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SHORT COMMUNICATION

Camel whey protein alleviates heat stress-induced liver injury by activating the Nrf2/HO-1 signaling pathway and inhibiting HMGB1 release

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Abstract

This study aimed to investigate the mechanism by which camel whey protein (CWP) inhibits the release of high-mobility group box 1 (HMGB1) in heat stress (HS)-stimulated rat liver. Administration of CWP by gavage prior to HS inhibited the cytoplasmic translocation of HMGB1 and consequently reduced the inflammatory response in the rat liver, and downregulated the levels of the NLR pyrin domain containing 3 (NLRP3) inflammasome, interleukin (IL)-1β, and tumor necrosis factor (TNF)-α. The use of N-acetyl-L-cysteine (NAC), an inhibitor of reactive oxygen species (ROS) production, indicated that this downregulation effect may be attributed to the antioxidant activity of CWP. We observed that CWP enhanced nuclear factor erythroid 2-related factor (Nrf)2 and heme-oxygenase (HO)-1 expression, which inhibited ROS production, nicotinamide adenine dinucleotide phosphate oxidase (NOX) activity, and malondialdehyde (MDA) levels, and increased superoxide dismutase 1 (SOD1) activity and reduced glutathione (GSH) content in the HS-treated liver, ultimately increasing the total antioxidant capacity (TAC) in the liver. Administration of Nrf2 or HO-1 inhibitors before HS abolished the protective effects of CWP against oxidative damage in the liver of HS-treated rats, accompanied by increased levels of HMGB1 in the cytoplasm and IL-1β and TNF-α in the serum. In conclusion, our study demonstrated that CWP enhanced the TAC of the rat liver after HS by activating Nrf2/HO-1 signaling, which in turn reduced HMGB1 release from hepatocytes and the subsequent inflammatory response and damage. Furthermore, the combination of CWP and NAC abolished the adverse effects of HS in the rat liver. Therefore, dietary CWP could be an effective adjuvant treatment for HS-induced liver damage.

Keywords Camel whey protein · HMGB1 · Heat stress · Liver damage · Nrf2

Introduction

Heat stress (HS) has emerged as an important factor affecting human and animal health during hot seasons worldwide; HS can increase body temperature and cause morbidity and mortality (Myers and Bernstein 2011). Furthermore, liver damage and subsequent refractory liver failure are common fatal complications in patients with HS and HS animal models (Geng et al. 2015; Wang et al. 2018). Studies have shown that the pathogenesis of HS-induced liver damage involves oxidative stress (OS) and inflammatory responses and is not merely a result of hyperthermia (Du et al. 2021a, 2021b; Geng et al. 2015).

High-mobility group box 1 (HMGB1), an important pro-inflammatory mediator of HS-induced liver damage, is a highly abundant nuclear protein in most cell types (Geng et al. 2015). HS promotes hepatic reactive oxygen species (ROS) production, which induces OS and contributes to the release of HMGB1 during the progression of HS-induced sterile inflammation (Geng et al. 2015; Tsung et al. 2007). Studies have demonstrated the crosstalk between ROS-evoked OS and HMGB1-mediated inflammation. The nuclear factor erythroid 2-related factor (Nrf)2 is a critical transcription factor (Sies et al. 2017), and pathways

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involving Nrf2 can control HS-induced hepatic OS by promoting the expression of a set of antioxidant enzymes, such as heme-oxygenase (HO)-1 (Chen et al. 2017; Du et al. 2021b; Song et al. 2018). Activation of the Nrf2/HO-1 pathway can inhibit HMGB1 release, resulting in protection against sterile inflammation-mediated tissue damage (Chen et al. 2017; Wang et al. 2013). These studies suggest that activating the Nrf2/HO-1 signaling axis and targeting HMGB1 may effectively prevent liver damage induced by HS.

Camel whey protein (CWP) is a major component of the total camel milk protein and is composed of α-lactalbumin (α-LA), serum albumin, lactoferrin (LF), immunoglobulin, and peptidoglycan recognition protein. In addition, CWP is similar in composition to human milk, both containing high levels of α-LA and LF, but lacking β-lactoglobulin that predisposes children and young animals to allergy. The antioxidant and anti-inflammatory activities of CWP are superior to those of bovine and other whey proteins (Badr et al. 2017b). We previously demonstrated that CWP alleviates HS-induced OS, damage, and apoptosis in hepatocytes via activation of the Nrf2/HO-1 signaling pathway using in vitro experiments (Du et al. 2021b). Subsequently, we demonstrated the anti-HS effect of CWP in vivo, which limited the NLR pyrin domain-containing 3 (NLRP3) inflammasome activation by inhibiting HMGB1 release, ultimately alleviating HS-induced liver damage (Du et al. 2021a). However, to the best of our knowledge, the mechanism by which CWP inhibits HMGB1 release has not been reported. Therefore, in the present study, we report a mechanism by which CWP inhibits HMGB1 release from hepatocytes after HS, blocks the inflammatory process, and alleviates liver damage by activating the Nrf2/HO-1 signaling pathway.

Methods

Isolation of camel whey protein

The isolation of CWP was performed based on our previously described method (Du et al. 2021a, 2021b). The CWP samples were lyophilized and stored at − 80 ℃.

Experiment design and treatments

Six-week-old male Sprague–Dawley rats (200 ± 20 g) were obtained from the Laboratory Animal Center of Inner Mongolia Medical University (Hohhot, China). The experimental protocols in this study were confirmed by the Scientific Research and Ethics Committee of Inner Mongolia Agricultural University. The rats were raised in a suitable temperature (21 ± 1 ℃) and humidity (60–65%) environment and allowed free access to clean water and standard pelleted food. The lights of the animal holding room were on at 8:00 AM and off at 8:00 PM. The rats were randomly divided into seven groups ($n = 6$) after 2 weeks of adaptive feeding. Throughout the test period, rats in the control, HS, CWP, N-acetyl-L-cysteine (NAC), and CWP + NAC groups were administered saline (1 ml), CWP (400 mg/kg), NAC (400 mg/kg; Beyotime Biotechnology, Shanghai, China), and CWP + NAC, respectively, by gavage once daily. In some rats, ML385 (MCE, New Jersey, USA) was administered intraperitoneally (i.p.; 30 mg/kg) prior to HS to inhibit the activity of Nrf2. Some rats were i.p. injected with zinc protoporphyrin (ZnPP; 5 mg/kg; MCE, New Jersey, USA) prior to HS to inhibit the activity of HO-1. Additionally, rats in the ML385 and ZnPP groups were intragastrically administered saline (1 ml) throughout the test. Rats in each group, except the control group, were housed in an artificial climate chamber (temperature: 40 ± 0.2 ℃; relative humidity: 60–65%) from the 15th day for 2 h per day for eight consecutive days. After the last HS treatment and 24-h recovery, the rats were anesthetized and serum and liver samples were collected.

