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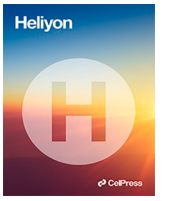

Intestinal alkaline phosphatase improves intestinal permeability and alleviates multiple organ dysfunction caused by heatstroke

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ABSTRACT

Objective: Heatstroke (HS) is a severe acute disease related to gastrointestinal barrier dysfunction, systemic inflammation and multiple organ injury. Many of the functions of Intestinal alkaline phosphatase (IAP) have been linked to gut homeostasis, gut barrier function and inflammation. However, the protective effect of IAP on heatstroke is not fully elucidated. This study aims to explore the protective effect of IAP on heatstroke by maintaining intestinal barrier and improving permeability.

Methods: Male C57BL/6 mice were placed in a controlled climate chamber (ambient temperature: 40.0 ± 0.5 °C; humidity: 60 ± 5 %) until the maximum core temperature (Tc, max) reached 42.7 °C (the received criterion of HS). Then heat exposed mice (n = 195) were divided into three groups: 0.2 mL of 0.9 % physiological saline (HS) or vehicle (HS + Vehicle) or 300 IU IAP (HS + IAP) by gavage at 0, 24, and 48 h after onset. Control group mice (Con) (n = 65) were not exposed to heat and were gavaged with 0.9 % physiological saline of the same volume at the same time.

Results: IAP treatment significantly reduced the levels of endotoxin, FD4, and D-lactate in the blood of heatstroke mice, reduced intestinal permeability and maintained the integrity of the intestinal barrier by increasing the expression of tight junction proteins. Meanwhile, IAP treatment alleviated liver and kidney damage caused by heatstroke, reduced serum levels of inflammatory cytokines, and thus improved survival rate of mice after heatstroke.

Conclusion: This study indicates that IAP can improve the intestinal barrier function and intestinal permeability by increasing intestinal tight junctions, reduce systemic inflammation and multiple organ injury and improving the survival rate of heatstroke. Therefore, we consider IAP may be added to enteral nutrition formulas as a potential means for diseases characterized by intestinal permeability disorders, including heatstroke.

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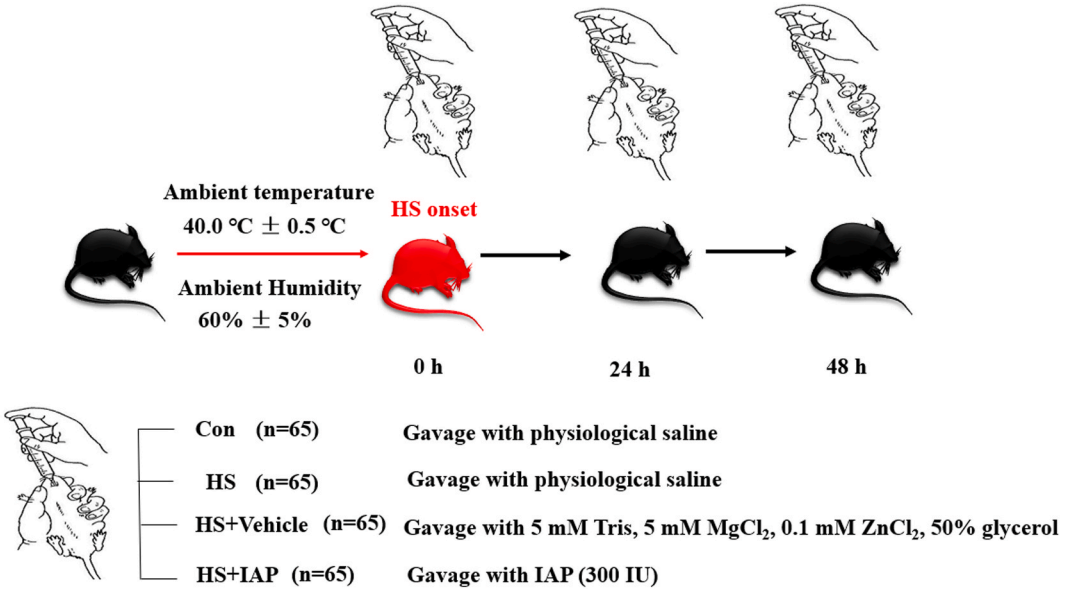

Fig. 1. Experimental flow chart.

1. Introduction

Climate change has increased the frequency and duration of extreme heat waves, and heatstroke often occurs during heat waves [1]. Symptoms of heatstroke include severe hyperthermia (Tc > 40 °C) and systemic inflammation, which can affect multiple organs [2]. Even in intensive care units, the mortality rates for classic and exertional heatstroke reached 26.5 % and 63.2 %, respectively [3]. Other than rapid cooling, there are no specific therapeutic drugs used in clinical practice.

The pathogenesis of heatstroke is still largely unknown [1]. Currently, a "sepsis-like" mechanism is widely recognized, which suggests that heat exposure causes vascular endothelial damage, intestinal barrier dysfunction, and endotoxemia, ultimately leading to systemic inflammation and multiple organ dysfunction [4,5]. Consequently, intestinal barrier function is closely related to heatstroke pathogenesis. As a result of heatstroke, intestinal barrier dysfunction occurs due to ischemi reperfusion, oxidative stress, inflammatory mediators, and cytokines. Dysfunction of the intestinal barrier and endotoxemia, ischemic shock, organ dysfunction, and other causes and consequences form a vicious cycle. Given the important role that intestinal barrier dysfunction plays in heatstroke occurrence and development, protecting intestinal barrier function may be an effective treatment option.

The endogenous glycoprotein intestinal alkaline phosphatase (IAP) is mainly distributed in the apical microvilli of the brush-like border of intestinal epithelial cells. For intestinal homeostasis, IAP is necessary [6]. IAP deficiency or lack of expression can worsen intestinal inflammation and damage in inflammatory bowel disease and metabolic syndrome, as well as increase intestinal permeability [7]. Animal experiments have shown that supplementation with IAP can alleviate these symptoms [8]. According to these studies, IAP inhibits inflammation and protects the intestinal barrier. We therefore hypothesize that IAP may also contribute to the development and occurrence of heatstroke. To determine if IAP protects against intestinal injury, systemic inflammation, and multiple organ dysfunction caused by heatstroke, this study examined mice with heatstroke. A theoretical basis for treating heatstroke will be provided by this study.

2. Materials and methods

2.1. Animals

The Animal Centre of the Army Medical University (Chongqing, China) provided male C57BL/6J mice (6–8 weeks, 17–20 g). In addition to standard chow and sterile water *at libitum*, mice were housed at an ambient temperature of 23±1 °C and humidity of 55±5 %. Army Medical University's Laboratory Animal Welfare and Ethics Committee approved all experimental protocols.

Many research teams have used different animals for heatstroke research, such as dogs [9], baboons [10], rats [11], mice [12], etc. Given that establishing heatstroke model requires sacrificing large numbers of animals to achieve stable and reproducible results, small mammals are typically used more frequently. Male C57BL/6 mice are commonly used model animals in the medical field. The heatstroke model established by male C57BL/6 mice has been very stable in our laboratory [13,14] and also been used in many literatures [15].

2.2. Induction of heatstroke and IAP treatment

In this study, the animal model was divided into two parts. The first part was induction of heatstroke. The second part was to treat heatstroke through intragastric administration of IAP.

Part I: The method of inducing the heatstroke model has been previously described [14]. For induction of heatstroke, mice were placed in a prewarmed specific environmental control smart chamber (HOPE-MED 8150E, Tianjin, China) maintained at 40 ± 0.5 °C and 60 ± 5 % relative humidity. An animal electronic thermometer (Alcbio, China) was used to directly measure the core temperature (Tc). The Tc reached 42.7 °C (heatstroke onset).

