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Dietary supplementation of camel whey protein attenuates heat stress-induced liver injury by inhibiting NLRP3 inflammasome activation through the HMGB1/RAGE signalling pathway

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ABSTRACT

Inflammasomes play an important role in promoting heat stress (HS) induced liver injury. Dietary supplementation of camel whey protein (CWP) has been shown to alleviate HS-induced tissue damage. However, whether and how dietary CWP supplementation can reduce HS-induced liver injury remains unclear. Thus, we evaluated the ability of rats supplemented with CWP to resist HS-induced liver injury. To induce liver injury, Sprague-Dawley rats were exposed to HS. The mechanism of action was confirmed using an antagonist of high mobility group box 1 (HMGB1) and the NLR pyrin domain containing 3 (NLRP3) inhibitor before HS. Histological changes in the livers of the HS rats were visualised using haematoxylin and eosin staining. The protein expression of HMG1, receptor for advanced glycation end products (RAGE), NLRP3, Caspase-3, and Bcl-2 were detected using immunohistochemistry or western blotting. The content of IL-1β, the activation of Caspase-1, and the level of alanine transaminase (ALT) were determined using commercial kits. We confirmed that HS activated NLRP3 in the liver, as evidenced by enhanced Caspase-1 activity and increased IL-1β content. Inhibition of NLRP3 activation reversed abnormal expression and nuclear translocation of Caspase-3 and Bcl-2, and alleviated apoptosis, necrosis, and subsequent liver injury. HS-induced hepatocyte NLRP3 activation is dependent on elevated extracellular HMGB1 levels. Glycyrrhizic acid, an antagonist of HMGB1, which is also an anti-inflammatory agent, inhibited HS-induced NLRP3 activation. Interestingly, CWP also reversed HS-induced abnormal expression of HMGB1, RAGE, NLRP3, IL-1β, ALT, Bcl-2, and Caspase-3, inhibited Caspase-1 activity, and alleviated apoptosis and liver histological changes. More importantly, CWP combined with glycyrrhizic acid completely prevented HS-induced hepatocyte apoptosis and injury. These results indicate that CWP ameliorates HS-induced liver injury by inhibiting the HMGB1/RAGE/NLRP3 axis. Dietary supplementation with CWP may be an effective strategy to prevent serious complications from HS.

1. Introduction

Heat stress (HS) is one of the most serious stress factors that endanger human and animal health in hot seasons and regions of the world. Persistent exposure to HS can lead to a variety of psychological and physiological stress reactions, such as irritability, rapid heart rate, and syncope, and in severe cases, may lead to death (Myers & Bernstein, 2011). Liver injury has been identified as a fatal complication in clinical cases and animal models of HS (Geng et al., 2015; Kew, Bersohn, Seftel, & Kent, 1970; Hassanein, Razack, Gavaler, & Van Thiel, 1992; Wang et al., 2018). Even if treatments to lower body temperature are utilized, they can fail to prevent the occurrence of liver damage in HS cases, which in turn leads to death (Kew et al., 1970; Weigand, Riediger, Stremmel, Flechtenmacher, & Encke, 2007). Recent studies have shown that in HS-treated rat models, liver injury is not observed during HS, but gradually becomes severe during recovery from HS (Geng et al., 2015; Leon, 2007). Although the mechanism of HS-induced liver injury is still poorly understood, previous studies have speculated that it might be

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mediated by inflammation rather than the direct result of hyperthermia (Geng et al., 2015; Leon, 2007).

Evidence suggests that liver inflammation and subsequent injury induced by adverse stressors, including HS, depend on the activation of the NLR pyrin domain-containing 3 (NLRP3) inflammasome (Geng et al., 2015; Szabo & Csak, 2012; Yan et al., 2012). Activated NLRP3 inflammasome promotes the maturation and secretion of pro-inflammatory cytokines such as interleukin-1β (IL-1β) by activating Caspase-1 (Geng et al., 2015), while synergistically activating Caspase-3 to mediate apoptosis (Chen & Chen, 2018; Lebeaupin et al., 2015). A recent study showed that in mice overexpressing NLRP3, persistently activated NLRP3 inflammasome causes severe liver inflammation and injury (Wree et al., 2014). Recent evidence suggests that high mobility group protein B1 (HMGB1), a damage-associated molecular pattern (Seong & Matzinger, 2004), plays an important role in mediating sterile inflammation in HS patients (Tong et al., 2011) and rats (Hagiwara, Iwasaka, Goto, et al., 2010; Hagiwara, Iwasaka, Shingu, et al., 2010; Tong et al., 2013), and can be released into the bloodstream in the early stages of HS (Wang et al., 1999). Geng et al. further confirmed that in HS rats, HMGB1 activates the NLRP3 inflammasome through the receptor for advanced glycation end products (RAGE), which in turn leads to liver inflammation and injury, while intraperitoneal injection of HMGB1 antibody or RAGE inhibitor reverses these changes (Geng et al., 2015). These studies suggest that targeting the HMGB1/RAGE/NLRP3 signalling pathway may be an effective way to prevent HS-induced liver injury.

To the best of our knowledge, no safe and effective therapies have been developed yet for HS-induced liver injury. Natural anti-inflammatory agents have been used as adjuvant strategies for the treatment of a variety of diseases because of their safety, effectiveness, and inexpensiveness (Gamal Badr, Ramadan, Sayed, Badr, & Selamoglu, 2017). Camel milk (CM) is the main source of milk in desert and semi-desert areas. Due to its anti-oxidation, anti-inflammation, immune regulation, and anti-apoptosis characteristics, it has been considered a natural Chinese patent medicine, which can be used to prevent and treat a variety of diseases; it is also known as “desert platinum” (Arab et al., 2014; G. Badr, Ramadan, Abdel-Tawab, Ahmed, & Mahmoud, 2018; Zhu et al., 2016). All the above studies show that CM has high nutritional and medicinal value, and one of its functions is to help young camels survive in harsh environments. Camel whey protein (CWP) derived from CM is similar in composition to bovine whey protein and consists of α-lactalbumin, serum albumin, lactoferrin, immunoglobulin, peptidoglycan recognition protein, etc. However it lacks β-lactoglobulin (the major cause of milk allergies in children), and has stronger antioxidant and anti-inflammatory activities than bovine and other whey proteins (Gamal Badr et al., 2017). Similarly, we also confirmed in our previous in vitro study that CWP suppresses the inflammatory response by reducing the phosphorylation of NF-κB p65 and the expression of NLRP3 and caspase-1, and alleviates HS-induced hepatocellular injury (Du et al., 2021). These results suggest that CWP has an inhibitory effect on the assembly of the NLRP3 inflammasome, a crucial inflammatory signalling factor. As mentioned earlier, the NLRP3 inflammasome-mediated inflammatory response is essential for promoting HS-induced liver injury (Geng et al., 2015). Thus, due to the strong anti-inflammatory effect of CWP, it has potential medical value against HS-induced liver injury. However, the potential protective effects and molecular mechanisms of CWP in HS diseases in humans and other animals are still unclear, and to the best of our knowledge, its regulatory effects on the HMGB1/RAGE/NLRP3 signalling pathway have not been reported. Therefore, this study focused on elucidating the mechanism of CWP in preventing HS-induced liver injury.