Histopathologic examination

Following the routine hematoxylin and eosin (HE) staining protocol, the 4-μm sections were stained and visualized using a microscope for morphological analysis. The histological score of liver was assessed according to the Ishak scoring system by the method described in a reference (Ishak et al. 1995).

Evaluation of liver function and inflammation

The activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in serum were used to assess liver function. The assay kits were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Rat interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) ELISA kit (HCUSABIO, Wuhan, China) were used to detect the content of IL-1β and TNF-α in serum. Detection methods and procedures were done following the operating instructions of the kits.

Determination of OS-related factors

The levels of ROS in rat liver were detected with a fluorescent probe dihydroethidium (DHE; Beyotime Biotechnology, Shanghai, China). Briefly, cryosections of fresh liver tissues (within 1 h after surgical resection) were prepared following routine procedures. The sections were coverslipped with 5 μM DHE solution and protected from light and incubated at 37 ℃ for 30 min. After that, the slides were washed three times for 5 min in phosphate-buffered saline

(PBS, pH 7.4). Finally, images were visualized by using a fluorescence microscope at wavelengths of 535 nm (excitation) and 610 nm (emission) after the slides were sealed with an antifade mounting medium (Beyotime Biotechnology, Shanghai, China). The fluorescence intensity was examined by using ImageJ software. Hepatic nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase, NOX) activity, reduced glutathione (GSH) levels, and malondialdehyde (MDA) levels were measured using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The total antioxidant capacity (TAC) of liver was assessed by diammonium 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonate) (ABTS) method according to the operation of kit instruction (Beyotime Biotechnology, Shanghai, China). Superoxide dismutase 1 (SOD1) content of liver tissues was detected by a rat extracellular superoxide dismutase (Cu/Zn) ELISA kit (HCUSABIO, Wuhan, China).

Immunohistochemistry (IHC)

IHC was performed as previously described (Du et al. 2021a). Primary antibodies used in this study included anti-HMGB1 (1:200; Abcam), anti-NLRP3 (1:500; GeneTex), and anti-Nrf2 (1:200; Abcam) antibodies. The secondary antibody was goat anti-rabbit IgG (H + L)-HRP (1:1000; Tianjin Sungene Biotech, China). The sections were visualized using a DAB Substrate Kit (Solarbio, Beijing, China). The ratio of the positive cells was calculated using ImageJ software. Scores of 0 (< 5%), 1 (5–25%), 2 (25–50%), 3 (50–75%), and 4 (> 75%) were counted according to the positive cell rate. The staining intensity was scored according to a four-step grading: score of 0, negative; score of 1, weakly positive; score of 2, moderately positive; and score of 3, strongly positive. The final IHC score = score of positive cells × score of staining intensity.

Western blotting analysis

Cytoplasmic or nuclear proteins were obtained using a commercial Mammalian Nuclear Extraction Kit (Sangon Biotech, Shanghai, China). Total cellular protein was obtained using a Mammalian Protein Extraction Reagent (Thermo Fisher Scientific). Total, cytoplasmic, or nuclear proteins (20 μg) from cells were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 10%) and then blotted onto a polyvinylidene difluoride (PVDF) membrane according to our previously described procedures (Du et al. 2021a, 2021b). After blocking with 5% skim milk (Beyotime Biotechnology, Shanghai, China), blots were incubated with anti-HMGB1 (1:1000), anti-Nrf2 (1:2000), and anti-HO-1 (1:1000; GeneTex, Inc., USA) antibody. The protein bands were visualized using Super ECL Plus (US Everbright Inc., Suzhou, Jiangsu, China). The optical density values of protein bands were analyzed using Image Pro Plus 6.0 software. TATA binding protein (TBP) or β-actin was used for normalization.

Statistical analyses

Results were represented as the mean ± standard error of the mean (SEM). Statistical analysis was performed using SPSS 25.0. Normal distribution of all the variables was evaluated using the Kolmogorov–Smirnov and the Shapiro–Wilk normality test. For data which did not conform to assumptions of normality, the Kruskal–Wallis nonparametric analysis of variance (ANOVA, for multiple comparisons) was used. Data were analyzed using one-way ANOVA followed by Tukey post-test when equal variances were assumed, and Tamhane's T2 when equal variances not assumed.

Results

CWP inhibits the cytoplasmic translocation of HMGB1 in the HS-treated liver by decreasing ROS production

Our previous study confirmed that CWP reduced HS-induced liver damage by suppressing the cytoplasmic translocation of HMGB1 (Du et al. 2021a). To explore whether CWP inhibited the cytoplasmic translocation of HMGB1 in hepatocytes after HS-induced OS, we pre-treated rats with NAC, a commonly used antioxidant and ROS production inhibitor (Halasi et al. 2013). The IHC results showed that HS increased HMGB1 levels in the cytoplasm (black arrows) and decreased HMGB1 levels in the nucleus (red arrows; Fig. 1A). NAC partially reversed the cytoplasmic translocation of HMGB1, which was consistent with the role of CWP. These results were verified using western blotting (Fig. 1B, C). This finding suggests that CWP, like NAC, suppressed HMGB1 expression in the cytoplasm by relieving HS-induced OS. Additionally, the combination of CWP and NAC completely restored the nuclear localization of HMGB1 in rat hepatocytes after HS.