Part II: There were four groups (Fig. 1). Mice with heatstroke (HS) were given 0.2 mL of 0.9 % physiological saline by gavage at 0, 24, and 48 h after onset. Heatstroke plus vehicle (HS + Vehicle) mice received a 50 % glycerol solution (5 mM Tris, 5 mM $MgCl_2$, 0.1 mM $ZnCl_2$) by gavage at 0, 24, and 48 h after the onset of heatstroke. In the heatstroke plus IAP (HS + IAP) group, the mice were given 300 IU of IAP (Sigma-Aldrich, USA) solution prepared with 50 % glycerol solution (5 mM Tris, 5 mM $MgCl_2$, 0.1 mM $ZnCl_2$) by gavage at 0, 24, and 48 h after the onset of heatstroke. In the control group (Con), mice were not exposed to heat and were given 0.9 % physiological saline by gavage at the same time. The dose and duration of IAP has been previously described [16]. We set the dosing time based on the survival rate of HS mice. In our study, after three doses, the survival rate of HS + IAP group mice were significantly higher than that of the HS group.

2.3. Survival rate

Heat-exposed mice were orally gavaged with 0.9 % physiological saline (HS) or 300 IU IAP or vehicle without IAP at 0, 24, and 48 h after exposure to the smart chamber. Within 120 h, the survival rate of each group of mice was calculated.

2.4. Fecal IAP activity

The IAP assay has been previously described [17]. After homogenizing 0.1 g of stool in water and incubating it on ice for 30 min, the sample was analyzed. As a result, the homogenates and supernatants were centrifuged for 15 min at 4 °C at 15,000 g to determine the IAP activity and protein concentration. Aidlab Biotechnologies provided the BCA (bicinchoninic acid) Protein Assay kit for protein quantification. In the IAP assay, 25 μL of supernatant was mixed with 175 μL of phosphatase assay reagent containing 5 mM p-nitrophenyl phosphate (pNPP, Sigma-Aldrich, USA). Enzyme specific activity is measured in picomoles of pNPP hydrolyzed per minute per gram of protein.

2.5. Biochemical markers and cytokines in serum

The mice were anesthetized with pentobarbital (40 mg/kg body weight) intraperitoneally after 72 h of heat exposure [13,14]. The serum was separated by centrifugation at 3000 rpm for 30 min at 4 °C (Eppendorf, Germany) after blood samples were collected. With a special automatic biochemical analyzer for animals (MNCHIP, China), the liver function indices alanine transaminase (ALT) and aspartate transaminase (AST), as well as the renal function indices blood urea (BUN) and creatinine (CREA), were detected.

For this study, an enzyme-linked immunosorbent assay (ELISA) kit (TNF-α, IL-1β, IL-6, Beyotime, China) was used, and 96-well plates were incubated for 60 min at 37 °C. Specifically, serum samples and enzyme labeling reagents were added to wells coated with TNF-α/IL-1β/IL-6 antibodies. For 20 min, streptavidin labeled with horseradish peroxidase was incubated at room temperature in the dark. IFor termination of the reaction, tetramethylbenzidine reagent was added, and absorbance was measured at 450 nm using a spectrophotometer (Bio-Rad, USA).

2.6. Histological examination

Following blood withdrawal, liver, kidney, and ileum samples were collected and preserved in 10 % neutral buffered formalin. We examined all preserved tissues microscopically (Leica DM 2000, Germany) in a blinded manner after paraffin embedding, sectioning, staining, and microscopic staining.

2.7. Endotoxin analysis in serum

The Pierce™ Chromogenic Endotoxin Quant Kit (Thermo Fisher Scientific, USA) was used for the endotoxin bioassay in duplicate. No pyrogenic micropipette tips, tubs, or other instruments were used. Reconstituted Amebocyte Lysate Reagent was added in non-pyrogenic tubes and incubated at 37 °C for 15 min. Before addition of 25 % acetic acid, a chromogenic substrate solution was added and incubated for 6 min at 37 °C. The 405 nm OD value was measured with a microplate spectrophotometer (Bio Rad, USA). The results are expressed as EU/mL.

2.8. Intestinal permeability assay

2.8.1. FD4 detection

For determination of gastrointestinal permeability, a solution of fluorescein isothiocyanate (FITC)-dextran (FD4, Sigma Aldrich

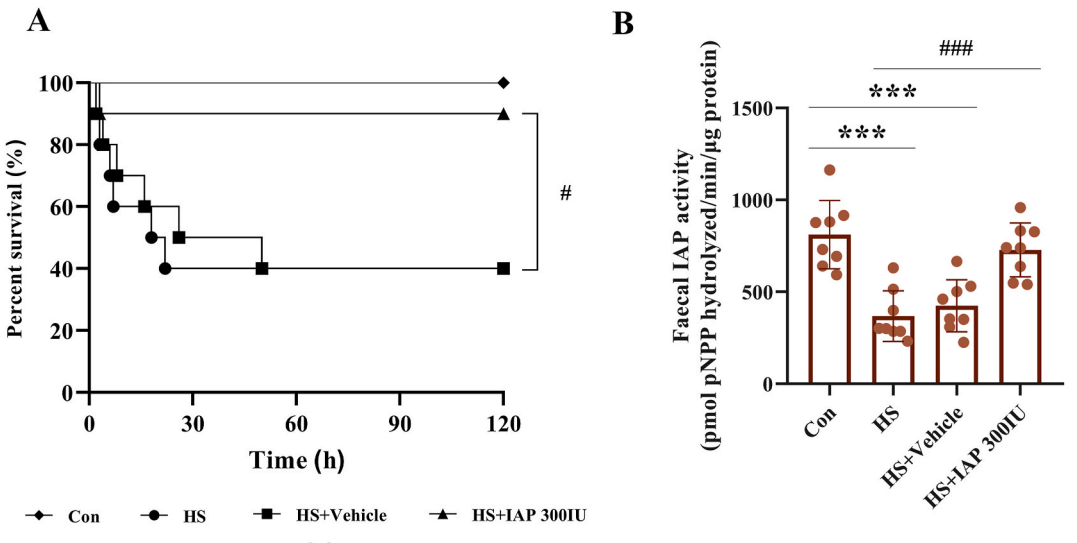

Fig. 2. Survival rates and fecal IAP activity induced by heatstroke were alleviated by IAP. (A) Survival rates (%) of mice in the Con group, HS group, HS + Vehicle group, and HS + IAP group are shown at 120 h after HS. The sample size was 10 mice/group. IAP treatment significantly improved the survival rate after heatstroke. (B) IAP activity in fecal samples in the Con group, HS group, HS + Vehicle group, and HS + IAP group was measured. IAP treatment increased the low levels of IAP activity after heatstroke. Values are expressed as the means ± SDs, n = 8 per group, vs. Con ***, $p < 0.001$; vs. HS #, $p < 0.05$; ###, $p < 0.001$. Con, control; HS, heatstroke; IAP, intestinal alkaline phosphatase.

USA) was used. For protection from light, Eppendorf tubes wrapped in silver paper were used to prepare FD4 (20.8 mM). By gavage, mice were administered 10 ml/g of FD4 solution. Plasma was obtained from blood samples (0.5 ml–1 ml). Microplate spectrophotometers with excitation wavelengths of 485 nm and emission wavelengths of 535 nm were used to measure plasma FITC concentrations. Furthermore, FITC was diluted serially and used as a standard curve. Control group plasma was used as a negative control.