2. Materials and methods

2.1. Preparation of camel whey protein

Fresh Bactrian camel milk was collected, and CWP was prepared as described previously (Du et al., 2021). Briefly, fresh Bactrian camel milk (CM) was aseptically collected from a camel farm in Alashan, Inner Mongolia, China. The samples were immediately refrigerated at 4 °C and transported to the laboratory. Raw milk was spun at 1400*g* at 4 °C for 30 min for degreasing. Defatted CM was heated at 80 °C for 20 min and cooled to 43–45 °C immediately for pasteurisation. After adjusting the pH of the skim CM to 4.3 with 1 M HCl, it was centrifuged at 11 000*g* for 10 min at 4 °C. The casein precipitated after centrifugation was discarded and the supernatant was the CWP sample. Subsequently, an ammonium sulphate assay was used to precipitate CWP. That is, an equal volume of saturated ammonium sulphate solution was added to the CWP sample and mixed using a magnetic stirrer for 6 h. Finally, a dialysis bag with a molecular weight cut-off of 6 000–8 000 kDa was employed for 48 h (the ratio of CWP to dH_2O was 1:20). The dialysate was freeze-dried and stored at −80 °C until further use.

2.2. Construction of HS model in rats

Sprague-Dawley rats (weight, 200 ± 20 g; age, 6 weeks; sex, male) were obtained from the Laboratory Animal Center of Inner Mongolia Medical University (Hohhot, China) and kept in the Animal Center of Inner Mongolia Agricultural University. The laboratory animal production license number was SCXK (Meng) 2015-0001, and the laboratory animal use license number was SYXK (Meng) 2015-0001. All experimental procedures were approved by the Scientific Research and Ethics Committee of Inner Mongolia Agricultural University. The rats had free access to a standard pellet diet and clean water. They were acclimatised for 2 weeks at a constant temperature (21 ± 1 °C), constant humidity (60–65%), and 12 h light/dark cycle (lights on at 8:00 AM and off at 8:00 PM) environment before HS treatment. To establish the HS model, rats were placed in an artificial climate chamber with a temperature of 40 ± 0.2 °C and relative humidity of 60–65% from 10:00 AM to 12:00 PM each day. After each HS treatment, the rats were immediately removed from the artificial climate chamber and returned to the initial feeding environment for 22 h. After the 2nd, 4th, 6th, and 8th HS, the sensors of a digital thermometer (TH212; Hong Ou Cheng Yun Instrument Co., Ltd, Beijing, China) were inserted into the rectum of rats at 6.5 cm to record the core temperature (Tc). After HS treatment, systolic blood pressure (SBP) was measured using a tail artery blood pressure measurement system (ZH-HX-Z; Huaibei Zhenghua Biologic Apparatus Facilities Co., Ltd, Anhui, China). Liver injury was evaluated based on histopathological morphology and serum alanine aminotransferase (ALT) content.

2.3. Groups and treatment

Rats were randomly divided into 12 groups, each group contained six rats (n = 6). Rats in the HS group were administered saline by oral gavage for 1 h and then placed in an artificial climate chamber with a temperature of 40 ± 0.2 °C and relative humidity of 60–65% for 2 h per day for 8 days. Rats in the low-dose group were administered CWP (dissolved in 1 ml saline) by oral gavage at a dose of 100 mg/kg body weight 1 h before each HS. Rats in the middle-dose group were administered CWP by oral gavage at a dose of 200 mg/kg body weight. Rats in the high-dose group were administered CWP by oral gavage at a dose of 400 mg/kg body weight. To block the effect of HMGB1, rats were intraperitoneally injected with glycyrrhizic acid (3 mg/kg body weight; MCE, NJ, USA; Cat. No.: HY-N0184), an antagonist of HMGB1, before each HS treatment. To inhibit the assembly and activation of the NLRP3 inflammasome, some rats were intraperitoneally injected with CY-09 (3 mg/kg body weight; MCE, NJ, USA; Cat. No.: HY-103666), an inhibitor

of NLRP3, before HS. To evaluate the effect of CWP combined with anti-inflammatory drugs, some rats were administered both CWP (400 mg/kg body weight) and glycyrrhizic acid (3 mg/kg body weight) before HS treatment. The rats in the control groups were orally administered 1 ml of saline or CWP (400 mg/kg body weight) or an intraperitoneal injection of glycyrrhizic acid (3 mg/kg body weight), CY-09 (3 mg/kg body weight), or a combination of glycyrrhizic acid and CWP, but without HS treatment, and underwent the entire test under the above standard laboratory conditions.

2.4. Haematoxylin and eosin (HE) staining

HE staining was performed on rat liver tissues after 8 days of HS treatment and 22 h of recovery under standard environmental conditions. According to the standard HE staining procedure, fresh liver tissue was fixed in 4% paraformaldehyde overnight and then transferred to 70% ethanol until it was embedded in paraffin. Paraffin-embedded specimens were cut into 4 μm sections and stained with haematoxylin and eosin for general cell morphology analysis. The degree of inflammation and necrosis of the rat liver was independently evaluated according to the Ishak scoring system (Ishak et al., 1995) by three investigators blinded to treatment assignments.

2.5. Immunohistochemistry

Paraffin sections were used for immunohistochemical (IHC-P) staining after standard dewaxing and rehydration procedures. Briefly, the sections were placed in EDTA Antigen Repair Buffer (pH8.0; Solarbio, Beijing, China; Cat. No.: C1034) and boiled at 95 °C for 20 min to repair the antigen, placed in phosphate-buffered saline (PBS) (pH 7.4), and washed three times on a shaker for 5 min each time. The sections were then incubated in PBS containing 3% hydrogen peroxide for 10 min to inhibit endogenous peroxidase activity. Sections were blocked for 1 h at room temperature in PBS containing 5% normal goat serum. Next, the blocking solution was gently shaken off and rabbit anti-rat HMGB1 (1:200; Abcam, Camb, UK; Cat. No.: ab18256), Caspase-3 (cleaved Asp175; 1:50; GeneTex, Inc., USA; Cat. No.: GTX86952), and Bcl-2 (1:200; GeneTex, Inc., USA; Cat. No.: GTX100064) antibodies diluted with PBS were dripped onto the sections, which were flat in a wet box and incubated overnight at 4 °C. Next, the sections were washed three times with PBS and incubated with goat anti-rabbit IgG (H + L)-HRP (1:1 000; Tianjin Sungene Biotech, Tianjin, China; Cat. No.: LK2001) for 50 min at room temperature. Finally, the sections were stained using a DAB Substrate Kit (Solarbio, Beijing, China; Cat. No.: DA1010) for 3–5 min, and the nuclei were counterstained with haematoxylin for 5 min. After being dehydrated, transparent, and mounted with Canada balsam (neutral mounting medium), the sections were visualised under a microscope.

Immunostaining was scored by considering account both the staining intensity and the percentage of positive cells. Staining intensity was scored as follows: 0, negative; 1, weakly positive; 2, moderately positive; and 3, strongly positive. Image J software was used to quantify the percentage of positive cells: 0 (<5%), 1 (5–25%), 2 (25–50%), 3 (50–75%), and 4 (>75%). The value of the positive cell percentage score multiplied by the value of the staining intensity score was the final immunohistochemical score, which ranged from 0 to 12. At least six randomly selected high-power fields (HPFS) were analysed per immunostained section.

2.6. TUNEL staining

Hepatocyte apoptosis was assessed using a One Step TUNEL Apoptosis Assay Kit (Beyotime Biotechnology, Shanghai, China; Cat. No.: C1088) according to the manufacturer's instructions. After the nuclei were counterstained with DAPI, they were visualised by using a fluorescence microscope (Zeiss, AX10, Jena, Germany).

