C (P < 0.01), and the expression of TNF-α and IL-1β in group A was significantly higher than that in group B (P < 0.01) (**Table 3** and **Figure 2A**, **2B**).

The activity of Na^+/K^+-ATPase in rat liver tissue

We detected the activities of Na^+/K^+-ATPase in the liver tissues of three groups of rats. The activity of Na^+/K^+-ATPase in group A and group B was lower than that in group C (P < 0.01), and the activity of NA^+/K^+-ATPase in group A was higher than that in group B (P < 0.01) (**Table 4** and **Figure 3**).

The expression of HSP60 mRNA in rat liver tissue

We used RT-PCR to detect the expression of HSP60 mRNA in the liver tissues of three

Table 3. Expression of TNF-α and IL-1β in serum of rats

Group	A group (n = 10)	B group (n = 10)	C group (n = 10)	F value	*P* value
TNF-α (μg/L)	2.38 ± 0.32[a,b]	1.71 ± 0.35[a]	0.72 ± 0.15	74.730	0.000
IL-1β (μg/L)	1.32 ± 0.26[a,b]	0.82 ± 0.20[a]	0.18 ± 0.05	79.818	0.000

Note: [a]indicates differences compared with group C. (P < 0.05). [b]indicates differences compared with group C. (P < 0.05).

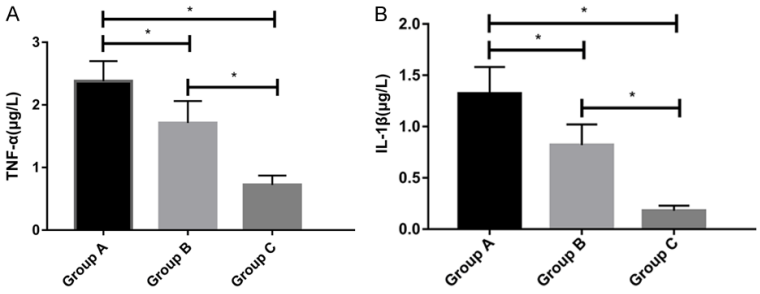

Figure 2. The expression of TNF-α and IL-1β in the serum of three groups of rats.

Table 4. Activity of NA^+, K^+-ATPase in liver tissue of rats

Group	NA^+, K^+-ATPase (μmol/mg/h)	F value	*P* value
A group (n = 9)	2.36 ± 0.42[a,b]	152.464	0.000
B group (n = 10)	1.21 ± 0.26[a]		
C group (n = 9)	4.63 ± 0.59		

Note: [a]indicates differences compared with group C. (P < 0.05). [b]indicates differences compared with group C. (P < 0.05).

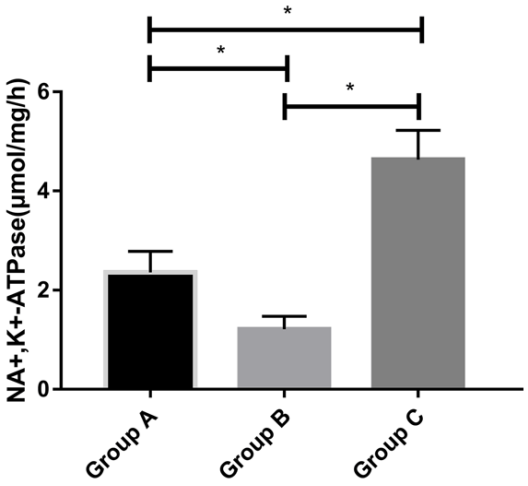

Figure 3. The activity of Na^+/K^+-ATPase in rat liver tissue.

groups of rats. It was found that the expression of HSP60 mRNA in group A and group B was significantly higher than that in group C (P < 0.01). There was no difference in the expression of HSP60 mRNA between group A and group B (P > 0.05) (**Table 5** and **Figure 4**).

The expression level of HSP60 protein in rat liver tissue

We used western blot to detect the expression of HSP60 protein in the liver tissues of the three groups. The expression of HSP60 protein in group A and group B was significantly higher than that in group C (P < 0.01), and there was no difference in the expression of HSP60 protein between group A and group B (P > 0.05) (**Table 6**, **Figures 5** and **6**).

Hepatocyte apoptosis in the rats

We detected the hepatocyte apoptosis in the rats by flow cytometry. It was found that the apoptosis of hepatocytes in group A and group B was higher than that in group C (P < 0.01); there was no difference in the hepatocyte apoptosis between group A and group B (P > 0.05) (**Table 7** and **Figure 7**).