2.8.2. D-lactate detection

With a BioVision D-lactate colorimetric assay kit (USA), serum D-lactate levels were measured. Ninety-six -well plates were filled with serum samples, detection buffer, and reaction mixture provided by the reagent kit. D-lactic acid was used as a standard curve. With the standard curve, the D-lactic acid content in the test sample was calculated after 30 min of incubation at room temperature.

2.9. Western blotting

After washing tissues in 0.9 % saline, we lysed them with ice-cold radioimmunoprecipitation assay (RIPA) buffer (Beyotime China) and a protease inhibitor "cocktail". BCA Protein Assay Kit (Aidlab Biotechnologies, China) was used to measure protein concentrations. After sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), equal amounts of each sample were transferred onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad, USA). For 24 h at 4 °C, anti-occludin (1:200, Santa Cruz, USA), anti-ZO-1 (1:200, Santa Cruz, USA) and anti-GAPDH (1:1,000, Cell Signaling Technology, USA) antibodies were incubated with the samples. A horseradish peroxidase-conjugated secondary antibody (1:1,000, ZsBio, China) was incubated after three washes in Tris-buffered saline Tween-20 (TBST). A ChemiDoc MP gel imaging system (Bio-Rad, USA) was used to analyze the protein bands visualized by enhanced chemiluminescence reagent (Bio-Rad, USA).

2.10. Immunofluorescence assay

From specimens fixed in Richard-Allan Scientific™ Neg-50™ Frozen Section Medium (Thermo Fisher Scientific, USA) that were retrieved during euthanasia, terminal ileum samples were examined histologically. This staining was carried out using an anti-occludin antibody and an Alexa Fluor 488 goat anti-rabbit IgG secondary antibody from Thermo Fisher Scientific. An anti-DAPI stain was used (Beyotime, China) to stain the nuclei. A white light laser was used to capture images with a Leica TCS SP8 confocal laser scanning microscope (Leica Microsystems). Image scanning analysis system was used to analyze the changes in fluorescence intensity of occludin in the intestine.

2.11. Quantitative real-time PCR

Ileal tissue was extracted with TRIzol reagent to obtain total RNA. A plate reader (Biotek Epoch, USA) was used to measure the RNA concentration. Real-time quantitative RT-PCR was performed using Primer 3.0 and a Bio-Rad CFX Connect™ Real-Time PCR Detection System (Bio-Rad, USA) and a KAPA SYBR® FAST qPCR kit (Kapa Biosystems, USA). As per the program, 95 °C was held for 3 min,

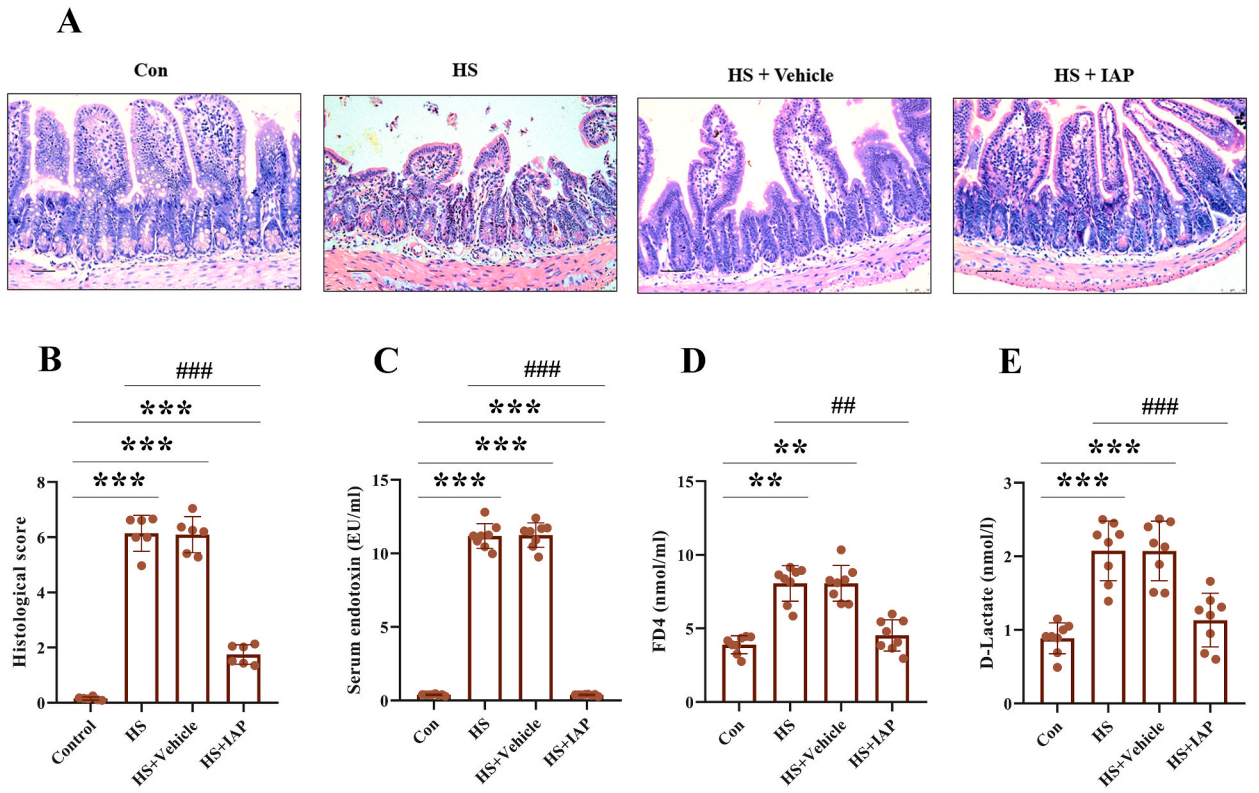

Fig. 3. High intestinal permeability and intestinal injury induced by heatstroke were alleviated by IAP. (A) Representative pathological images of the ileum in the Con group, HS group, HS + Vehicle group, and HS + IAP group stained with H&E at 200 × magnification. Histological scores of intestines (B) were counted and plotted. Blood levels of endotoxin (C), FD4 (D), and D-lactate (E) were measured. IAP treatment decreased the high levels of endotoxin, FD4 and D-lactate induced by heatstroke. Values are expressed as the means ± SDs, n = 8 per group, vs. Con **, $p < 0.01$; ***, $p < 0.001$; vs. HS ##, $p < 0.01$; ###, $p < 0.001$. Scale bar = 50 μm. Con, control; HS, heatstroke; IAP, intestinal alkaline phosphatase; FD4, a 4 kD fluorescein isothiocyanate (FITC)-dextran; H&E, hematoxylin and eosin.

followed by 40 cycles of 15 s at 95 °C, 30 s at 60 °C, and 10 s at 70 °C. We analyzed the relative expression levels of the target genes with the $2^{-\triangle\triangle T}$ method.

2.12. Statistical analysis

The results were graphically visualized using GraphPad Prism software (GraphPad Software v8) after performing all statistical analyses with SPSS (IBM, version 23.0). The significance of differences between more than two test groups was determined by one-way analysis of variance with Tukey's multiple-comparison posttests. Statistical significance was defined as a two-tailed p value of 0.05 or less.

3. Results

3.1. IAP can improve mortality from heatstroke and significantly improve fecal IAP activity

IAP improved the survival rates of mice with heatstroke. Mice with heatstroke were treated with 300 IU of IAP after 0 h, 24 h, and 48 h, and their survival rates were determined. The survival rates of mice in the HS group and the HS + IAP group were 40 % and 90 %, respectively, within 120 h. The survival rate in the HS + IAP group was significantly higher than that in the HS group [$p = 0.0262$, log-rank (Mantel–Cox) test, Fig. 2A]. The survival rate in the HS + IAP group was also significantly increased compared to that in the HS + Vehicle group [$p = 0.0289$, log-rank (Mantel–Cox) test, Fig. 2A].