2.7. Determination of Caspase-1, IL-1β, and biomarkers for liver function

The activity of Caspase-1 was determined using a Caspase-1 Activity Assay Kit (Beyotime Biotechnology, Shanghai, China; Cat. No.: C1102). IL-1β in cells and serum was detected using a Rat IL-1β ELISA Kit (HCUSABIO, Wuhan, China; Cat. No.: CSB- E08055r). The serum alanine aminotransferase (ALT) activity was measured using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.8. Western blotting analysis

The assay was performed as our previously reported (Du et al., 2021). Briefly, equal amounts of total cellular protein (20 μg) were separated by 10% SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane. After the PVDF membranes were blocked with QuickBlock™ Blocking Buffer (Beyotime, Shanghai, China) for 1 h, they were incubated with rabbit anti-NLRP3 (1:1 000; GeneTex, Inc., USA; Cat. No.: GTX00763), rabbit anti-RAGE (1:1 000; Abcam, Camb, UK; Cat. No.: ab216329), and rabbit anti-β-actin antibody (1:10 000; Abcam, Camb, UK; Cat. No.: ab8227) overnight at 4 °C. After the PVDF membranes were washed three times (10 min each) with Tris-buffered saline, 0.1% Tween 20 (TBST), they were incubated in goat anti-rabbit IgG (H + L)-HRP (1:5 000; Tianjin Sungene Biotech, Tianjin, China; Cat. No.: LK2001) for 45 min at room temperature. The membranes were then washed three times with TBST. After visualisation using Super ECL Plus (US Everbright Inc., Suzhou, Jiangsu, China; Cat. No.: S6009), pictures were taken with an Odyssey infrared imaging system (LI-COR Biosciences) and analysed using Image Pro Plus 6.0 software (Media Cybernetics, Maryland, USA).

2.9. Statistical analyses

SPSS 25.0 software was used to analyse the data. Data are presented as the mean ± standard error of the mean (SEM). Differential analysis was performed using the nonparametric Wilcoxon rank sum test for samples that did not conform to a normal distribution. For samples meeting normal distribution, the one-way ANOVA and Tukey post-test were used for comparison when variances were equal; otherwise, the Kruskal-Wallis test was used for analysis.

3. Results

3.1. Tc, SBP, and serum ALT response to HS in rats

The Tc of animals suffering from HS is usually elevated to 43 °C, and SBP is decreased (Geng et al., 2015). Therefore, we monitored the Tc and SBP in the HS rats. As shown in Fig. 1, treatment with a temperature of 40 ± 0.2 °C and 60–65% humidity for 8 days could cause the Tc of rats to increase to 43 °C (Fig. 1A), while the SBP began to decrease (Fig. 1B). In addition, the serum ALT activity of rats increased gradually with prolonged HS time (Fig. 1C), indicating that the integrity of the hepatocyte membrane had been lost.

3.2. HS-induced histological changes in rat liver

As shown in Fig. 1D, the histological structure of the liver from rats that were not subjected to HS was normal, and no pathological changes such as swelling, necrosis, and inflammatory infiltration were observed. After 2 days of HS treatment, parts of the central veins of the liver were observed to be filled with pink proteinaceous mucus (red arrows). Diffuse or focal infiltration of inflammatory cells such as lymphocytes (black arrows) and neutrophils (grey arrows) could found around some central veins and within the hepatic sinusoids. Some hepatocytes showed swelling and ballooning degeneration (orange arrows), visible low cytoplasmic staining, shrinkage of the nucleus, and deeper staining (pyknosis). Some hepatocytes underwent steatosis, and intracytoplasmic

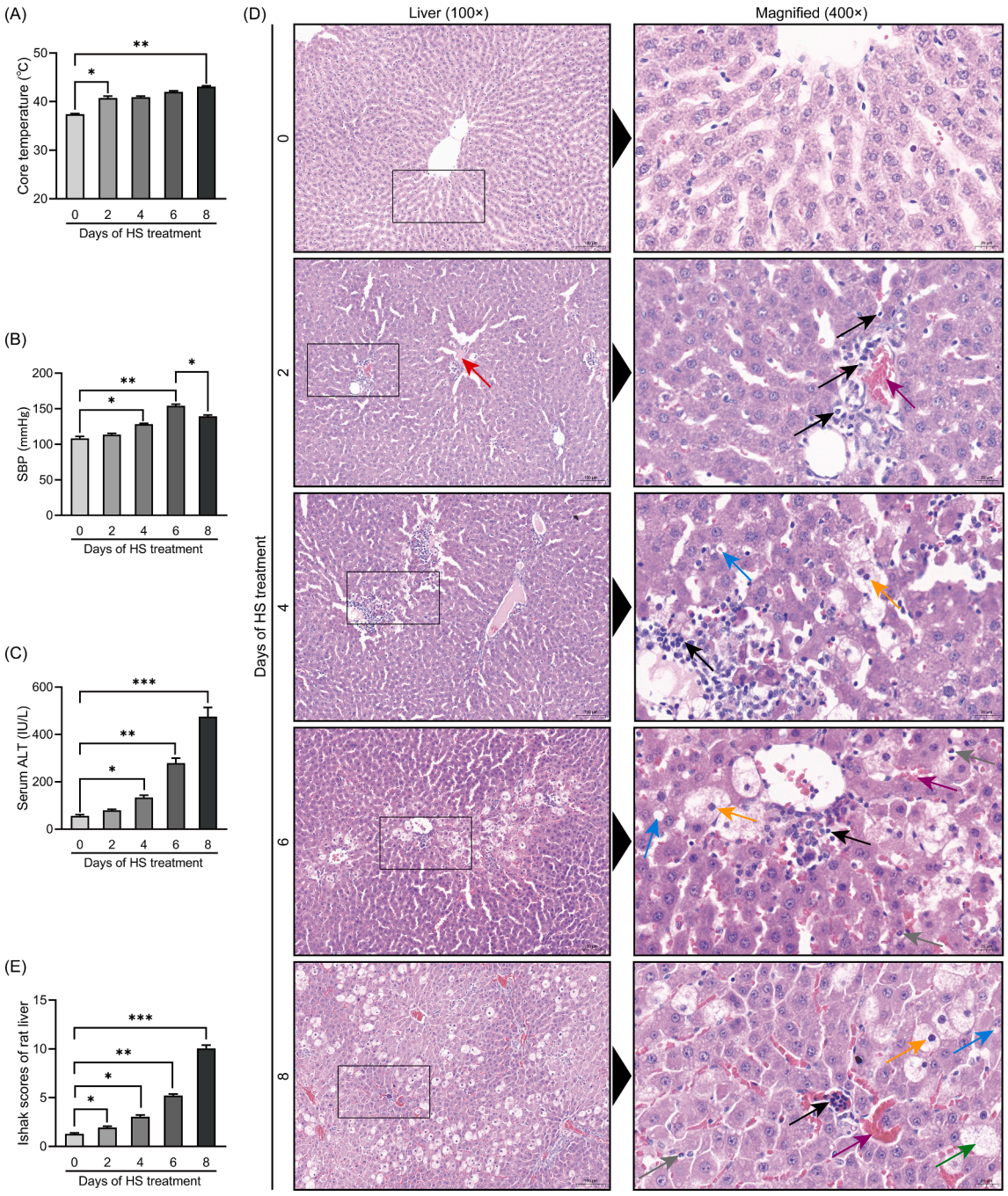

Fig. 1. Effect of heat stress (HS) on core temperature (Tc), systolic blood pressure (SBP), alanine transaminase (ALT) levels, and histological changes in rats. Sprague-Dawley rats were exposed to the HS environment for 2 h per day for eight consecutive days. Tc (A), SBP (B), and serum ALT (C) levels were recorded before (0 days) and 2, 4, 6, and 8 days after HS treatment. The livers of the rats were collected, and histological sections were prepared and subjected to haematoxylin and eosin staining (100× magnification; magnified images were taken at 400× magnification). Representative images are shown (n = 6). The figures represent each time point of HS treatment (D). Histopathological scores were evaluated using the Ishak scoring system (E). Red arrows indicate pink proteinaceous mucus. Black arrows indicate lymphocytes. Grey arrows indicate neutrophils. Orange arrows indicate ballooning degeneration. Blue arrows indicate steatosis. Green arrows indicate karyorrhexis and karyolysis. Purple arrows indicate liver haemorrhage. Data are presented as the mean ± standard error of six measurements. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