CWP suppresses HMGB1-mediated hepatic inflammatory responses by decreasing ROS production

HMGB1-mediated NLRP3 inflammasome activation and subsequent inflammatory responses are essential to liver damage (Du et al. 2021a; Geng et al. 2015). We observed that rats pretreated with NAC had suppressed NLRP3 inflammasome expression (Fig. 2A, B) and activity, as evidenced by the reduced IL-1β levels (Fig. 2C). Moreover, NAC suppressed the expression of another important pro-inflammatory factor,

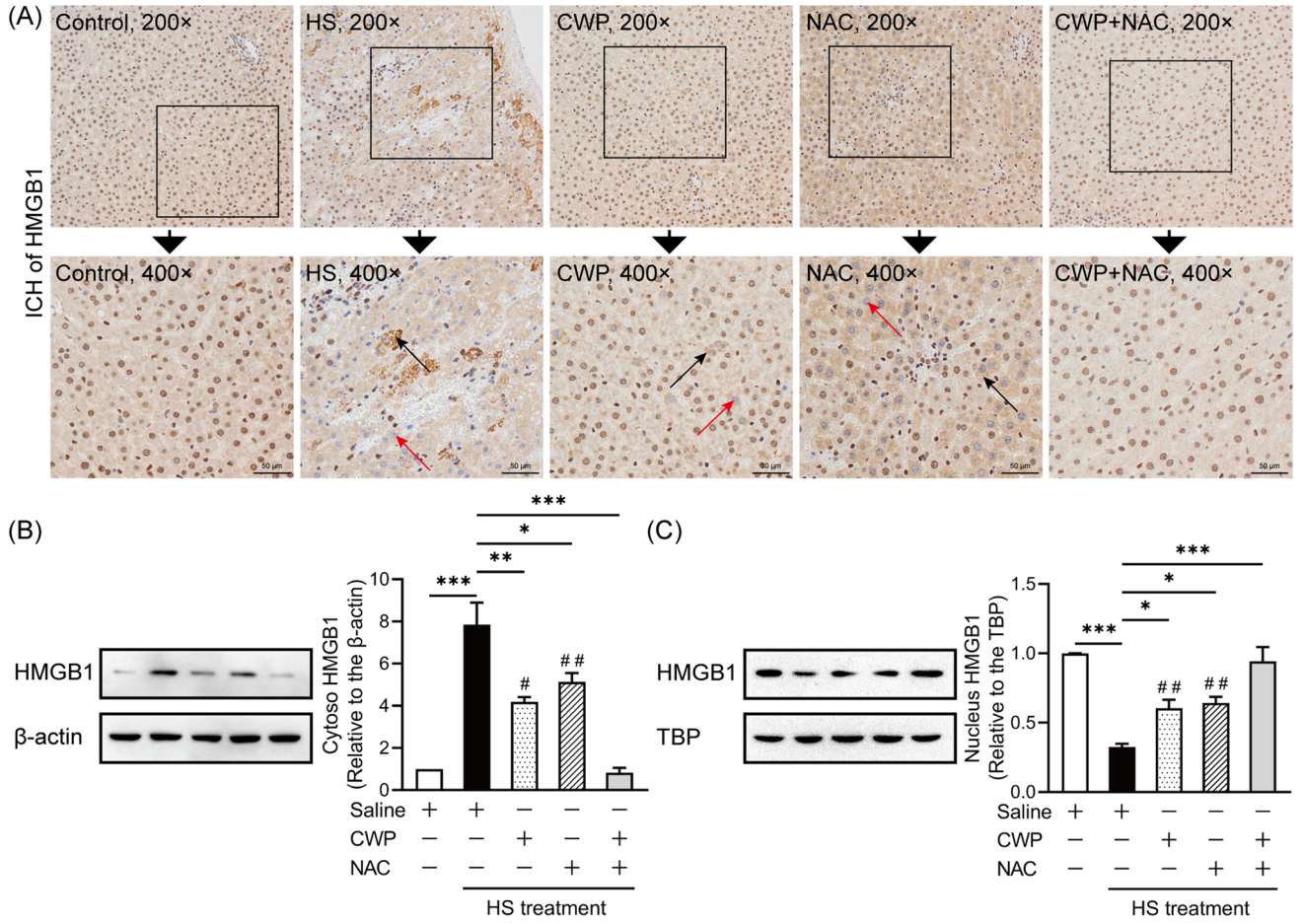

Fig. 1 CWP inhibited the cytoplasmic translocation of HMGB1 in the liver of HS rats. **A** Representative figures of IHC results of HMGB1 (×200 and ×400 magnification). Red arrows indicate attenuated HMGB1 staining intensity in the nucleus. Black arrows indicate enhanced HMGB1 staining intensity in the cytoplasm. **B** The expression of HMGB1 in the cytoplasm of HS rat hepatocytes was assessed using a western blotting assay. β-Actin was used as an internal reference protein. **C** The expression of HMGB1 in the nucleus of HS rat hepatocytes was investigated using a western blotting assay. TBP was used as an internal reference protein. *Representative significance is based on the comparison with the HS group, whereas # representative significance is based on the comparison with the control group. Data are presented as the mean of at least three determinations ± SEM. * or # $p < 0.05$, ** or ## $p < 0.01$, and *** or ### $p < 0.001$

TNF-α, in the livers of HS-treated rats (Fig. 2D). Interestingly, CWP treatment more effectively reduced the IHC scores of the NLRP3 inflammasome and the levels of IL-1β and TNF-α in HS-treated livers compared with that in NAC-treated rats. The combination of CWP and NAC blocked HS-induced hepatic inflammatory responses. These results suggest that OS and inflammation are inextricably linked in HS-treated livers and both may act in a positive reciprocal feedback loop. Additionally, it implies that the anti-HS-induced liver damage effects of CWP may be attributed to its powerful antioxidant properties.

Antioxidant protects against liver damage induced by HS

The present study further evaluated the role of ROS-mediated OS in HS-induced liver damage. As shown in Fig. 3A, HS-treated rat livers displayed an infiltration of inflammatory cells such as lymphocytes (black arrows) could be 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). In addition, the central vein and hepatic sinusoids were filled with numerous red blood cells (purple arrows). HS-stimulated rats had higher histopathological scores than rats in the control group (Fig. 3B). In addition, elevated serum ALT (Fig. 3C) and AST (Fig. 3D) levels indicated abnormal liver function. Interestingly, CWP or NAC administration alleviated liver histological changes and ALT and AST levels induced by HS. More importantly, the combined application of CWP and NAC blocked HS-induced liver damage.