From the proximal small intestine to the distal colon, IAP moves within the intestine. With the feces, it passes out. In this way, fecal IAP levels can be directly related to IAP levels. There was a significant difference in fecal IAP levels among the groups after 72 h ($p < 0.01$, one-way ANOVA, Fig. 2B). There was a significant decrease in the HS group compared to the Con group ($p < 0.01$, Dunn's test, Fig. 2B). Compared to those in the HS group, the mice that received IAP supplementation had higher fecal IAP levels ($p = 0.0004$, Dunn's test, Fig. 2B).

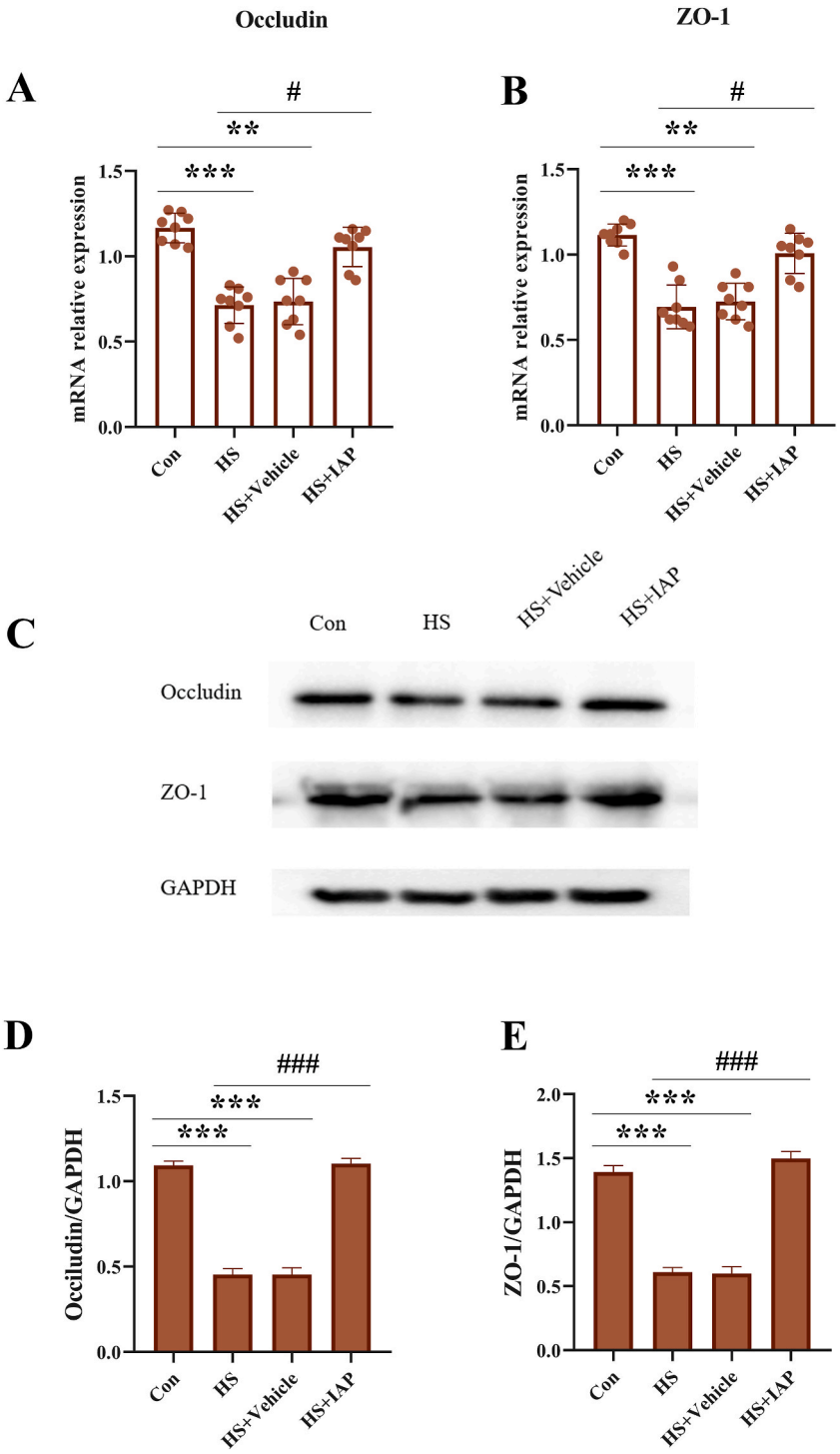

Fig. 4. IAP enhanced tight junction protein expression. (A) The mRNA expression of occludin and ZO-1 was measured by real-time PCR. Relative abundance was normalized to that of GAPDH. (B) Representative Western blot images showing occludin and ZO-1 in the ileum. Increased expression of occludin (C) and ZO-1 (D) was observed in HS + IAP mice. Relative abundance was normalized to that of GAPDH. Values are expressed as the means ± SEMs, n = 3 per group, vs. Con **, $p < 0.01$; ***, $p < 0.001$; vs. HS #, $p < 0.05$; ###, $p < 0.001$. Con, control; HS, heatstroke; IAP, intestinal alkaline phosphatase; ZO-1, Zonula occludins-1.