scattered fatty vacuoles could be seen (blue arrows). After 8 days of HS treatment, the number of ballooned and necrotic hepatocytes was significantly increased, showing pyknotic nuclei and even fragmented which led to the failure of HE staining (green arrows). In addition, the central vein and hepatic sinusoids filled with numerous red blood cells (purple arrows). The histopathological scores further confirmed that the degree of liver injury in the rats was aggravated with the extension of HS treatment time (Fig. 1E). In short, these results indicate that we successfully established a model of HS-induced liver injury in rats.

3.3. CWP alleviated HS-induced liver injury and inflammation in rats

The results showed that no pathological changes were observed in the liver tissue sections of the control and control + CWP rats, indicating that CWP (400 mg/kg body weight) had no hepatotoxicity (Fig. 2A). However, there were typical histological features of liver injury in the

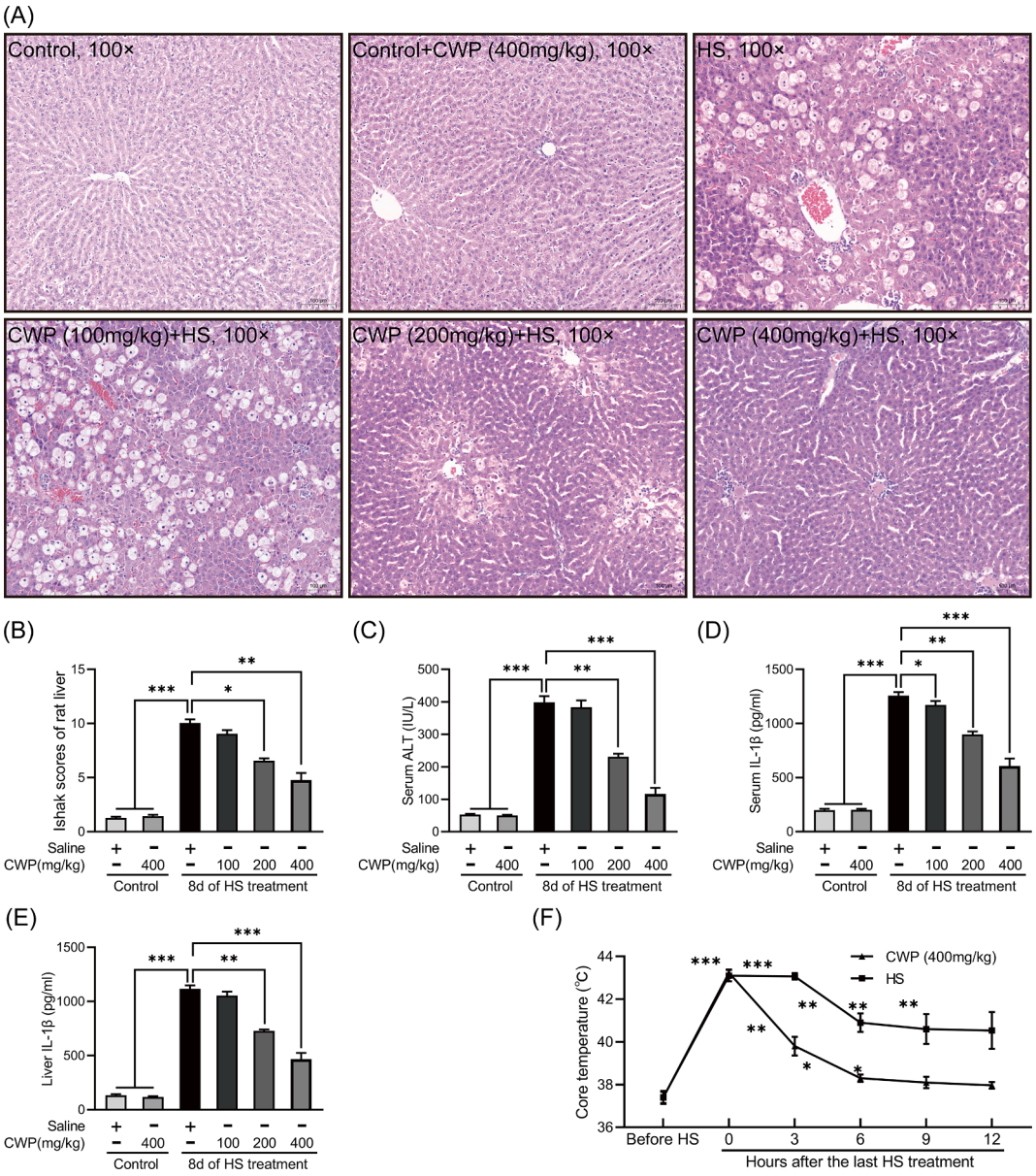

Fig. 2. Camel whey protein (CWP) alleviated heat stress (HS)-induced liver injury and inflammation in rats. (A) Preventive effects of CWP on HS-induced liver injury in rats were evaluated by haematoxylin and eosin staining (100×). The pictures are representative of rat liver histological changes in each group (n = 6). (B) Histopathological scores were evaluated according to the Ishak scoring system. (C) Liver injury was assessed by detecting serum alanine transaminase (ALT) activity. (D & E) Serum and liver IL-1β levels of rats were detected by using ELISA. (F) Core temperature (Tc) was recorded from the rats in the HS and CWP groups before HS treatment and at 0, 3, 6, 9, and 12 h after the last HS treatment (significance is based on comparison to before HS). Data are presented as the mean of six determinations ± standard error of the mean (SEM). *p < 0.05, **p < 0.01 and ***p < 0.001.

rats treated with HS for 8 days compared with those in the control or control + CWP rats. Liver tissue sections from the rats subjected to HS exhibited extensive ballooning degeneration, necrosis, inflammatory infiltration, and haemorrhage (Fig. 2A), which were accompanied by elevated histopathological scores (Fig. 2B). Furthermore, the serum ALT activity (Fig. 2C) and serum and hepatic IL-1β levels (Fig. 2D & E) of the rats were significantly increased after HS treatment, further indicating that HS induced a liver injury process accompanied by inflammatory responses. Interestingly, CWP alleviated HS-induced liver injury in the rats in a dose-dependent manner. Notably, liver tissue sections from the rats in the high-dose group showed that the histological changes were almost reversed, except for a mild inflammatory cell infiltration in the portal area and sinusoids. Correspondingly, CWP reduced serum ALT activity and serum and liver IL-1β levels in a dose-dependent manner. We also investigated the effects of CWP on Tc in rats after HS treatment. The results showed that although CWP failed to prevent Tc elevation in the rats after HS, it effectively promoted its decline and reduced it to normal levels within 12 h after HS (Fig. 2F). These results indicated that dietary supplementation with CWP reduced HS-induced hepatic inflammation and restored Tc in rats, thereby alleviating liver injury.