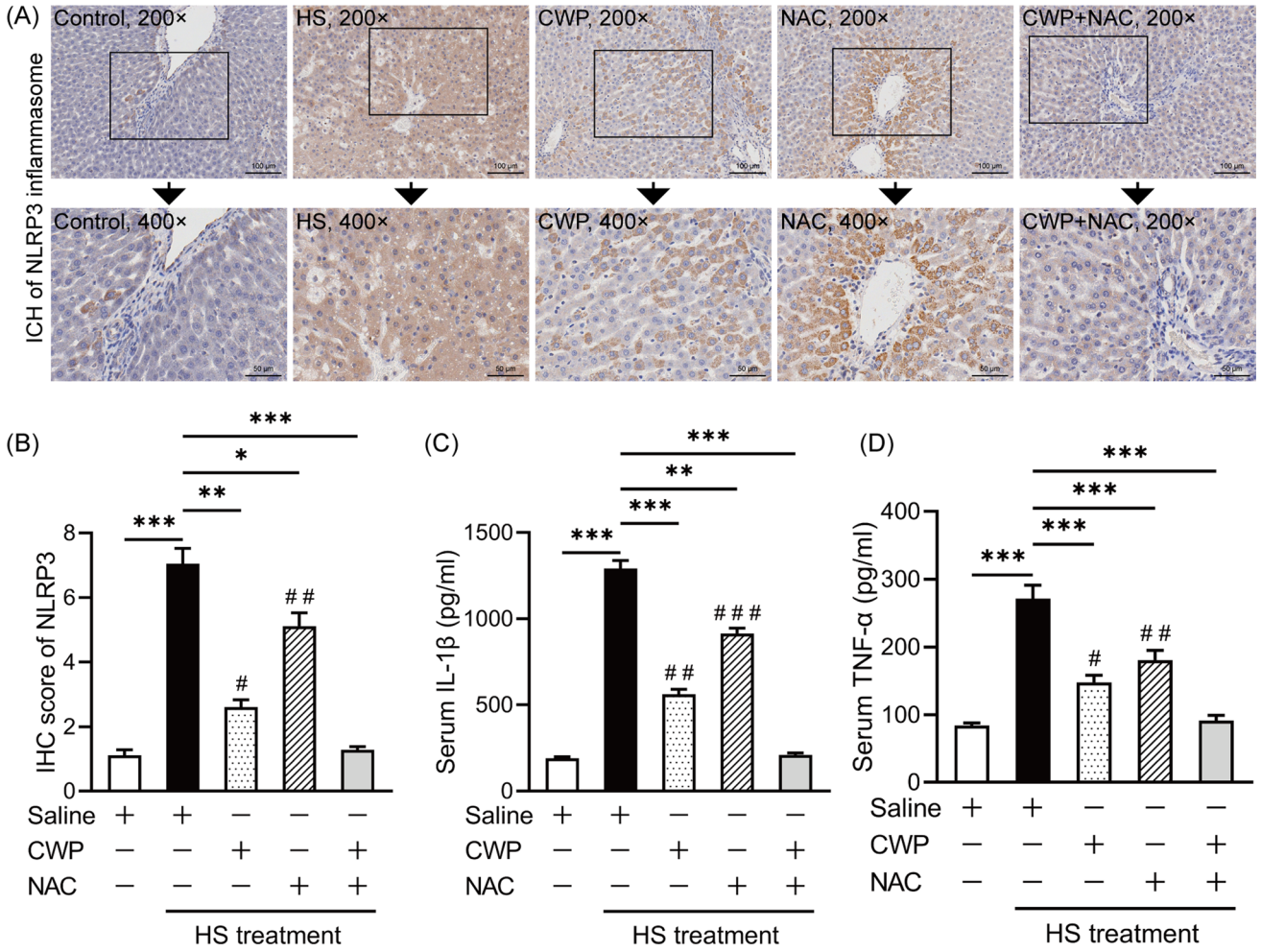

Fig. 2 CWP suppressed HMGB1-mediated hepatic inflammation. **A** Representative figures of IHC results of NLRP3 inflammasome (×200 and×400 magnification). **B** IHC scores of NLRP3 inflammasome. **C** IL-1β content in serum. **D** TNF-α content in serum. *Representative significance is based on the comparison with the HS group, whereas # representative significance is based on the comparison with the control group. Data are presented as the mean of at least six determinations ± SEM. * or # $p < 0.05$, ** or ## $p < 0.01$, and *** or ### $p < 0.001$

CWP alleviates HS-induced hepatic OS in rats

To evaluate the effects of CWP on HS-induced hepatic OS, we examined OS-related factors. Our results showed that HS promoted hepatic ROS production (Fig. 4A, B) and enhanced NOX activity (Fig. 4C) and MDA levels (Fig. 4D) in rats, indicating that HS mediated the progression of hepatic OS. Successively, OS reduced the TAC (Fig. 4E) of the HS-stimulated rat liver, as evidenced by decreased SOD1 (Fig. 4F) and GSH (Fig. 4G) levels. Interestingly, CWP treatment exhibited antioxidant effects superior to those of NAC treatment. Importantly, CWP combined with NAC completely restored hepatic ROS production; MDA, SOD1, and GSH levels; and NOX activity, and enhanced hepatic TAC in HS-treated rats compared with that in the control group. These results suggest that CWP has powerful antioxidant activity.

CWP activates the Nrf2/HO-1 signaling pathway in the liver of HS-treated rats

Nrf2 is an important factor in maintaining intracellular redox homeostasis, and HO-1, which is known to be regulated by it, plays a dual role in cellular OS and inflammation (Audrey and Diana 2016; Cuadrado et al. 2014). The results showed that the expression of Nrf2 in rat liver after HS was decreased compared with that in the control group, which was verified by IHC (Fig. 5A, B) and western blotting (Fig. 5C, D). Furthermore, HO-1 expression was reduced by HS (Fig. 5E, F). Interestingly, CWP not only reversed the reduction of Nrf2