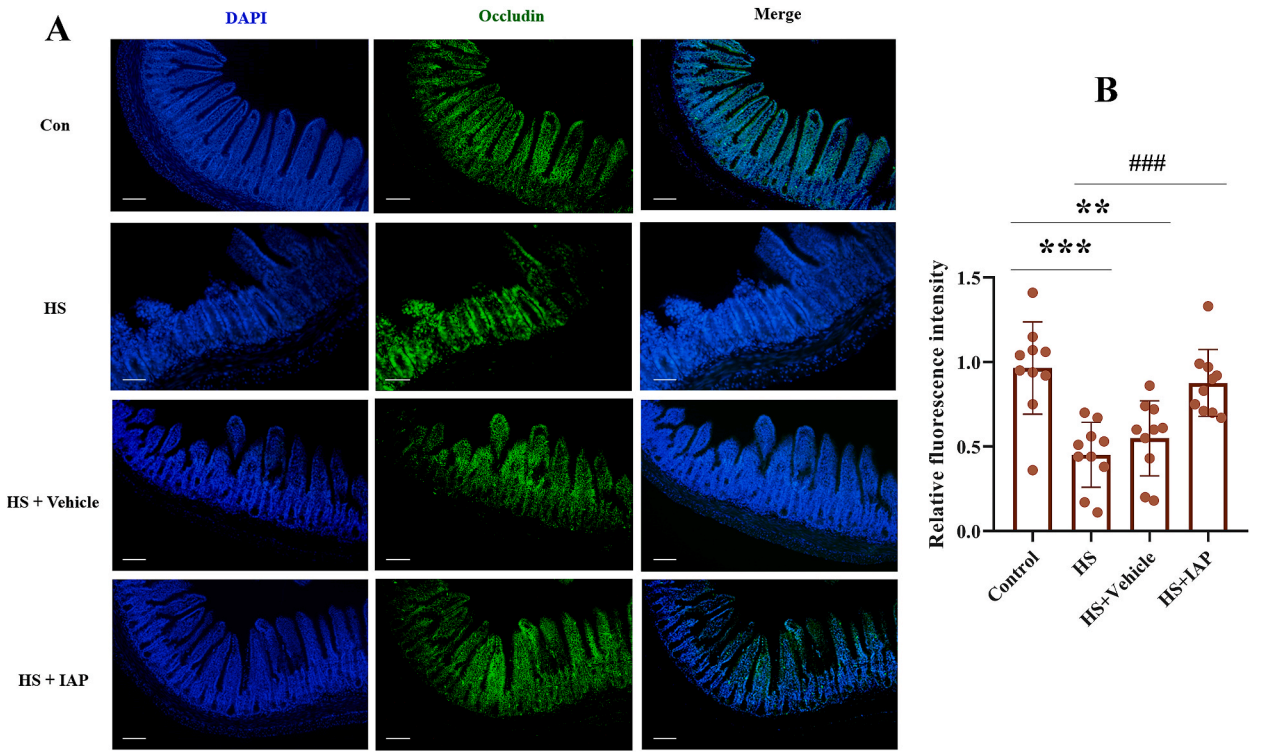

Fig. 5. IAP enhanced occludin protein expression. (A) Representative laser confocal images of ileum samples in the Con group, HS group, HS + Vehicle group, and HS + IAP group, which were immunostained with occludin antibodies. IAP treatment enhanced occludin protein expression after heatstroke. Scale bar = 50 μm. (B) Quantitative analysis of the fluorescence intensity of occludin was performed in ten randomly chosen fields from each section. Values are expressed as the means ± SDs, n = 10 per group, vs. Con **, $p < 0.01$; ***, $p < 0.001$; vs. HS ###, $p < 0.001$. Con, control; HS, heatstroke; IAP, intestinal alkaline phosphatase.

3.2. IAP improves intestinal barrier dysfunction caused by heatstroke

Intestinal barrier dysfunction is a common symptom of heatstroke. Histopathological sections of the intestinal tract of mice with heatstroke showed significant expansion of capillaries, hyperemia of red blood cells, and surface loss of epithelial cells (Fig. 3A). The intestinal tissue of the IAP-treated mice showed only slight villous capillary dilation (Fig. 3A). Normal intestinal villi were present in the control group (Fig. 3A). There was a significant difference in the histological score among the groups ($p < 0.01$, Welch one-way ANOVA test, Fig. 3B). The histological score in the HS group was significantly higher than in the Con group ($p < 0.01$, Tukey HSD test, Fig. 3B). There was a significant decrease in the HS + IAP group compared to the HS group ($p < 0.01$, Tukey HSD test, Fig. 3B). IAP reduced serum endotoxin levels in mice with heatstroke. In each group, limulus lysate dynamic turbidimetry was used to measure endotoxin levels. There was a significant difference between the groups ($p < 0.01$, Welch one-way ANOVA test, Fig. 3C). The endotoxin level in the HS group was significantly higher than that in the Con group ($p < 0.01$, Tukey HSD test, Fig. 3C). There was a significant decrease in the HS + IAP group compared to the HS group ($p < 0.01$, Tukey HSD test, Fig. 3C). To further evaluate intestinal permeability, we administered FD4 solution to the mice by gavage. When the intestinal barrier is destroyed, FD4 can leak into the blood from the intestine. The plasma concentration of FD4 significantly differed between the groups ($p < 0.01$, Kruskal–Wallis test, Fig. 3D). The plasma FD4 level in the HS group was significantly higher than that in the Con group ($p = 0.001$, Dunn's test, Fig. 3D). Compared with that in the HS group, the plasma FD4 level in the IAP-supplemented mice was decreased ($p = 0.0069$, Dunn's test, Fig. 3D). D-lactic acid concentrations in mammalian serum are usually very low. A dysfunctional intestinal barrier contributes to the accumulation of D-lactic acid in serum. We measured the changes in the levels of D-lactic acid in the serum of mice in each group, and the changes were similar to those of the FD4 levels ($p < 0.01$, one-way ANOVA, Fig. 3E). IAP treatment resulted in a significant decrease in serum D-lactic acid compared to that in the HS group ($p < 0.01$, Tukey HSD test, Fig. 3E). In summary, heatstroke increases intestinal permeability, but IAP treatment can improve the intestinal barrier.

Intestinal barrier integrity is maintained by tight junction proteins. The expression of tight junction proteins was examined to determine whether IAP protects the intestinal barrier by increasing the mRNA and protein expression of tight junction proteins. The PCR results showed that there was a significant difference in the expression of occludin among the groups ($p < 0.01$, Kruskal–Wallis test, Fig. 4A). The expression in the HS group was significantly lower than that in the control group ($p = 0.0005$, Dunn's test, Fig. 4A). Compared with that in the HS group, the expression of occludin in the intestinal tract of the IAP-treated mice was significantly increased ($p = 0.0155$, Dunn's test, Fig. 4A). There was a significant difference in the expression of ZO-1 among the groups ($p < 0.01$,

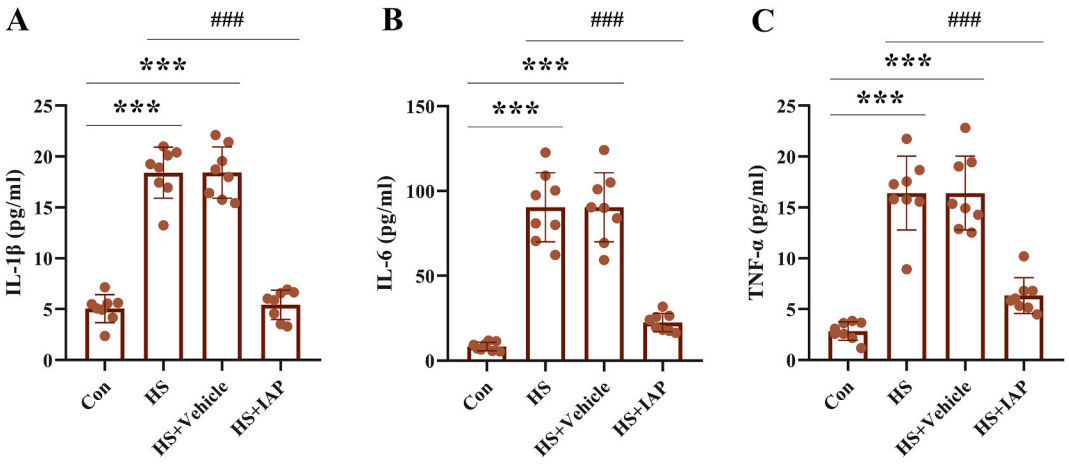

Fig. 6. High levels of systemic inflammatory cytokines induced by heatstroke were alleviated by IAP. Plasma levels of IL-1β (A), IL-6 (B), and TNF-α (C) were measured. IAP treatment decreased the high levels of IL-1β, IL-6 and TNF-α induced by heatstroke. Values are expressed as the means ± SDs, n = 8 per group, vs. Con ***, $p < 0.001$; vs. HS ###, $p < 0.001$. Con, control; HS, heatstroke; IAP, intestinal alkaline phosphatase; IL-1β, interleukin 1 beta; IL-6, interleukin 6; TNF-α, tumor necrosis factor alpha.