3.4. CWP alleviated HS-induced hepatocyte apoptosis by inhibiting Caspase-3 and enhancing Bcl-2 expression

HS-induced inflammatory responses can lead to apoptosis and, subsequently, liver dysfunction. Thus, we investigated the potential effects and mechanisms of CWP against HS-induced hepatocyte apoptosis in rats. First, we evaluated the expression of Caspase-3, a key executor of apoptosis, and Bcl-2 protein, which inhibits apoptosis, using IHC-P. The results showed that HS resulted in an elevation of the IHC scores for

Caspase-3 (Fig. 3A & B) and Bcl-2 (Fig. 3C & D). We also observed the nuclear translocation of Caspase-3 in response to HS stimuli (Fig. 3A). Moreover, the TUNEL assay showed that TUNEL-positive cells (green) were increased in the liver of the HS-treated rats, indicating that HS induced DNA strand breaks in hepatocytes (Fig. 3E & F). Interestingly, supplementation with CWP before HS treatment restored the IHC scores of Caspase-3 and Bcl-2, and the cytosolic distribution of Caspase-3 in a dose-dependent manner compared with those in the control group. Correspondingly, CWP also decreased the number of TUNEL-positive hepatocytes in the HS rats in a dose-dependent manner. Taken together, these results indicate that CWP has a strong anti-hepatic apoptotic effect in a rat model of HS.

The above results also indicated that 400 mg/kg body weight CWP had the best anti-HS-induced hepatic injury and apoptosis effects in rats; therefore this dose was used in subsequent tests.

3.5. CWP alleviated HS-induced liver injury and apoptosis in rats by inhibiting NLRP3 inflammasome activation

The NLRP3 inflammasome promotes the maturation of pro-IL-1β by activating Caspase-1. Therefore, the activation of the NLRP3 inflammasome can be confirmed by the elevation of Caspase-1 activity and IL-1β content (Szabo & Csak, 2012; Yan et al., 2012). In the present study, we demonstrated that HS activated the NLRP3 inflammasome in rat liver, as manifested by enhanced Caspase-1 activity (Fig. 4A) and increased IL-1β levels (Fig. 4B & C) in serum and liver tissues. The activated NLRP3 inflammasome-induced hepatocyte injury manifested as increased serum ALT activity (Fig. 4D), which in turn aggravated liver injury. CY-09, a specific NLRP3 inhibitor, inhibited NLRP3 inflammasome assembly and activation, as indicated by decreased Caspase-1 activity and serum and liver IL-1β content, which in turn suppressed serum ALT activity and alleviated HS-induced liver injury and histopathological scores (Fig. 4E & F). Moreover, CY-09 decreased Caspase-3 (Fig. 4I & J) and increased the IHC score of Bcl-2 (Fig. 4I & K), which in turn alleviated HS-induced hepatocyte apoptosis (Fig. 4G & H). These results suggest that NLRP3 inflammasome activation is involved in HS-induced hepatocyte apoptosis and injury in rats. Interestingly, CWP exhibited effects consistent with those of CY-09. Rats supplemented with CWP before HS treatment showed decreased hepatic Caspase-1 activity, IL-1β content, and ALT activity. CWP similarly decreased Caspase-3 and enhanced the IHC score of Bcl-2, which in turn decreased HS-induced apoptosis. Furthermore, CWP reversed the HS-induced nuclear translocation of Caspase-3. The above results indicated that CWP alleviated HS-induced rat hepatocyte apoptosis and injury by inhibiting the activation of the NLRP3 inflammasome.

3.6. CWP inhibited NLRP3 activation and alleviated HS-induced liver injury through the HMGB1/RAGE axis

HS-induced enhancement of cytosolic and extracellular HMGB1 expression is required to mediate hepatic inflammasome activation and subsequent liver injury (Geng et al., 2015; Hagiwara, Iwasaka, Goto, et al., 2010; Hagiwara, Iwasaka, Shingu, et al., 2010; H.-S. Tong et al., 2011; Tong et al., 2013). Therefore, we examined the expression and distribution of HMGB1 in HS rat hepatocytes using IHC. The results showed that HMGB1 was localised to the nucleus in normal hepatocytes, whereas its expression increased in the cytoplasm (black arrows) and decreased in the nucleus (red arrows) of HS hepatocytes (Fig. 5 A). The IHC score of HMGB1 was higher than that of the control group (Fig. 5B).

To further elucidate the underlying mechanism of HMGB1 in mediating HS-induced activation of the hepatic inflammasome and subsequent liver injury, its effect was blocked with glycyrrhizic acid, an antagonist of HMGB1. Glycyrrhizic acid promoted the nuclear localization of HMGB1 and decreased the IHC score of HMGB1. Correspondingly, Glycyrrhizic acid reduced hepatic Caspase-1 activity (Fig. 5C) as well as IL-1β (Fig. 5D) and NLRP3 inflammasome (Fig. 5E & G) levels, and alleviated histological changes (Fig. 5H & I) and apoptosis (Fig. 5J & K) in the HS rats. Interestingly, CWP showed the same effect as glycyrrhizic acid. Moreover, reports have shown that the RAGE receptor is involved in the signal transduction by which HMGB1 activates the NLRP3 inflammasome. Therefore, we examined the role of CWP in RAGE receptor expression. The results showed that CWP inhibited the HS-induced increase in RAGE expression in the rat liver (Fig. 5F & G). In conclusion, our results confirm the regulatory effects of CWP on the hepatic HMGB1/RAGE/NLRP3 signalling pathway in HS rats. More importantly, the combined application of CWP and glycyrrhizic acid almost completely restored the intranuclear localisation of HMGB1 and the activation of the NLRP3 inflammasome, which in turn completely blocked HS-induced histological changes and apoptosis in rat liver.

4. Discussion

Liver injury is the most common histological change in all cases of HS and is one of the most lethal complications (Geng et al., 2015; Weigand, Riediger, Stremmel, Flechtenmacher, & Encke, 2007; Kew et al., 1970; Hassanein et al., 1992). Although the mechanism of HS-induced liver injury has not been fully elucidated, there is evidence that it may be mediated by an inflammatory response rather than a direct consequence of hyperthermia acting on the organism (Leon, 2007). Furthermore, studies have shown that HS-induced extracellular expression of HMGB1 can activate the NLRP3 inflammasome through RAGE receptors, which in turn triggers sterile inflammation, leading to liver injury (Geng et al., 2015). The obvious anti-inflammatory effect of CWP has been previously demonstrated (G. Badr, Ramadan, et al., 2018; Gamal Badr et al., 2017; G. Badr et al., 2017; Ebaid, Ahmed, Mahmoud, & Ahmed, 2013; Ramadan et al., 2018). Our previous in vitro studies also demonstrated the inhibitory effects of CWP on the NLRP3 inflammasome in HS hepatocytes (Du et al., 2021). The current study demonstrated in a rat model of HS that CWP alleviated HS-induced histological changes in the liver, including hepatocyte ballooning degeneration, apoptosis, necrosis, and inflammatory cell infiltration.