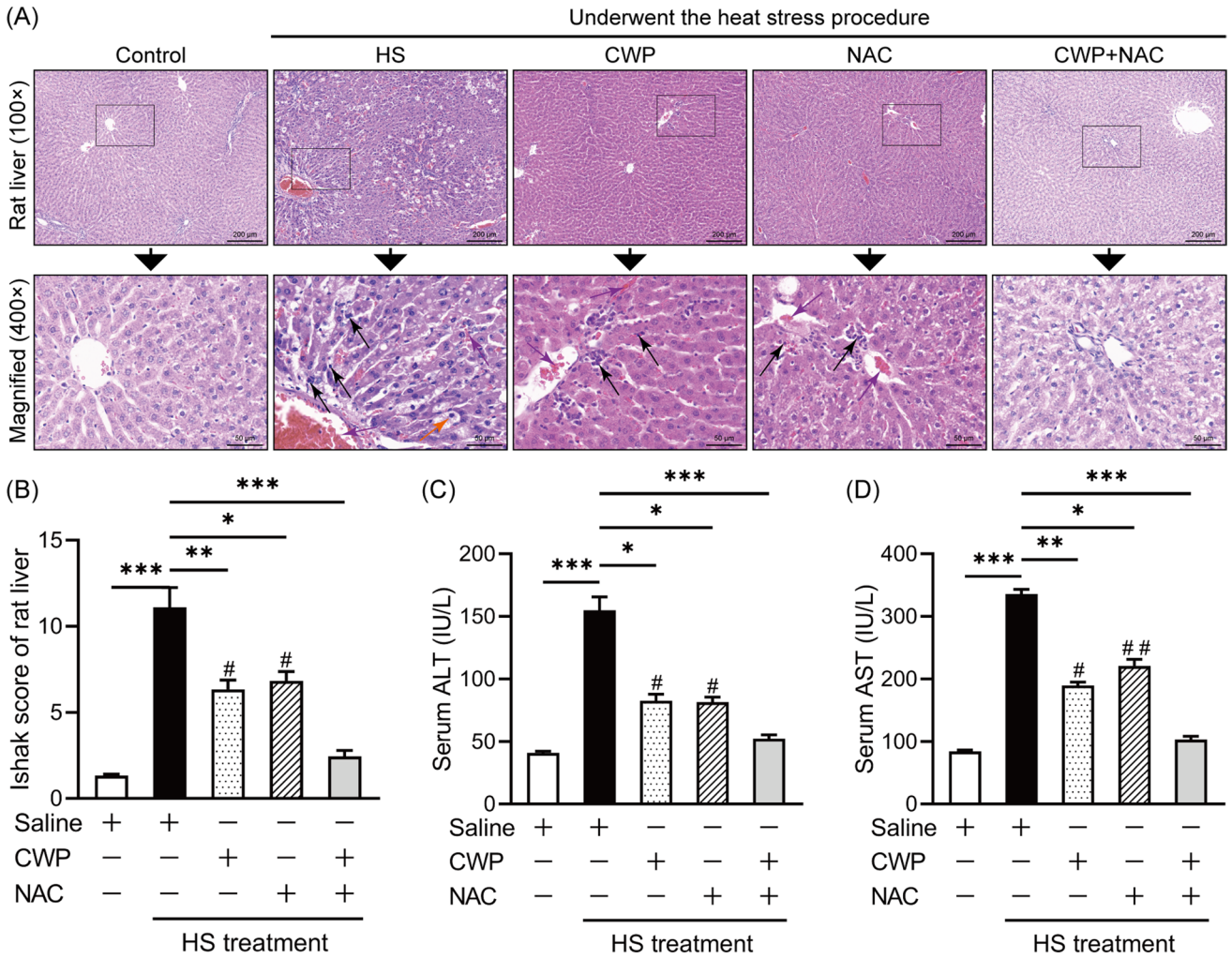

Fig. 3 CWP alleviated HS-induced liver damage, which may be attributed to its antioxidant activity. Consistent effects of CWP and the antioxidant NAC were confirmed by in vivo tests, which alleviated hepatic histopathological changes (**A**) and reduced histopathological scores (**B**) and serum ALT (**C**) and AST (**D**) activities in HS rats. *Representative significance is based on the comparison with the HS group, whereas # representative significance is based on the comparison with the control group. Data are presented as the mean of at least six determinations ± SEM. * or # $p < 0.05$, ** or ## $p < 0.01$, and *** or ### $p < 0.001$

expression in HS-treated livers compared that in the NAC group, but also promoted nuclear translocation (green arrows) and IHC scores of Nrf2 (Fig. 5B) and markedly enhanced HO-1 expression. Importantly, CWP combined with NAC significantly enhanced nuclear translocation, IHC scores, and expression of Nrf2 and HO-1 compared with that in the control group. These results indicate that CWP increased hepatic Nrf2 transcriptional activity in HS-treated rats and suggest that CWP in combination with other antioxidants is more likely to contribute to this effect.

CWP inhibits ROS production and the cytoplasmic translocation of HMGB1 in the liver of HS-treated rats via activation of the Nrf2/HO-1 axis

To investigate the role of Nrf2 and HO-1 in inhibiting HMGB1 release induced by HS in rat livers, we i.p. injected rats with ML385 (a specific Nrf2 inhibitor) or ZnPP (a competitive HO-1 inhibitor) prior to HS treatment. The results showed that ML385 and ZnPP reversed the beneficial effects of CWP on HS-induced liver damage