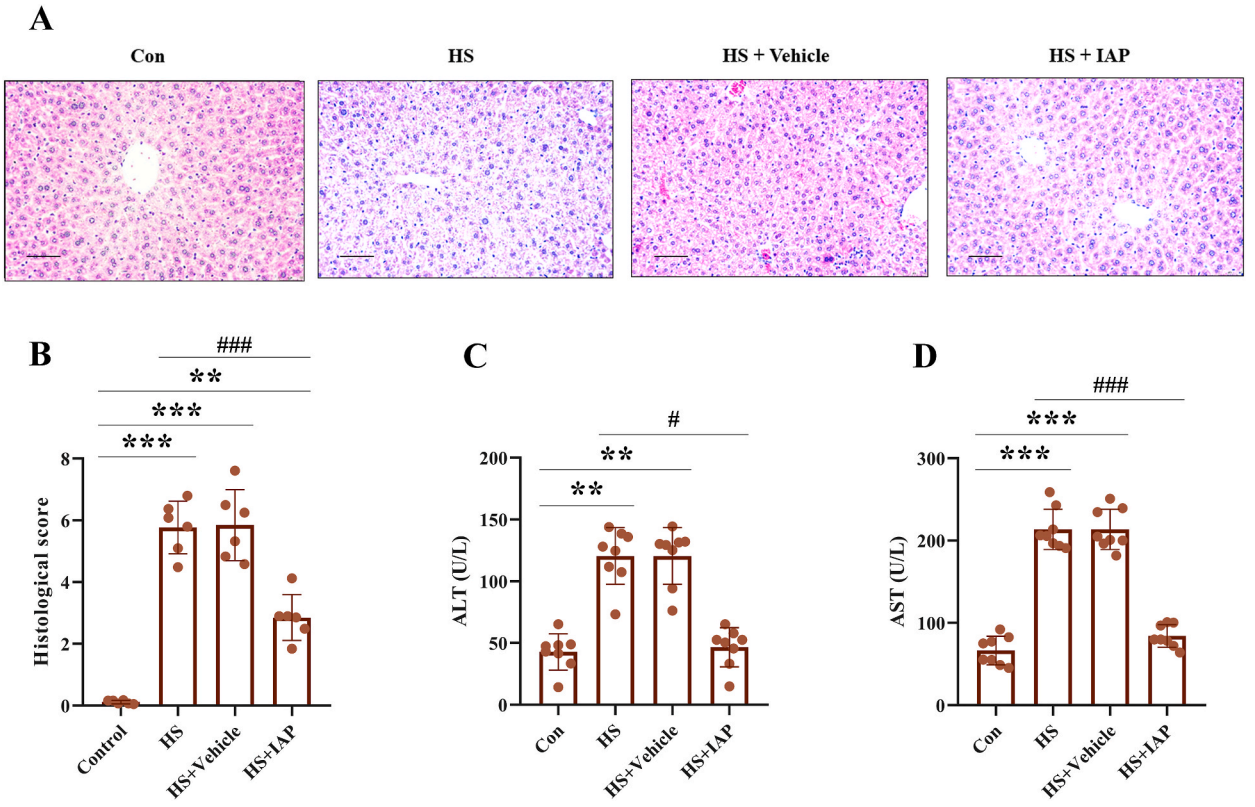

Fig. 7. High serum levels of liver biomarkers and liver injury induced by heatstroke were alleviated by IAP. (A) Representative pathological images of livers in the Con group, HS group, HS + Vehicle group, and HS + IAP group stained with H&E at 200 × magnification. Histological scores of livers (B) were counted and plotted. Serum levels of ALT (C) and AST (D) are shown. Serum biomarkers are expressed as the means ± SDs, n = 8 per group, vs. Con **, $p < 0.01$; ***, $p < 0.001$; vs. HS #, $p < 0.05$; ###, $p < 0.001$. Scale bar = 50 μm. Con, control; HS, heatstroke; IAP, intestinal alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; H&E, hematoxylin and eosin.

Kruskal–Wallis test, Fig. 4B). The expression in the HS group was significantly lower than that in the control group ($p = 0.0004$, Dunn's test, Fig. 4B). Compared with that in the HS group, the expression of ZO-1 in the intestinal tract of the IAP-treated mice was significantly increased ($p = 0.0305$, Dunn's test, Fig. 4B). Consistent with the PCR results, the Western blot results showed that there was a

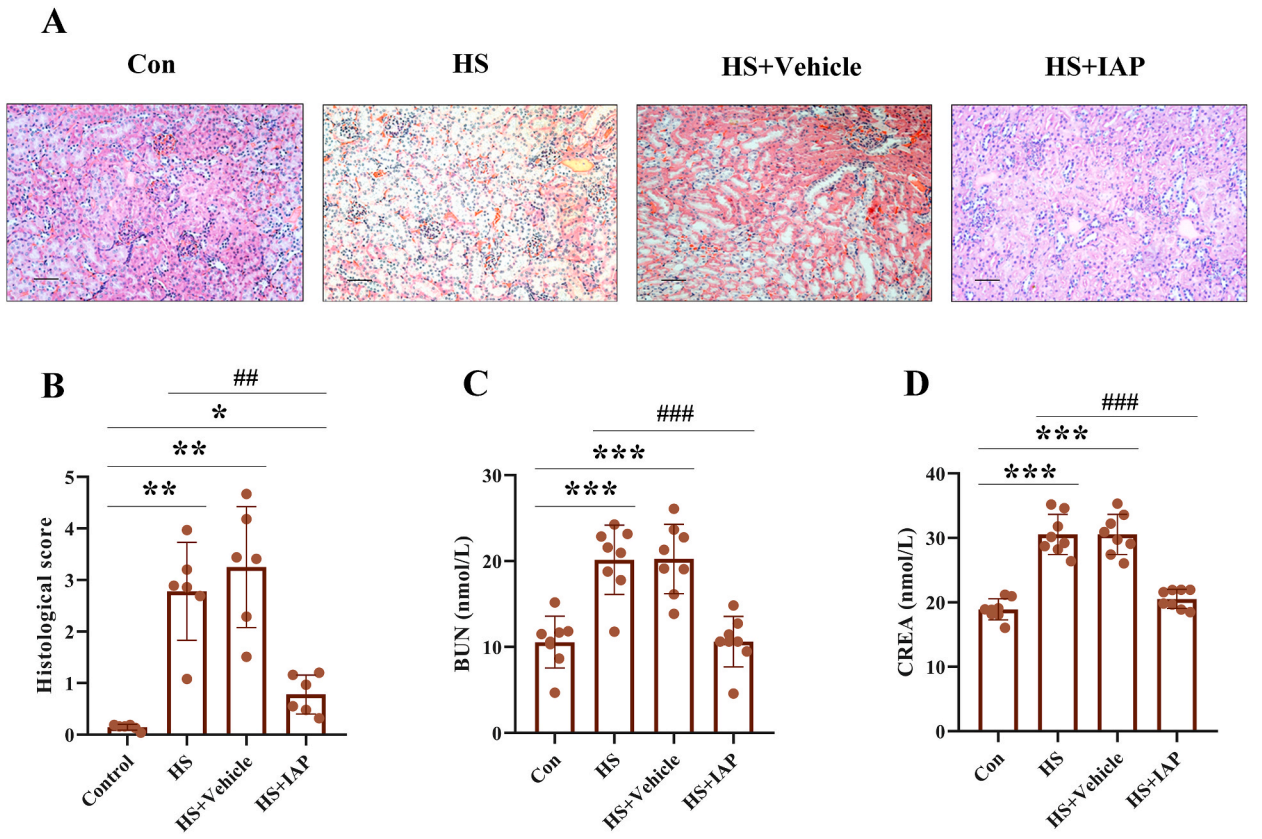

Fig. 8. High serum levels of kidney biomarkers and kidney injury induced by heatstroke were alleviated by IAP. (A) Representative pathological images of kidneys in the Con group, HS group, HS + Vehicle group, and HS + IAP group stained with H&E at 200 × magnification. Histological scores of kidneys (B) were determined and plotted. Serum levels of BU (C) and CREA (D) are shown. Serum biomarkers are expressed as the means ± SDs, n = 8 per group, vs. Con *, $p < 0.05$, **, $p < 0.01$; ***, $p < 0.001$; vs. HS ##, $p < 0.01$; ###, $p < 0.001$. Scale bar = 50 μm. Con, control; HS, heatstroke; IAP, intestinal alkaline phosphatase; BU, blood urea; CREA, creatinine; H&E, hematoxylin and eosin.

significant difference in the protein expression of occludin ($p < 0.01$, one-way ANOVA, Fig. 4B and C) and ZO-1 ($p < 0.01$, one-way ANOVA, Fig. 4B, D) in intestinal tissue between the groups. Among them, the expression of occludin in the HS group was significantly lower than that in the control group ($p < 0.01$, Tukey HSD test, Fig. 4B and C). The expression of occludin in the intestinal tract of the IAP-treated mice was significantly higher than that in the HS group. The expression of ZO-1 in the HS group was significantly lower than that in the control group ($p < 0.01$, Tukey HSD test, Fig. 4B, D). Compared with that in the HS group, the expression of ZO-1 in the intestinal tract of the IAP-treated mice was significantly increased ($p < 0.01$, Tukey HSD test, Fig. 4B, D).