Previous studies have shown that HS can induce activation of the hepatic NLRP3 inflammasome in rats (Song et al., 2018). The persistently activated NLRP3 inflammasome may synergistically activate Caspase-3 to mediate hepatocyte apoptosis, which in turn leads to liver injury (Chen & Chen, 2018; Lebeaupin et al., 2015; Wree et al., 2014). The NLRP3 inflammasome is an intracellular multiprotein complex assembled from NOD-like receptors (NLRs), apoptosis-associated speck-like protein containing CARD (ASC), and Caspase-1. After its assembly, pro-caspase-1 can be recruited and cleaved into activated Caspase-1 by the CARD domain of ASC. Then, pro-IL-1β is cleaved to mature IL-1β by activating Caspase-1 (Conesa et al., 2008; Martine et al., 2019). An additional type of inflammatory form of programmed cell death, pyroptosis, can be induced by activated Caspase-1. Furthermore, studies have shown that inflammasome-induced apoptosis is accompanied by the activation of Caspase-3 and is dependent on Caspase-1 (Tsuchiya et al., 2019). This suggests that activated Caspase-1 or NLRP3 inflammasomes mediate two types of programmed cell death, apoptosis and pyroptosis (Lebeaupin et al., 2015). In addition, elevated IL-1β may contribute to cell death, and silencing of NLRP3 in rats with siRNA significantly reduces Caspase-1 cleavage in hepatocytes and ameliorated HS-induced liver injury (Geng et al., 2015). Consistent with these studies, the results of the present study suggest that HS activates the NLRP3 inflammasome in rat liver, manifested by increased Caspase-1 activity and IL-1β levels. However, hepatic NLRP3 activity was suppressed in the rats supplemented with CWP before HS treatment, which in turn decreased Caspase-1 activity and IL-1β levels. These data also show the potential inhibitory roles of CWP in pyroptosis.

Additionally, we observed that activated NLRP3 inflammasomes induced hepatocyte apoptosis, as CY-09, a specific inhibitor of NLRP3, alleviated hepatocyte apoptosis. Caspase-3 participates in both the mitochondrial and death receptor apoptotic pathways, and is the final

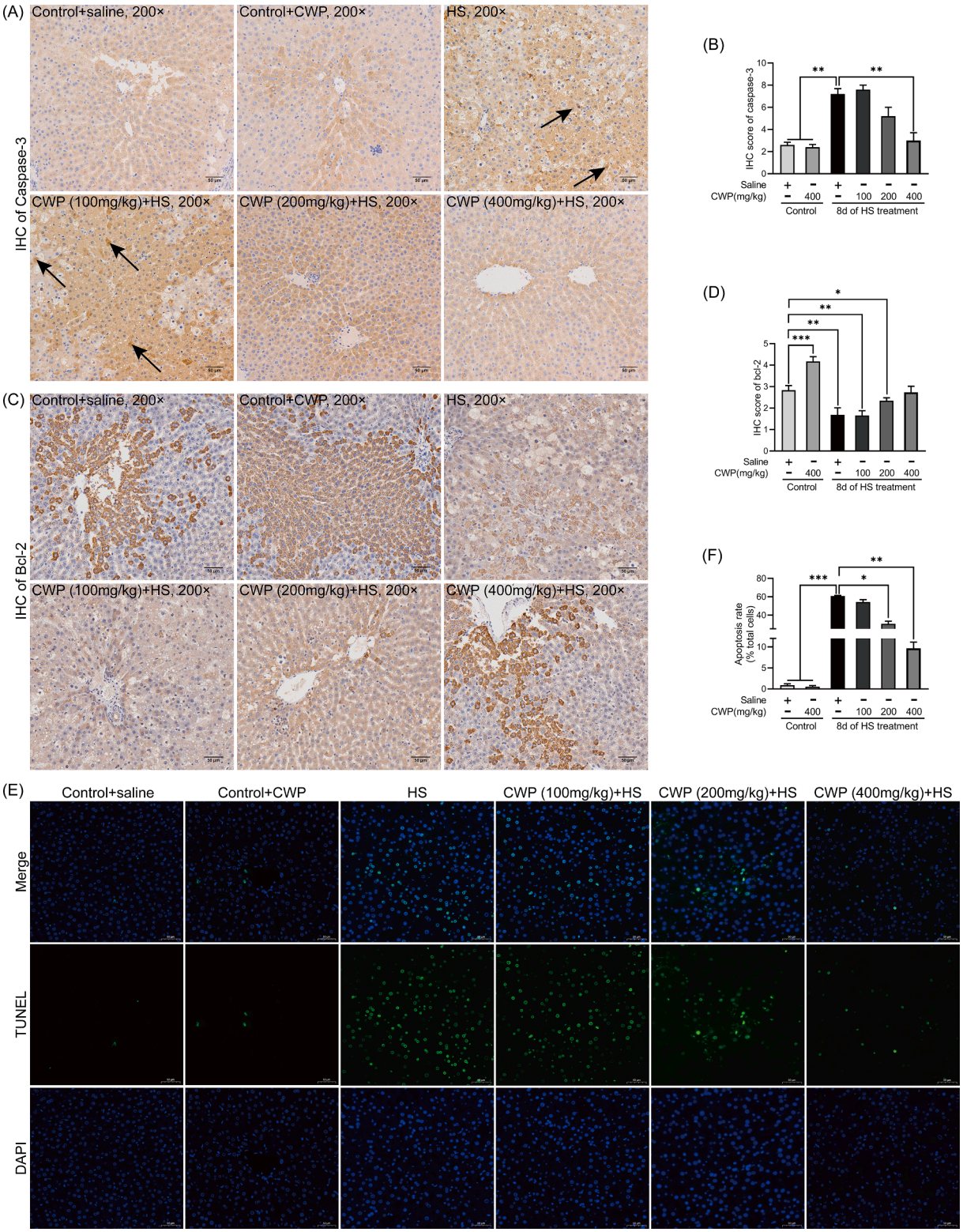

Fig. 3. Camel whey protein (CWP) alleviated heat stress (HS)-induced hepatocyte apoptosis by enhancing Bcl-2 and inhibiting Caspase-3 expression. (A) Representative figures of immunohistochemistry-paraffin (IHC-P) staining of Caspase-3 (200× magnification). (B) IHC scores of Caspase-3. (C) Representative figures of IHC-P staining of Bcl-2 (200× magnification). (D) IHC scores of Bcl-2. (E & F) Representative figures of *in situ* TUNEL assay for HS-induced hepatocyte apoptosis and the anti-apoptotic effect of CWP (200× magnification). Nuclei were counterstained with DAPI (blue) and DNA fragmentation was measured by TUNEL (green). Black arrows indicate Caspase-3 protein expression in the nucleus. Data are presented as the mean of at least six determinations ± standard error of the mean (SEM). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