Correlation analysis between HSP60 mRNA and HSP60 protein and Na^+/K^+-ATPase in rat liver tissue

We analyzed the expression of HSP60 mRNA and HSP60 protein and Na^+/K^+-ATPase in the liver tissues of rats in group A and group B by the Pearson's test (**Figure 8A**, **8B**). HSP60 mRNA and HSP60 protein in A and B groups were positively correlated with Na^+/K^+-ATPase activity (**Figure 8C**, **8D**).

Discussion

Reperfusion injury (RI) is a pathological phenomenon in which the organ damage is aggravated after IRI occurs within a certain period of time [13]. According to the nature of RI, it can be divided into cold IRI and heat IRI. When the patient undergoes liver resection, the blood flowing into and out of the liver channel needs

Table 5. The expression of HSP60 mRNA in rat liver

Group	HSP60 mRNA	F value	*P* value
A group (n = 9)	1.694 ± 0.528[a]	10.717	0.000
B group (n = 10)	1.784 ± 0.421[a]		
C group (n = 9)	1.011 ± 0.049		

There was significant difference between group A and group C (P < 0.05). [a]P<0.05 compared with C group.

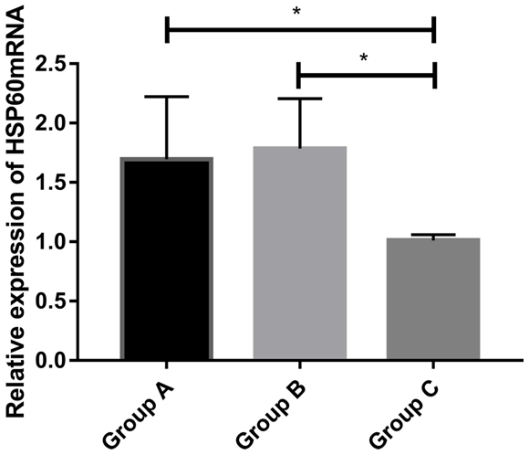

Figure 4. The expression of HSP60 mRNA in rat liver tissue.

Table 6. Expression of HSP60 protein in rat liver

Group	HSP60 protein expression	F value	*P* value
A group (n = 9)	1.231 ± 0.084[a]	185.190	0.000
B group (n = 10)	1.286 ± 0.080[a]		
C group (n = 9)	0.684 ± 0.052		

There was significant difference between group A and group C (P < 0.05). [a]P<0.05 compared with C group.

to be blocked, or the hepatic flow should be half-blocked to avoid large bleeding during the operation to avoid unnecessary harm [14]. In addition, with the advances in liver transplant surgery, it has become the best treatment for various end-stage liver diseases. However, the donor liver needs to be preserved by cold perfusion before surgery, which will aggravate cold IRI injury in the liver [15]. Related studies have shown that [16] cold-heat IRI is an important factor for liver dysfunction, hepatic congestion and non-function after surgery.

HSP60 is an important class of molecular chaperones, which are widely distributed in prokaryotes and eukaryotes. Studies have shown that

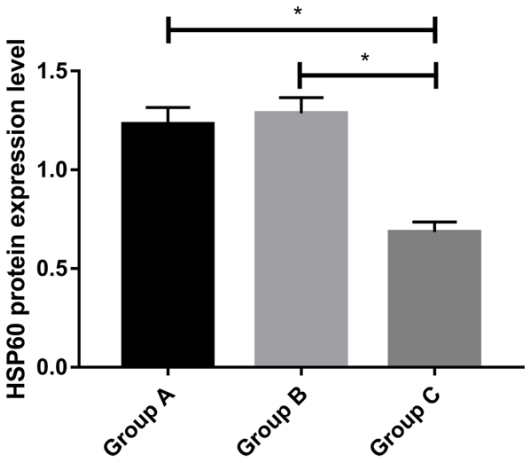

Figure 5. The expression level of HSP60 protein in rat liver tissue.