Our immunofluorescence analysis further clarified the role of IAP in maintaining the integrity of the intestinal barrier through tight junction proteins (Fig. 5A). There was a significant difference in occludin intensity among the four groups ($p < 0.01$, Welch one-way ANOVA test, Fig. 5B). The intestinal expression of occludin in the HS group mice was significantly lower than that in the control group ($p < 0.01$, Tukey HSD test, Fig. 5B). After treatment with IAP, the expression of occludin partially recovered ($p < 0.01$, Tukey HSD test, Fig. 5B). Heatstroke increases intestinal permeability, which may be alleviated by IAP promoting the expression of tight junction proteins.

3.3. IAP improves SIRS caused by heatstroke

SIRS is triggered by both intestinal barrier dysfunction and endotoxin release from the damaged intestinal barrier that is the result of heatstroke. Heatstroke-induced inflammatory cytokines were reduced in the mice treated with IAP. Inflammatory factors were measured by ELISAs in each group. There was a significant difference in serum IL-1β concentrations among the groups ($p < 0.01$, one-way ANOVA, Fig. 6A). Compared with that in the control group, the serum IL-1β concentration in the HS group was significantly increased ($p < 0.01$, Tukey HSD test, Fig. 6A). IAP treatment significantly reduced the serum IL-1β concentration compared with that in the HS group ($p < 0.01$, Tukey HSD test, Fig. 6A). There was a significant difference in serum IL-6 concentrations among the groups ($p < 0.01$, Welch one-way ANOVA test, Fig. 6B). The serum IL-6 concentration in the HS group was significantly higher than that in the control group ($p < 0.01$, Tukey HSD test, Fig. 6B). IAP treatment significantly reduced the serum IL-6 concentration compared with that in the HS group ($p < 0.01$, Tukey HSD test, Fig. 6B). There was a significant difference in serum TNF-α concentrations among the

groups ($p < 0.01$, Welch one-way ANOVA test, Fig. 6C). The serum TNF-α concentration in the HS group was significantly higher than that in the control group ($p < 0.01$, Tukey HSD test, Fig. 6C). IAP treatment significantly reduced the serum TNF-α concentration compared with that in the HS group ($p < 0.01$, Tukey HSD test, Fig. 6C).

3.4. IAP ameliorates multiple organ failure caused by heatstroke

IAP (300 IU) was administered by gavage at 0 h, 24 h, and 48 h after heatstroke, and blood and tissue were collected for analysis. In the HS group, there was significant panlobular sinus congestion in the liver (Fig. 7A). There were no substantial abnormalities in the liver of the mice that were treated with IAP except for platelets adhering to the intradermal sinus (Fig. 7A). The livers in the Con group were normal (Fig. 7A). There was a significant difference in histological score between the groups ($p < 0.01$, Welch one-way ANOVA test, Fig. 7B). The histological score in the HS group was significantly higher than that in the Con group ($p < 0.01$, Tukey HSD test, Fig. 7B). There was a significant decrease in the HS + IAP group compared to the HS group ($p < 0.01$, Tukey HSD test, Fig. 7B). We detected serum levels of biomarkers (ALT, AST) of liver function. There was a significant difference in serum ALT levels among the groups ($p < 0.01$, Kruskal–Wallis test, Fig. 7C). Compared with that in the control group, the serum ALT concentration in the HS group increased significantly ($p = 0.0019$, Dunn's test, Fig. 7C). IAP treatment significantly reduced the serum ALT concentration compared with that in the HS group ($p = 0.0109$, Dunn's test, Fig. 7C). There was a significant difference in serum AST concentrations among the groups ($p < 0.01$, one-way ANOVA, Fig. 7D). The serum AST concentration in the HS group was significantly higher than that in the control group ($p < 0.01$, Tukey HSD test, Fig. 7D). IAP treatment significantly reduced the serum AST concentration compared with that in the HS group ($p < 0.01$, Tukey HSD test, Fig. 7D).

The typical renal pathological damage caused by heatstroke mainly occurs in the cortex and is characterized by hyaline degeneration (Fig. 8A). There was a significant difference in histological score between the groups ($p < 0.01$, Welch one-way ANOVA test, Fig. 8B). The histological score in the HS group was significantly higher than that in the Con group ($p < 0.01$, Tukey HSD test, Fig. 8B). There was a significant decrease in the HS + IAP group compared to the HS group ($p < 0.01$, Tukey HSD test, Fig. 8B). We measured serum levels of biomarkers (BU, CREA) of renal function. There was a statistically significant difference in serum BU concentrations among the groups ($p < 0.01$, one-way ANOVA, Fig. 8C). The serum BU concentration in the HS group was significantly higher than that in the control group ($p < 0.01$, Tukey HSD test, Fig. 8C). IAP treatment significantly reduced the serum BU concentration compared with that in the HS group ($p < 0.01$, Tukey HSD test, Fig. 8C). There was a statistically significant difference in serum CREA levels between the groups ($p < 0.01$, one-way ANOVA, Fig. 8D). Compared with that in the control group, serum CREA in the HS group was significantly increased ($p < 0.01$, Tukey HSD test, Fig. 8D). IAP treatment significantly reduced the serum CREA concentration compared with that in the HS group ($p < 0.01$, Tukey HSD test, Fig. 8D). In summary, IAP protected against multiple organ damage caused by heatstroke.

4. Discussion

Due to various factors, such as extremely high temperatures, urban heat island effects, and global aging, heat-induced diseases have become a global challenge affecting public health [18]. As an acute severe illness, heat stroke poses a serious threat to human health. However, thus far, there are no specific therapeutic drugs other than cooling down [19]. According to this study, heat stroke caused a decrease in intestinal IAP activity and pathologic injuries in intestine. The levels of endotoxin, FD4, and D-lactate in the blood of heatstroke mice significantly increased. Heatstroke mice had significantly reduced mRNA and protein levels of ZO-1 and occludin, indicating damage to the intestinal barrier. Additionally, we detected increased serum biomarkers levels of liver and kidney, and visible pathologic injuries in liver, kidney in HS mice. However, IAP significantly reduced the levels of endotoxin, FD4, and D-lactate in the blood of heatstroke mice, reduced intestinal permeability and maintained the integrity of the intestinal barrier by increasing the expression of tight junction proteins. At the same time, IAP alleviated liver and kidney damage caused by heatstroke and reduced serum inflammatory cytokine levels. We demonstrated that IAP can obviously decrease systemic inflammatory response and protect against multiple organ damage via increasing tight junction proteins expression and maintaining the intestinal barrier integrity during heatstroke.