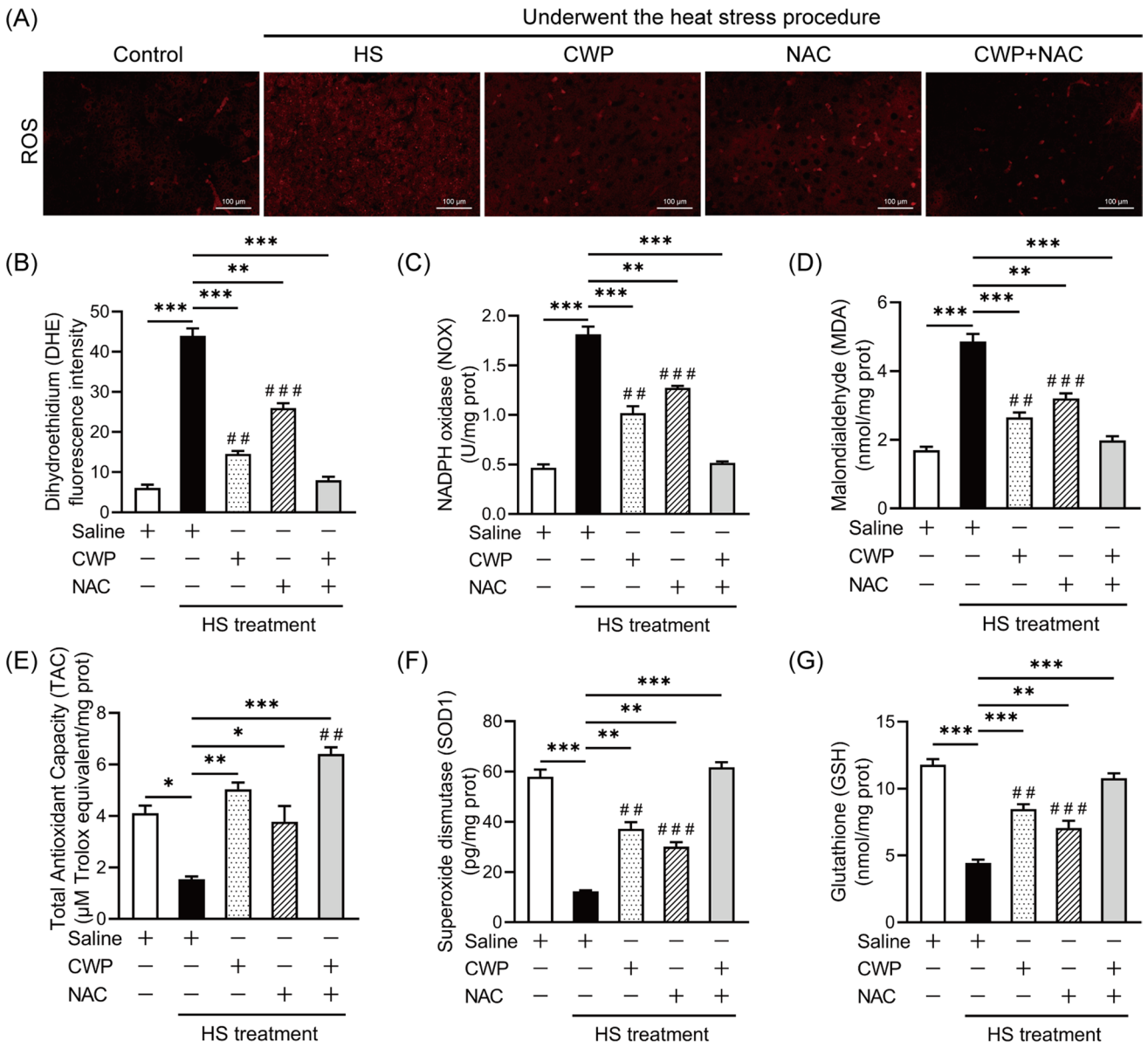

Fig. 4 CWP alleviated HS-induced hepatic oxidative stress in rats. **A** Effect of CWP on the production of ROS in HS rat liver was detected using a fluorescent probe DHE. **B** The fluorescence intensity was evaluated by using ImageJ software. **C** Activity of NOX in rat liver. **D** Content of MDA in rat liver. **E** The TAC of liver was assessed by ABTS method. **F** SOD1 content of liver tissues. **G** GSH levels. *Representative significance is based on the comparison with the HS group, whereas # representative significance is based on the comparison with the control group. Data are presented as the mean of at least six determinations ± SEM. * or # $p < 0.05$, ** or ## $p < 0.01$, and *** or ### $p < 0.001$

in rats, manifesting as worsened histopathological changes (Fig. 6A, B). Moreover, the inhibitory effects of CWP on ROS production in HS-stimulated livers were also reversed by ML385 and ZnPP (Fig. 6C, D). In HS-treated rat livers, ML385 and ZnPP antagonized the inhibition of HMGB1 release by CWP, manifesting as an increase in HMGB1 expression in the cytoplasm (Fig. 6E, F). Furthermore, ML385 and ZnPP abolished the alleviating effects of CWP on HMGB1 mediated inflammatory responses, as manifested by increased levels of IL-1β (Fig. 6G) and TNF-α (Fig. 6H). Overall, these results indicate that the Nrf2/HO-1 signaling pathway plays a critical role in the antioxidant effects of CWP, including the inhibition of HMGB1 release from the nucleus of hepatocytes under HS conditions.