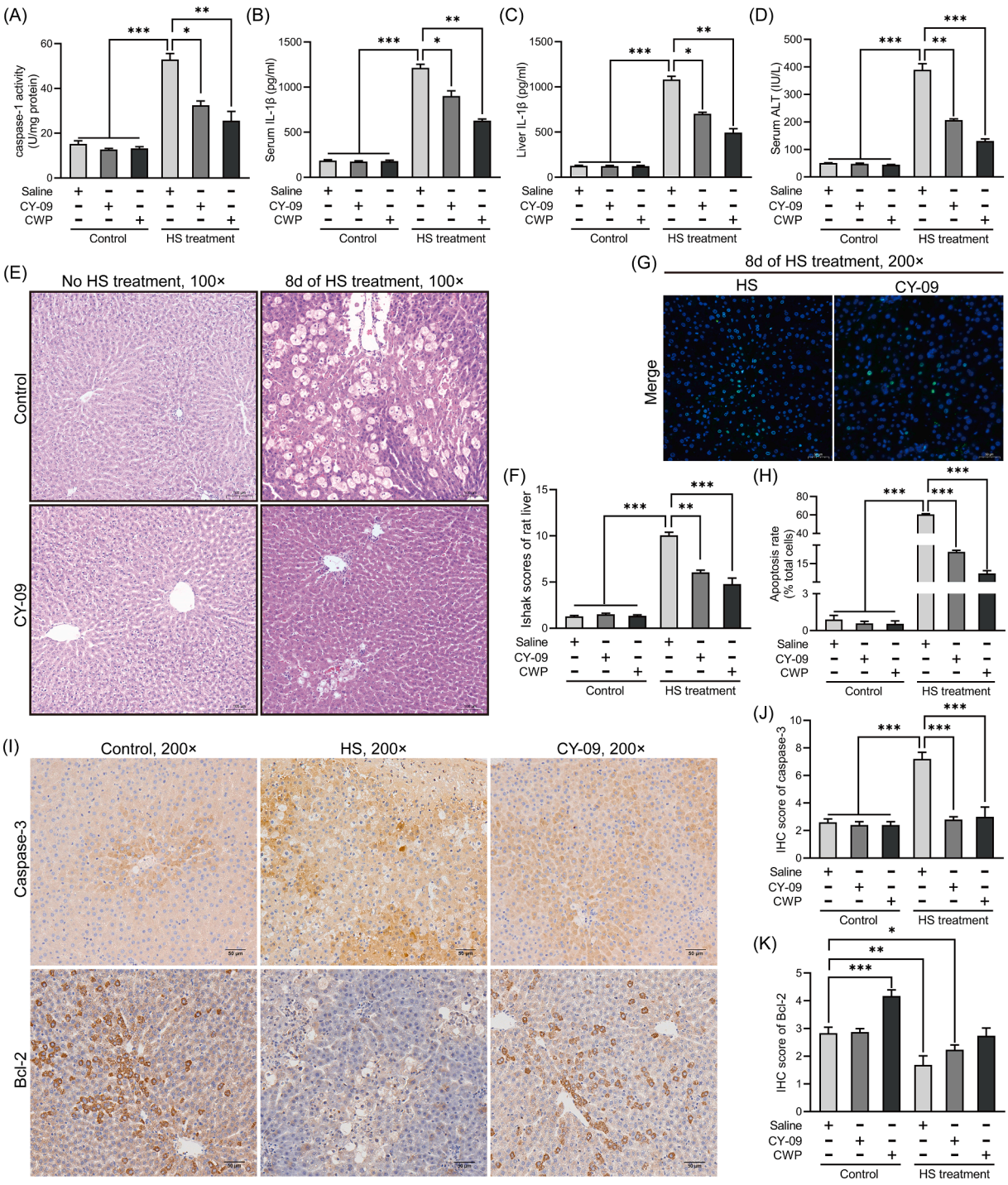

Fig. 4. Camel whey protein (CWP) alleviated heat stress (HS)-induced liver injury and apoptosis in rats by inhibiting NLRP3 inflammasome activation. In vivo tests confirmed that CWP inhibited HS-induced NLRP3 inflammasome activity in rat liver, as indicated by decreased Caspase-1 activity (A), and IL-1β content in serum (B) and liver tissues (C). (D) CWP suppressed HS-induced elevation of serum alanine transaminase (ALT) activity in rats. (E) Representative figures of CY-09 alleviated HS-induced liver injury. (F) Histopathological scores were evaluated according to the Ishak scoring system. (G & H) Representative figures of CY-09 alleviated HS-induced apoptosis. (I) Caspase-3 and Bcl-2 expressions were evaluated using immunohistochemistry-paraffin (IHC-P) assay. (J) IHC scores for Caspase-3. (K) IHC scores for Bcl-2. Data are presented as the mean of at least six determinations ± standard error of the mean (SEM). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

executioner of apoptosis (Sudo & Minami, 2010). Although the inactivated zymogen form of Caspase-3 is localised in the cytoplasm, its activated form executing the apoptotic effects is within the nucleus, as its substrates are mostly found in the nucleus (Takemoto, Nagai, Miyawaki, & Miura, 2003). The present study revealed that HS promoted the relocation of Caspase-3 from the cytoplasm into the nucleus, which in turn triggered hepatocyte apoptosis, resulting in DNA damage manifested as positive TUNEL staining. A previous study showed that CWP can inhibit lymphocyte apoptosis by regulating Caspase-3 (Ramadan et al., 2018). The current study further confirmed that CWP inhibited hepatic Caspase-3 expression and apoptosis in an HS rat model. In contrast, there is an anti-apoptotic Bcl-2 within the cell, and its reduced expression may affect the apoptosis of cells after HS (G. Badr, Abdel-Tawab, Ramadan, Ahmed, & Mahmoud, 2018; Ramadan et al.,