According to a growing body of evidence, heatstroke causes the intestinal barrier to malfunction, allowing endotoxins to leak. This change leads to a systemic inflammatory response [15]. In the study, serum endotoxin concentrations and the inflammatory factors TNF-α, IL-1β, and IL-6 were significantly elevated in the serum of mice with heatstroke. Clinical and animal evidence suggests that the intestine is sensitive to heat and can be damaged in the early stages of heat stroke [20,21]. Moreover, the degree of intestinal damage in heat stroke patients is highly correlated with their prognosis [22]. The leakage of intestinal endotoxins into the bloodstream caused by intestinal injury leads to the efficient release of inflammatory cytokines, thereby inducing systemic inflammatory response syndrome. Heat stroke models indicate that the gut is the main source of endotoxemia and systemic inflammation. Previous studies have shown that gastrointestinal injury induced by heat stroke may depend on signal transduction through the TLR4 pathway. LPS is an effective TLR4 agonist, and IAP can relieve its toxicity by dephosphorylating the lipid A portion of LPS [23]. IAP has been proven to have an anti-inflammatory effect in a mouse atherosclerosis model and shows a protective effect on the intestinal barrier [24]. In our model, supplementation with IAP significantly increased intestinal IAP activity and reduced intestinal pathological damage and barrier permeability, possibly by limiting the downregulation of tight junction proteins expression. Simultaneous, the decrease endotoxin and TNF-α, IL-1 β, IL-6 inflammatory factor levels in serum may be due to reduced stimulation of the TLR4 pathway by LPS.

Importantly, excessive inflammation is considered an important cause of multiple organ dysfunction induced by heat stroke [25]. As a result of heat exposure, skin blood flow can increase, leading to a decrease in visceral perfusion. Persistent visceral ischemia, as

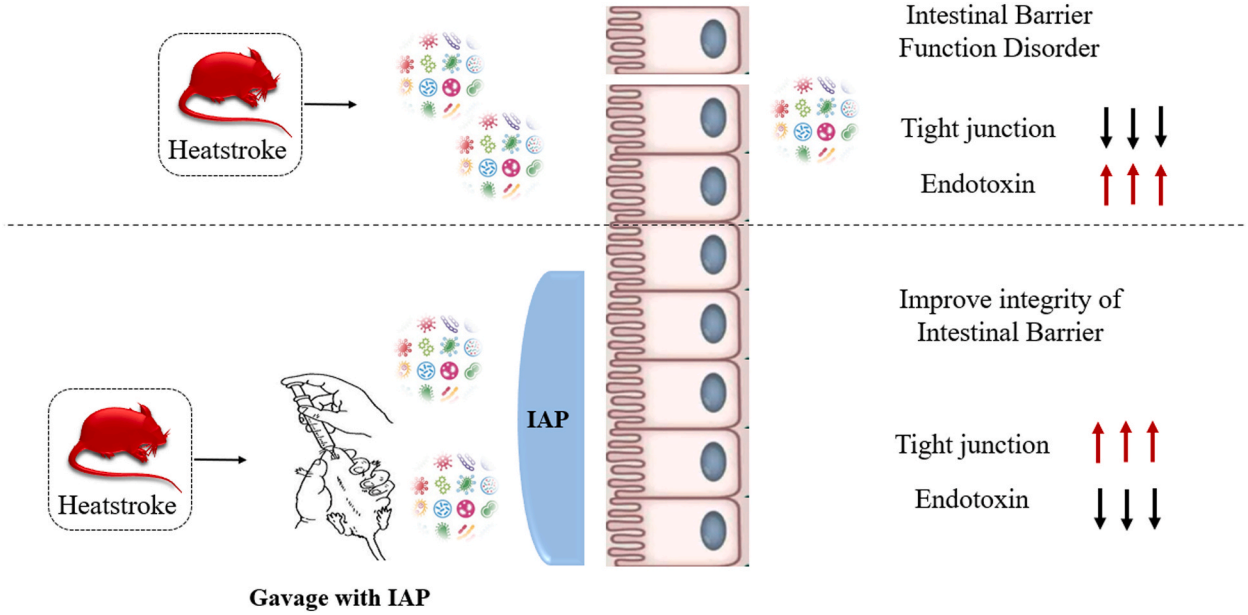

Fig. 9. IAP improves intestinal permeability and alleviates multiple organ dysfunction caused by heatstroke.
Heat stroke has a high mortality rate and causes a decrease in IAP activity. Supplementing intestinal alkaline phosphatase (IAP) can improve the survival rate of heatstroke mice. IAP mainly plays a role in the intestinal cavity. Therefore, it mainly improves the function of multiple organs by reducing the intestinal barrier damage caused by heat stroke disease, strengthening the tight connection of the intestine, improving the intestinal permeability, and reducing endotoxin entry into the systemic circulation and systemic inflammation. IAP may be added to enteral nutrition formulas as a potential means of protecting the intestinal barrier in patients with heat stroke.

well as thermal cell toxicity and inflammatory reactions, further lead to multiple organ dysfunction [26]. In this study, our experiment showed that the concentrations of the serum biomarkers ALT, AST, BUN and CREA in heatstroke mice with liver and kidney damage were significantly increased. Pathological damage to the liver and kidneys was also observed in the tissue sections. In this case, enteral IAP supplementation significantly alleviated these symptoms and improved liver and kidney function in heat stroke. Sepsis-related acute kidney injury and rheumatoid arthritis have been shown to be protected by IAP or recombinant human intestinal alkaline phosphatase [27,28]. In addition, enteral IAP has been shown to be beneficial for a group of patients with severe ulcerative colitis, and no side effects have been observed [29]. This promising discovery provides interesting evidence that may support the strategy of reducing the systemic inflammatory response and multiple organ dysfunction in the treatment of heat stroke patients in the future.

Although a variety of other compounds, including dexmedetomidine [15], eicosapentaenoic acid [30], ulinastatin [31], and Huoxiang Zhengqi Dropping Pills (HZPD) [32], had intestinal protective effects in the heatstroke, IAP is the unique because it is an endogenous enzyme secreted into the intestinal cavity by duodenal intestinal cells and is considered to play a role only in the intestinal cavity, rarely entering the systemic circulation [17]. Since most heat stroke patients are in a coma state, adding IAP to enteral nutrition formulas is feasible for early enteral nutrition in clinical settings.

Although this study provides interesting data, it also has certain limitations. In heat stroke models, IAP improves the intestinal barrier and increases survival, but its exact mechanism is unknown. The determination of this mechanism may lead to the development of additional therapies for intestinal barrier dysfunction following heat stroke. We only studied the short-term effects of IAP on HS injury, and further validation is needed for more doses and long-term effects. The pathogenesis of heat stroke differs between mice and patients, and further research is needed to determine its clinical application.

According to our research, IAP plays a crucial role in maintaining intestinal barrier function in heatstroke mice. Supplementing intestinal IAP can alleviate intestinal barrier damage, improve intestinal barrier function, maintain tight junctions of intestinal epithelial cells, and reduce endotoxin and systemic inflammation, thereby alleviating multiple organ damage, such as the liver and kidney (Fig. 9). Therefore, IAP may be added to enteral nutrition formulas as a potential means for diseases characterized by intestinal permeability disorders, including heatstroke.

Ethics statement

The animal study was reviewed and approved by the Experimental Animal Ethics Committee of Army Medical University.

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Data availability

Data included in article/supp. material/referenced in article.

CRediT authorship contribution statement

Zhen Luo: Data curation, Funding acquisition, Writing – original draft, Writing – review & editing. **Zeze Wang:** Data curation, Methodology. **Ping Li:** Formal analysis, Methodology. **Yulong Tan:** Formal analysis. **Genlin He:** Data curation, Methodology. **Xiaoqian Liu:** Data curation. **Tingting Shen:** Data curation. **Xuesen Yang:** Project administration, Supervision. **Xue Luo:** Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled.

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