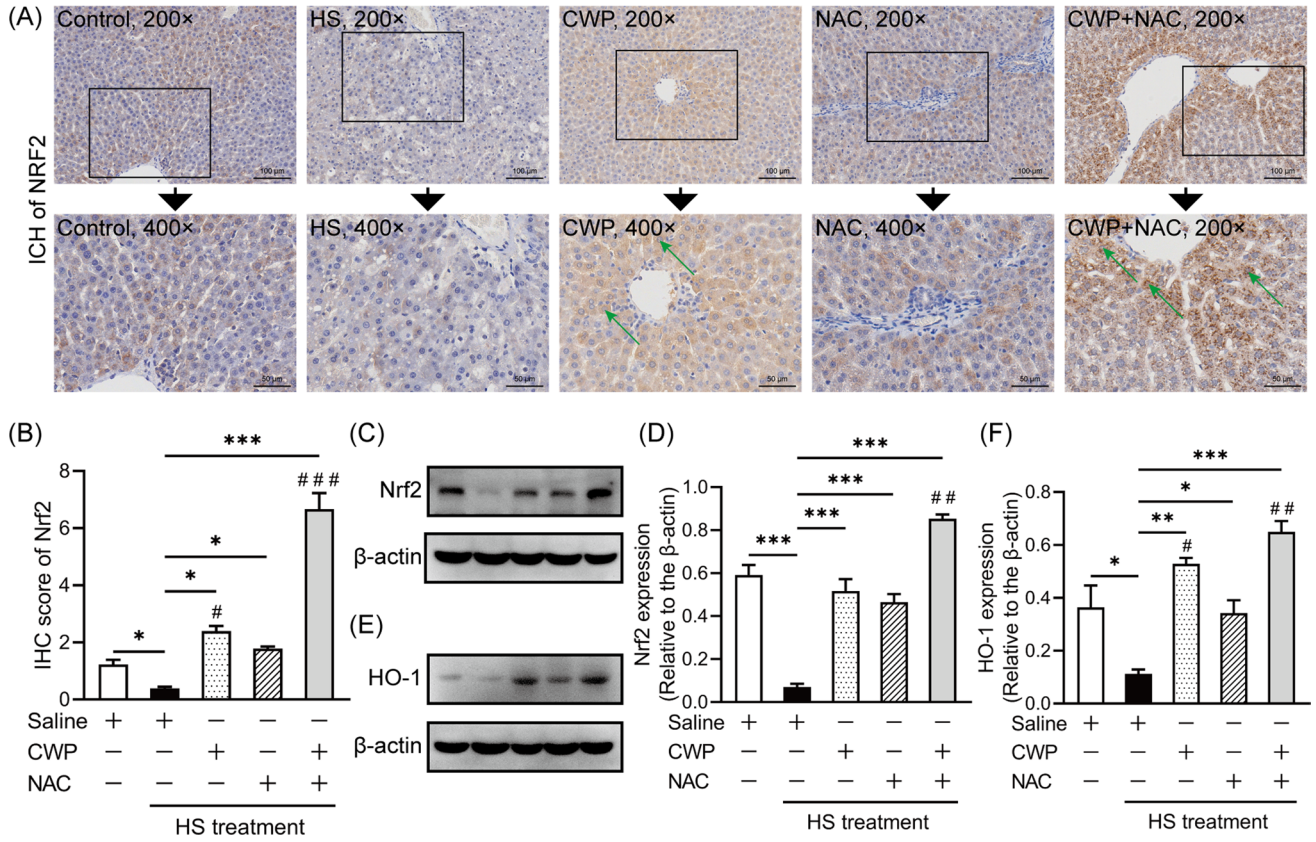

Fig. 5 CWP activated the Nrf2/HO-1 signaling pathway in HS rats. **A** Representative figures of IHC results of Nrf2. Green arrows indicate enhanced Nrf2 staining intensity in the nucleus. **B** IHC scores of Nrf2. **C** The expression of total Nrf2 in hepatocytes was assessed using a western blotting assay. β-Actin was used as an internal reference protein (**D**). **E, F** The expression of HO-1 was investigated using a western blot assay. * Representative significance is based on the comparison with the HS group, whereas # representative significance is based on the comparison with the control group. Data are presented as the mean of at least three determinations ± SEM. * or # $p < 0.05$, ** or ## $p < 0.01$, and *** or ### $p < 0.001$

Discussion

HS is a dangerous environmental stimulus that causes various stress responses such as anorexia, increased heart rate, and even fainting during summer, which increases the morbidity and mortality of humans and animals (Badr et al. 2018b; Crandall and Wilson 2015; He et al. 2019; Song et al. 2018). Numerous studies have shown that liver damage and failure are the most prevalent complications causing death in patients with HS and animal models of HS (Geng et al. 2015; Hassanein et al. 1992; Kew et al. 1970; Weigand et al. 2007). Although the mechanism by which HS leads to liver damage has not been fully elucidated, reports have shown that it is involved in OS and inflammatory responses (Akbarian et al. 2016; Badr et al. 2018b; Geng et al. 2015). Recent studies have shown that HMGB1 translocates from the nucleus to the cytoplasm at an early stage in patients with HS (Tong et al. 2011) and animal models (Hagiwara et al. 2010a, 2010b; Tong et al. 2013), which is a critical step for HMGB1 release into the extracellular space and bloodstream (Bonaldi et al. 2003; Scaffidi et al. 2002). HMGB1 is a target for treating and preventing of HS-induced hepatic inflammatory damage (Geng et al. 2015). CWP derived from camel milk has been shown to exhibit anti-HS-induced lymphoid organ (Badr et al. 2018b; Ramadan et al. 2018) and testicular damage properties (Badr et al. 2018a). Our previous study showed that CWP alleviates HS-induced hepatocyte damage by inhibiting HMGB1 release (Du et al. 2021b). To our knowledge, this study is the first to report the inhibitory effects of CWP on HMGB1 release. In corroboration with these findings, the present study demonstrated that CWP alleviated liver injury in HS-treated rats by inhibiting HMGB1 release by activating the Nrf2/HO-1 signaling pathway.

There is evidence that OS appears to be a central regulatory factor in mediating HMGB1 translocation and secretion and subsequent inflammatory responses (Tang et al. 2011; Yu et al. 2015). OS is defined as an imbalance of the oxidative and antioxidant systems, which occurs when the production of ROS exceeds the body's antioxidant defence capacity,