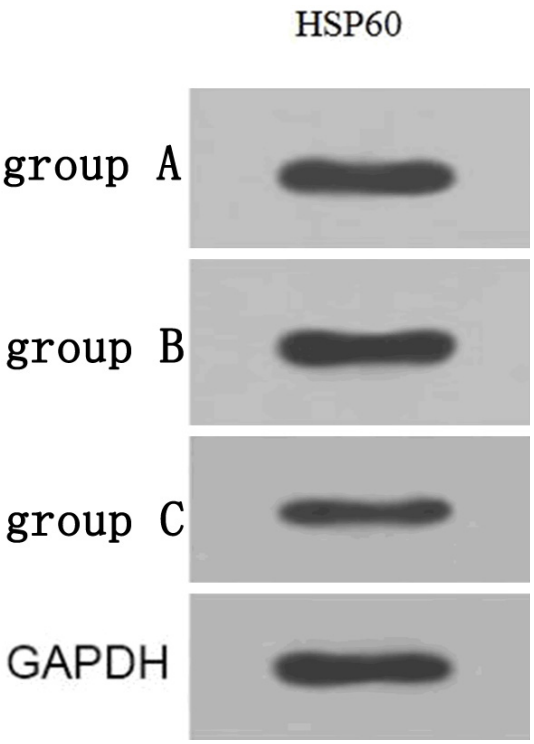

Figure 6. The expression level of HSP60 protein in rat liver tissue.

[17] the body can produce a large number of HSP60 after being stimulated by its environment, which can be evolved into a highly conserved antigenic exogenous molecule considered by the immune system. In recent years, studies have shown that [18, 19] HSP60 has a certain protective effect in IRI and is also expressed in various cancers. Na^+/K^+-ATPase, as an important enzyme in the tricarboxylic

Table 7. Hepatocyte apoptosis in rats

Group	Apoptosis rate	F value	*P* value
A group (n = 9)	21.36 ± 3.44[a]	70.933	0.000
B group (n = 10)	22.58 ± 4.28[a]		
C group (n = 9)	5.84 ± 1.84		

There was significant difference between group A and group C (P < 0.05). [a]P<0.05 compared with C group.

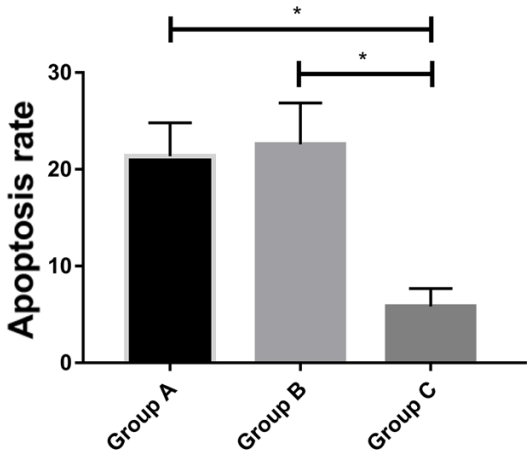

Figure 7. Hepatocyte apoptosis in the rats.

acid cycle, is a heterodimer composed of one α subunit and one β subunit [20]. As a transmembrane protein, the α subunit promotes extracellular K^+ and Na^+ exchange; thus, Na^+/K^+-ATPase regulates the balance between ions outside the body [21]. From the literature review, it was found that there were no reported studies on HSP60 and Na^+/K^+-ATPase in hepatic cold-heat IRI. Therefore, in this study, we established a rat model of the liver heat-cold IRI to detect the expression of HSP60 and NA^+/K^+-ATPase in rat liver tissue, and explored the relationship between them to provide a basis for clinical use.