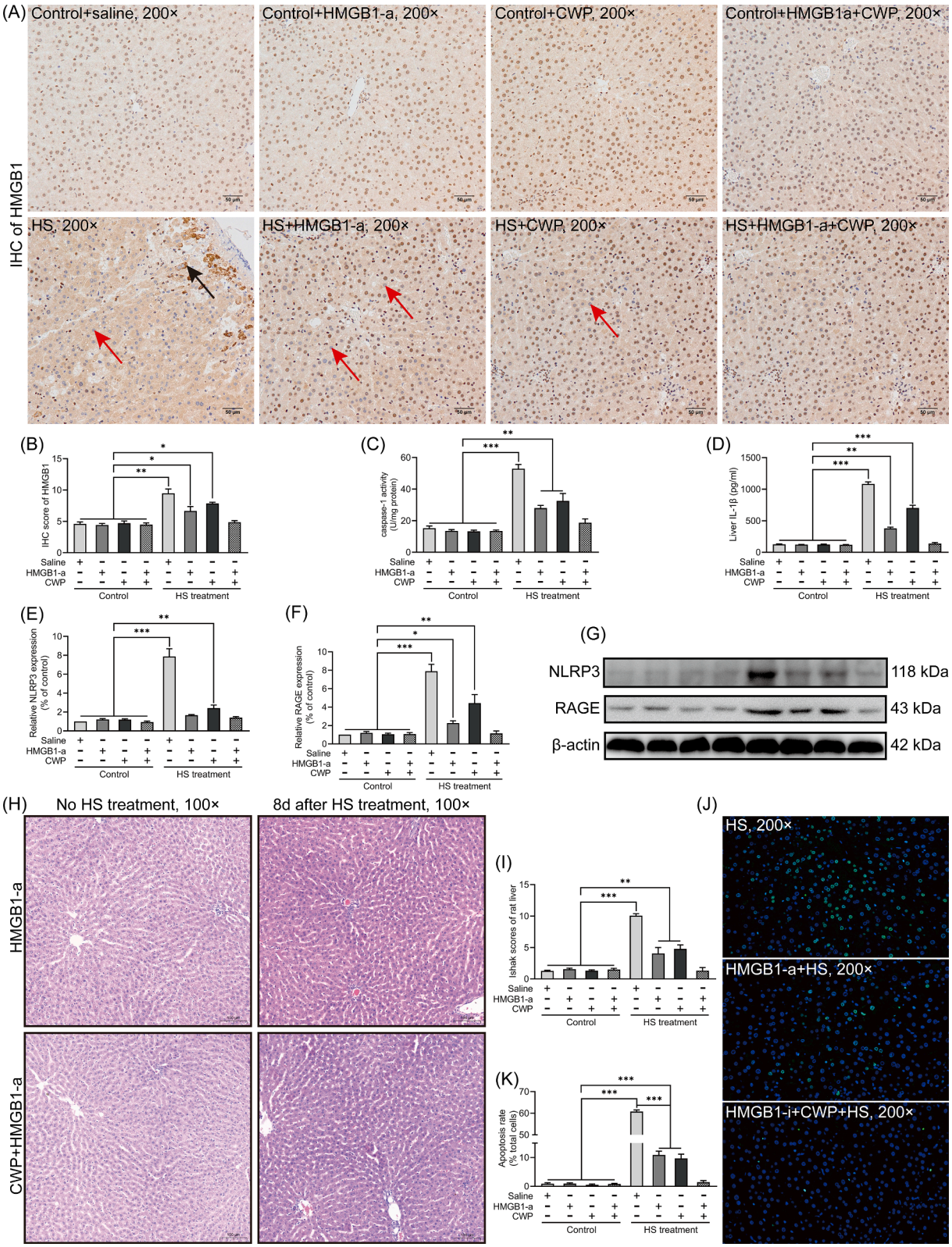

Fig. 5. Regulation of hepatic HMGB1/RAGE/NLRP3 signaling pathways by Camel whey protein (CWP) in heat stress (HS) rats. (A) Effect of CWP and high-mobility group box 1 (HMGB1) antagonist (HMGB1-a) on the localization of HMGB1 in HS rat hepatocytes was evaluated using an immunohistochemistry (IHC) assay. (B) IHC scores of HMGB1. (C) Caspase-1 activity in the rats' liver. (D) Content of IL-1β in the rats' liver. (E) Relative expression of the NLR pyrin domain containing 3 (NLRP3) inflammasome in the rats' liver. (F) Relative expression of the receptor for advanced glycation end products (RAGE) in the rats' liver. (G) Expression of the NLRP3 inflammasome and RAGE in rat liver tissues was examined using a western blot assay. (H & I) HMGB1 antagonist alleviated HS-induced liver histological changes histopathological scores, whereas its combined action with CWP completely prevented them. (J & K) Combined application of CWP and HMGB1 antagonist completely blocked HS-induced hepatocyte apoptosis. Data are presented as the mean of at least six determinations ± standard error of the mean (SEM). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

2018). This is consistent with our results showing that HS significantly downregulated the expression of Bcl-2, whereas CWP upregulated its expression in a dose-dependent manner. Importantly, we found that the degree of hepatocyte apoptosis was positively correlated with liver injury, as manifested by enhanced serum ALT activity and liver histological changes in the HS rats. Under physiological conditions, ALT is mainly distributed in the cytoplasm of hepatocytes, and its elevated activity proves that the membrane has been damaged, resulting in increased cell membrane permeability (Li et al., 2019). CY-09 alleviated ALT activity and liver histological changes, suggesting that NLRP3 activation is associated with HS-induced liver injury. Previous studies have shown that camel milk alleviates CCl_4- and ethanol-induced liver injury (Darwish, Abd Raboh, & Mahdy, 2012; Hamed, Bellassoued, El Feki, & Gargouri, 2019), but the effect of CWP on HS-induced liver injury is unknown. The present study demonstrates, for the first time, the protective effects of CWP against HS-induced liver injury in rats. We found that CWP alleviated HS-induced elevation of serum ALT activity and liver histological changes in rats in a dose-dependent manner. Taken together, these data suggest that CWP may alleviate HS-induced liver injury in rats by inhibiting NLRP3 inflammasome activation.

HMGB1, a representative damage-associated molecular pattern, plays a central role in mediating sterile inflammatory responses (Andersson & Tracey, 2011; Matzinger, 2002; Seong & Matzinger, 2004). With the discovery of its late pro-inflammatory role, HMGB1 has become one of the hot spots of clinical research in recent years. HMGB1 localises to the nucleus in normal cells, is actively secreted by hepatocytes when subjected to various pro-inflammatory stimuli (Wang, Yang, & Tracey, 2004; Yu et al., 2019), and can also be passively released by injured cells, which in turn induces inflammatory responses during cell necrosis (Andersson & Tracey, 2011). Previous studies have shown that the elevation of HMGB1 in blood circulation can be induced at an early stage by HS (Hagiwara, Iwasaka, Goto, et al., 2010; Tong et al., 2011; Tong et al., 2013). Recent evidence has also demonstrated that HS induces the translocation of rat hepatic HMGB1 from the nucleus to the cytoplasm, which in turn activates the NLRP3 inflammasome and mediates liver injury (Geng et al., 2015). In addition, RAGE, an endogenous ligand, has been confirmed to be involved in the signal transduction of HMGB1 to activate the NLRP3 inflammasome (Geng et al., 2015). The above studies suggest that HMGB1 can stimulate the secretion of pro-inflammatory cytokines by activating the NLRP3 inflammasome, which may be a major mechanism underlying HS-induced liver injury. In the present study, we further confirmed the role of HMGB1 in HS-induced liver injury, as an antagonist of HMGB1 (Glycyrrhizic acid) reduced RAGE expression, NLRP3 activity, and the rate of apoptosis, which in turn alleviated hepatic histological changes. Interestingly, the protective effects of CWP supplementation and intraperitoneal glycyrrhizic acid administration before HS treatment on HS-induced liver injury in rats were consistent, accompanied by a decrease in the IHC scores of HMGB1 and in the expression of RAGE and NLRP3 inflammasome. Taken together, our results suggest that the anti-HS-induced liver injury effect of CWP may be associated with its regulation of the HMGB1/RAGE/NLRP3 signalling pathway.

Glycyrrhizic acid, a triterpenoid saponin derived from the root of liquorice, has been confirmed as a direct antagonist of HMGB1 and has been clinically used to treat acute and chronic hepatitis-induced liver dysfunction (Yu et al., 2019). This role was similarly confirmed in the present study. More importantly, the combined application of CWP and glycyrrhizic acid completely restored the nuclear distribution and IHC score of HMGB1, the activity of Caspase-1, the content of IL-1β, and the expression of the NLRP3 inflammasome and RAGE receptor, and, in turn, completely blocked HS-induced hepatic apoptosis and liver histological changes in rats compared with those in the control rats. These data suggest that CWP may serve as an effective adjunctive strategy for the treatment of HS-induced liver injury.

5. Conclusion

In conclusion, the present study demonstrated that CWP has protective effects against HS-induced liver injury in rats. The protective effect of CWP is attributed to its ability to reduce the increase in ALT and IL-1β levels, restore core temperature, and block apoptosis and histopathological changes in the liver. The potential protective mechanism of CWP was associated with the inhibition of NLRP3 inflammasome activation through the regulation of HMGB1/RAGE, which in turn reduced HS-induced hepatic inflammation in rats. More importantly, CWP combined with anti-inflammatory agents almost completely prevented HS-induced liver injury. Thus, the current study provides a new strategy for adjuvant treatment of HS-induced liver injury with CWP. Furthermore, the reported effects of CWP on various inflammatory diseases are particularly exciting. Therefore, the exact mechanisms underlying the hepatoprotective effects of CWP warrant further exploration in future studies.

Ethics statement

Sprague-Dawley (SD) rats (weight, 200 ± 20 g; age, 6 weeks; sex, male) were obtained from Laboratory Animal Center of Inner Mongolia Medical University (Hohhot, China) and kept in the Animal Center of Inner Mongolia Agricultural University. Laboratory animal production license No.: SCXK (Meng) 2015–0001. Laboratory animal use license No.: SYXK (Meng) 2015–0001. All experimental procedures were approved by the Scientific Research and Ethics Committee of Inner Mongolia Agricultural University.

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CRediT authorship contribution statement

Donghua Du: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Visualization. **Wenting Lv:** Conceptualization, Validation, Formal analysis, Investigation, Writing - review & editing, Visualization. **Xiaoxia Jing:** Validation, Investigation, Data curation, Writing - review & editing, Visualization. **Xueni Ma:** Investigation, Writing - review & editing. **Jiya Wuen:** Formal analysis, Investigation. **Surong Hasi:** Conceptualization, Resources, Supervision, Funding acquisition, Writing - review & editing.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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