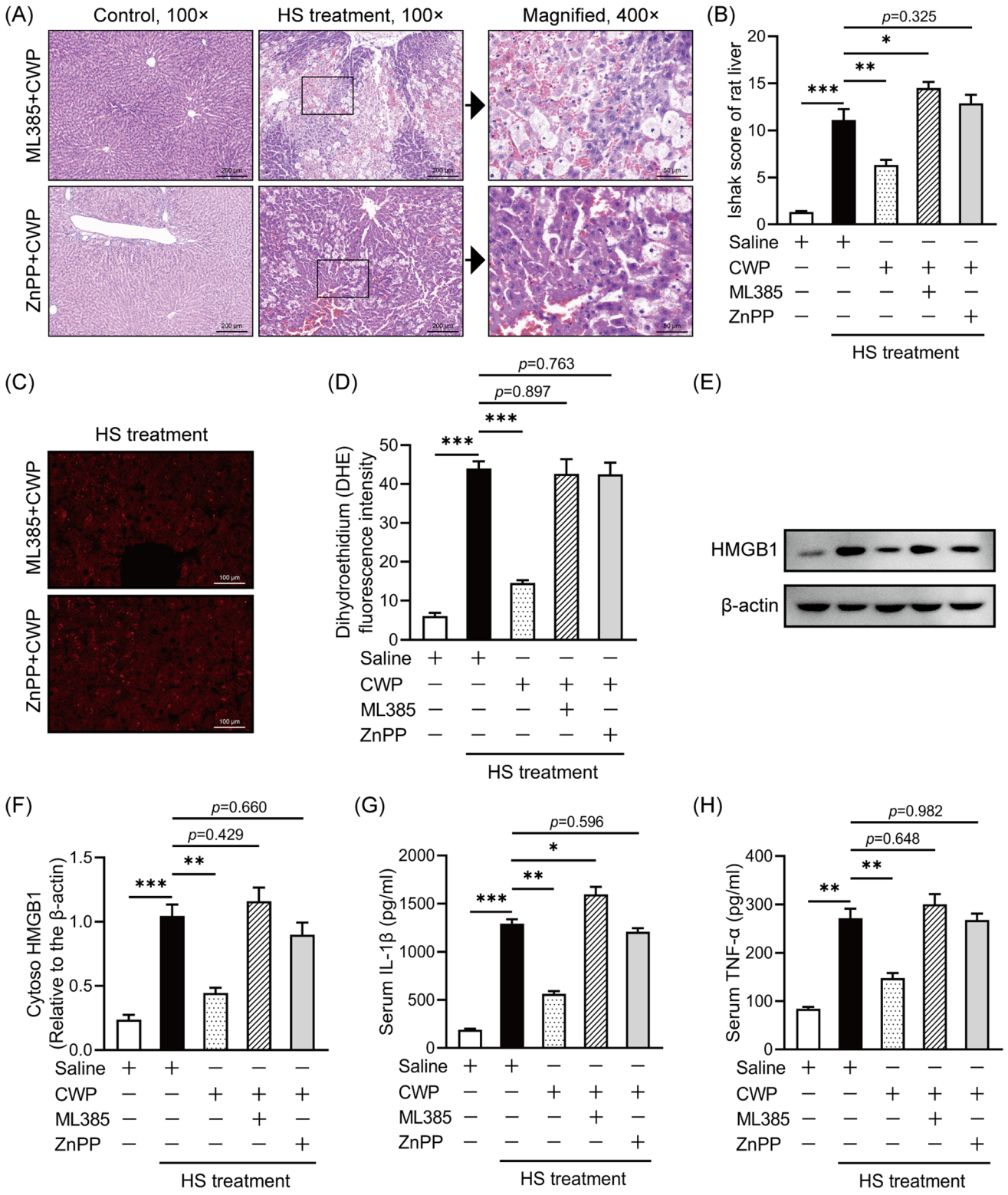

Fig. 6 CWP inhibited damage and the cytoplasmic translocation of HMGB1 in the liver of HS rats via activation of the Nrf2/HO-1 axis. ML385, an inhibitor of Nrf2, or ZnPP, an inhibitor of HO-1, reversed the inhibitory effects of CWP on histopathological changes (**A**), histological scores (**B**), ROS levels (**C**, **D**), cytoplasmic HMGB1 expression (**E**, **F**), and serum IL-1β (**G**) and TNF-α (**H**) contents in HS livers. Data are presented as the mean of at least three determinations ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$

and in severe cases, it can lead to oxidative damage and even cell death (Sies et al. 2017). A recent study showed that the administration of the ROS inhibitor NAC prior to acute HS treatment inhibited HMGB1 release in rat hepatocytes (Geng et al. 2015). The results were the same as those in the above study, and we further found that cytoplasmic translocation of HMGB1 in HS-treated rat hepatocytes was a ROS-dependent event. These results suggested that targeting HMGB1 with antioxidants is a feasible strategy to prevent or treat HS-induced liver damage. The potent antioxidant activity of CWP has been confirmed in our previous in vitro studies (Du et al. 2021b). The present study further confirmed that in an in vivo test CWP decreased ROS production by inhibiting NOX activity, which, in turn, decreased the level of the lipid peroxidation product MDA and increased the levels of the antioxidants SOD1 and GSH. Morover, we observed that CWP inhibited the cytoplasmic translocation of HMGB1 and alleviated the histopathological changes and liver failure in HS-treated rats. Interestingly, we observed that the anti-HS-induced hepatic OS, HMGB1 cytoplasmic translocation, inflammatory response, and damaging effects of CWP were superior to those of NAC. This is consistent with previous reports that NAC is unsatisfactory in treating OS and inflammatory diseases, which may be attributed to its effectiveness being limited to the early stage of the disease process, while the reactivity of ROS is faster by several orders of magnitude (Du et al. 2016; Wu et al. 2019). These results suggested that CWP consistently enhanced the antioxidant capacity of the rat livers subjected to HS. Importantly, CWP in combination with NAC completely reversed the abovementioned adverse effects induced by HS, indicating that the dietary addition of CWP has a favorable adjuvant therapeutic effect on HS-induced liver damage.

Overall, our results demonstrated that CWP increased the TAC of HS-treated rat livers. This may be attributed to its higher antioxidant amino acid content compared with that in bovine and other animal whey proteins (Badr et al. 2017a; Salami et al. 2010). Additionally, CWP may reduce OS and inflammatory responses by inducing the expression of GSH and antioxidant enzymes and stimulating the proliferative activation of immune cells, antibody production, cytokine secretion, and activity of phagocytes, granulocytes, and natural killer cells (Badr et al. 2018b). Most recent studies on CWP have focused on its unique antidiabetic effects, and CWP has been confirmed to regulate OS and inflammatory responses as an important factor in the treatment of diabetes (Badr et al. 2017a). In summary, the unique composition of CWP supports its therapeutic use in many OS diseases, but studies related to it are still scarce, limiting its clinical translation and application.

Next, we investigated the mechanism of action of CWP in OS. Nrf2 is a well-established core regulator of the antioxidant stress response, which translocates from the cytoplasm to the nucleus after activation and binds to antioxidant response elements, thereby initiating the expression of a set of cytoprotective enzymes, including HO-1 (Audrey and Diana 2016; Xu et al. 2018). HO-1, the rate-limiting enzyme involved in the heme catabolic process, has dual antioxidation and anti-inflammation effects (Xu et al. 2018). Our previous in vitro study demonstrated that CWP alleviates HS-induced OS, inflammation, and hepatocyte damage by activating Nrf2/HO-1 signaling (Du et al. 2021b). The present study further showed that CWP activated the Nrf2/HO-1 signalling axis in the livers of HS-treated rats, as evidenced by the nuclear translocation of Nrf2 and its increased expression. Interestingly, CWP exerted a superior upregulation effect on the Nrf2 signaling axis compared with NAC, which further demonstrated its excellent antioxidant activity. Additionally, previous studies have demonstrated crosstalk between OS and inflammation, and activation of the Nrf2 signalling pathway could suppress inflammatory diseases (Wu et al. 2019; Yu et al. 2015). We found that inhibition of Nrf2 or HO-1 abolished the beneficial effects of CWP on histopathological changes in the liver of HS-treated rat models and reversed the downregulating effects of CWP on ROS production and cytoplasmic HMGB1, IL-β, and TNF-α levels. This finding is consistent with previous studies showing that activating the Nrf2/HO-1 signaling pathway inhibits HMGB1 release in sepsis, ischemia, and reperfusion damage, thereby alleviating sterile inflammation-mediated tissue damage (Yu et al. 2015). In summary, the present study provides further evidence that the Nrf2/HO-1 signaling axis is an important mediator of the anti-inflammatory effects of CWP, including the inhibition of HMGB1 release from hepatocytes under HS conditions.

Conclusion

In conclusion, the present study suggests that the combined application of CWP and NAC is a preferable strategy for alleviating liver injury induced by HS. This mechanism was associated with activation of the Nrf2/HO-1 signaling pathway to enhance the antioxidant defense capacity of the liver, thereby inhibiting HMGB1-mediated inflammatory damage. Future studies should focus on the synergistic effects of CWP in novel combinations with other known antioxidants and observe whether CWP provides antioxidant benefits, and the bioactive peptides in CWP also need further identification.

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Author contributions Study concept and design: Donghua Du, Surong Hasi. Acquisition of data: Donghua Du, Wenting Lv, Xiaoxia Jing. Analysis and interpretation of data: Chunwei Yu, Jiya Wuen. Drafting

of the manuscript: Donghua Du, Wenting Lv, Xiaoxia Jing. Critical revision of the manuscript for important intellectual content: Surong Hasi. Administrative, technical, or material support, study supervision: Surong Hasi.

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Declarations

Conflict of interest The authors declare no competing interests.

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