In this study, we used Sprague-Dawley clean grade rats for the establishment of cold-heat ischemia RI. We examined the levels of ALT, AST, TNF-α, and IL-1β in the rat serum. ALT and AST are important indicators of liver function, whose difference in expression can directly indicate liver function. TNF-α and IL-1β act as cytokines with high expression in the liver upon IRI, and the interaction between endothelial cells and neutrophils can cause obstacles in the circulation of the liver [16, 22]. Here, we found that the expression of ALT, AST, TNF-α, and IL-1β was significantly increased and the expression of each indicator in group A was higher than that in group B, and the liver color of the two groups was changed from red to white and to red again. Subsequently, we detected the activity of Na^+/K^+-ATPase in rat liver tissue, and found that the activity of Na^+/K^+-ATPase in group A and group B decreased significantly after 3 h, which was significantly lower than that in group C. In addition, the activity of Na^+/K^+-ATPase in group B was significantly lower than that in group A. In a study by Huang [23], Na^+/K^+-ATPase activity was also reduced in rat brain tissue, which was established in a model of cerebral ischemia in rats. This was because the mitochondria cannot supply oxygen during hepatic ischemia in both the groups, which in turn reduced the synthesis of ATP. In addition, the energy consumption could not be reduced and hence the Na^+/K^+-ATPase activity was reduced. Furthermore, we detected the relative expression of HSP60 mRNA and HSP60 protein in rat liver tissue. The relative expressions of HSP60 mRNA and HSP60 protein in liver tissue of group A and group B were higher than that of group C, indicating that the liver cold or heat IRI can increase the expression of HSP60. In addition, we detected the apoptosis of the three groups of rats by flow cytometry, and found that the apoptosis of rats in group A and group B were significantly higher than that in group C; this is because the liver IRI produced a large number of oxygen free radicals, causing direct damage leading to apoptosis. This suggested that HSP60 was highly expressed in the presence of cold-heat IRI in the liver and may be a potential indicator of liver IRI. Moreover, in the study of Pan et al. [24], the expression of HSP60 was significantly increased after testicular ischemia-reperfusion in rats, indicating that HSP60 not only has high expression in liver IRI model but was also differentially expressed in other IRI models. At the end of the study, we found that the activity of NA^+/K^+-ATPase and expression of HSP60 mRNA and HSP60 were positively correlated, as determined by the correlation analysis. The related studies showed that [25], HSP60 played a role in maintaining mitochondrial integrity and ATP production capacity, indicating that Na^+/K^+-ATPase activity may have certain regulatory relationship with HSP60 expression. Further research is needed to understand the regulation between them.

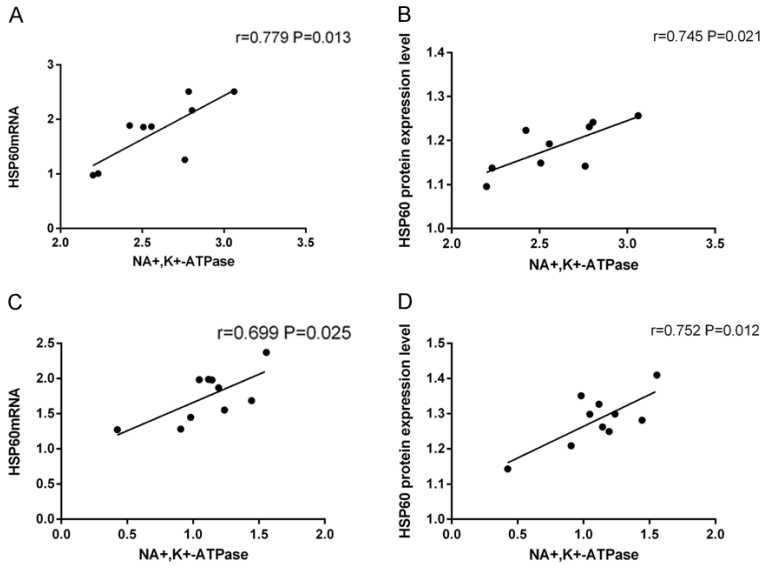

Figure 8. The correlation of HSP60 mRNA and HSP60 protein with Na^+/K^+-ATPase enzyme in rat liver tissue. There was a positive correlation of HSP60 mRNA and HSP60 protein levels in each group with their Na^+/K^+-ATPase enzyme activity. (A) Indicates that HSP60 mRNA vs Na^+/K^+-ATPase, (B) indicates HSP60 protein vs Na^+/K^+-ATPase, (C) indicates that Na^+/K^+-ATPase vs protein, (D) indicates HSP60 protein vs Na^+/K^+-ATPase.

There were certain limitations in this study. For example, we have not tested Ca^{2+}-ATPase in this study, and do not know how HSP60 expression and Na^+/K^+-ATPase activity are regulated. Hence, in future research, we plan to study the relationship between HSP60 and Na^+/K^+-ATPase, to verify the results of our study.

In summary, liver cold-heat IRI promotes HSP-60 expression, inhibits the activity of Na^+/K^+-ATPase, and HSP60 expression is positively correlated with Na^+/K^+-ATPase activity.

Disclosure of conflict of interest

None.

Address correspondence to: Xidong Wang, Hepatobiliary and Pancreatic Surgery, Inner Mongolia Autonomous Region People's Hospital, No. 20 Zhaowuda Road, Saihan District, Hohhot 010000, Inner Mongolia Autonomous Region, China. Tel: +86-0471-3286744; E-mail: wangxidong-2005@163.com

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