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Effects of Shark Hepatic Stimulator Substance on the Function and Antioxidant Capacity of Liver Mitochondria in an Animal Model of Acute Liver Injury

Qiu-Ling FAN[1,2], Cai-Guo HUANG[2]*, Yan JIN[2], Bo FENG[2], Hui-Nan MIAO[2], Wen-Jie LI[2], Bing-Hua JIAO[2], and Qin-Sheng YUAN[1]*

[1] *State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai 200237, China;*
[2] *Department of Biochemistry and Molecular Biology, Second Military Medical University, Shanghai 200433, China*

Abstract This study was carried out to investigate whether shark hepatic stimulator substance (HSS) can prevent acute liver injury and affect mitochondrial function and antioxidant defenses in a rat model of thioacetamide (TAA)-induced liver injury. The acute liver injury was induced by two intraperitoneal injections of TAA (400 mg/kg) in a 24 h interval. In the TAA plus shark HSS group, rats were treated with shark HSS (80 mg/kg) 1 h prior to each TAA injection. In this group, serum liver enzyme activities were significantly lower than those in the TAA group. The mitochondrial respiratory control ratio was improved, and the mitochondrial respiratory enzyme activities were increased in the TAA plus shark HSS group. The mitochondrial antioxidant enzyme activities and glutathione level were higher in the TAA plus shark HSS group than in the TAA group. These results suggest that the protective effect of shark HSS against TAA-induced acute liver injury may be a result of the restoration of the mitochondrial respiratory function and antioxidant defenses and decreased oxygen stress.

Key words shark hepatic stimulator substance; acute liver injury; mitochondrion; respiratory function; antioxidant capacity

There is ample growing evidence suggesting that the altered mitochondrial function and morphology are linked to liver diseases. The hepatic mitochondrial function is impaired in rats with CCl_4-induced cirrhosis as a result of the reduced mitochondrial volume and impaired metabolism of the remaining mitochondria [1]. Mitochondria extracted from the rat liver 24 h after treatment with CCl_4 were shown to have lost their respiratory control ability [2].

Energy required for all cellular processes is supplied by mitochondria through the process of oxidative phosphorylation, and reactive oxygen species (ROS) are formed as by-products [3]. The mitochondrion is a major source of ROS within eukaryotic cells [4]. ROS can react with cellular components, especially membrane lipids, and lead to cell damage [5]. In a normal liver, the level of ROS is low, and antioxidant defenses are adequate to protect the liver from oxidative damage [6]. However, this delicate balance can be broken easily, leading to cellular dysfunction [7,8]. Mitochondrial dysfunction is accompanied by the increase in the release of ROS [9,10], the oxidative alteration of membrane proteins and lipids [11] and the decrease in ATP production [8].

Hepatic stimulator substance (HSS) is a heat-stable, alcohol-precipitable extract, which was first extracted from the cytosol of regenerating adult rat livers and normal livers of weanling rats. HSS is a progression factor for replication of hepatocytes *in vivo* and *in vitro* [12–14]. In comparison with other growth factors, HSS is organ-specific, but not species-specific [15,16]. It has been found to be able to stimulate liver regeneration when injected intraperitoneally into partially (34%)

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*Corresponding authors:
Qin-Sheng YUAN: Tel, 86-21-64252255; Fax, 86-21-64252255; E-mail, qsyuan@ecust.edu.cn
Cai-Guo HUANG: Tel, 86-21-25071457; Fax, 86-21-65334344; E-mail, huangcaig@hotmail.com

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hepatectomized rats [13,17,18]. HSS plays an important role in the regenerative process triggered by acute hepatic injury or partial hepatectomy. Recently, HSS has been shown to protect the liver from failure induced by chemical poisons or drugs, such as CCl_4, *D*-galactosamine, cadmium, acetaminophen, thioacetamide (TAA) or ethanol [12,19–27]. Acute administration of TAA has been reported to cause hepatic centrilobular necrosis, triggering a regenerative process [28,29], while chronic administration of TAA may induce biliary carcinoma and liver cirrhosis in rats [30]. The administration of HSS enhances the hepatocyte proliferative capacity, induced by TAA treatment, and is dependent on the duration of its administration [25]. Compared with normal livers, cirrhotic livers have diminished oxidative phosphorylation capabilities caused by changes in nicotinamide adenine dinucleotide-reduced (NADH) and $FADH_2$-linked respiration as well as impaired antioxidant defenses following partial hepatectomy [3]. Shark HSS protects hepatocytes from acetaminophen-induced acute hepatic injury [21]. As liver regeneration requires a lot of energy and control of oxidative stress, we postulated that the administration of shark HSS could also influence liver mitochondrial function and antioxidant capacity.

In the present study, we investigated the effects of shark HSS on the function and antioxidant capacity of liver mitochondria in an animal model of acute liver injury.

Materials and Methods

Materials

Sucrose, *D*-mannitol and Tris-(hydroxymethyl)aminomethane were obtained from Amresco (Boise, USA). NADH, cytochrome c and rotenone were purchased from Sigma (St. Louis, USA). The ATPase assay kit, glutathione (GSH) assay kit, superoxide dismutase assay kit, glutathione peroxidase assay kit, glutathione reductase assay kit, and malondialdehyde assay kit were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Other chemicals were purchased from China National Medicine Group Shanghai Chemical Reagent Company (Shanghai, China).

Animals and treatment

Male Sprague-Dawley (SD) rats were obtained from Fudan University (Shanghai, China). The animals, weighing 180–220 g each, were fed ad libitum and kept at constant room temperature (22–25 ℃) with 12 h of light exposure (8:00–20:00) and 12 h of darkness (20:00–8:00). All animals received humane care in compliance with the International Guiding Principles for Animal Research. All procedures were performed under diethylether anesthesia between 7:00 and 10:00, and the animals were fasted for 12 h before the procedures. The animals were grouped as following: (1) control group, intraperitoneal administration of 0.9% sodium chloride; (2) TAA group, two intraperitoneal injections of TAA (400 mg/kg body weight) in a 24 h interval; (3) TAA plus shark HSS group, two intraperitoneal injections of shark HSS (80 mg/kg body weight) in a 24 h interval followed by a TAA intraperitoneal injection (400 mg/kg body weight) 1 h after each injection of shark HSS; and (4) shark HSS group, intraperitoneal injection of one dose of shark HSS. The animals in each group were killed at 0 h, 12 h, 24 h, 36 h or 48 h after the final injection. Immediately after exsanguination, each liver was removed, cleaned and weighed. A small portion of each liver was then immersed in buffered Formalin solution for histological examination, and the remaining portion was immediately used for mitochondria preparation.

Determination of serum enzyme activities

Serum enzyme activities of aspartic aminotransferase (AST) and alanine aminotransferase (ALT) were assayed by an automated analysis system (Hitachi 7600-020; Hitachi, Japan).

Preparation of liver mitochondria

The livers were quickly removed, chopped into small pieces and placed in ice-cold isolation buffer for mitochondria (10 mM Tris-HCl, pH 7.4, 250 mM sucrose, 0.5 mM EDTA and 0.5% bovine serum albumin). After being homogenized, the homogenate was centrifuged at 750 *g* for 10 min. Next, 2 ml of the supernatant was stored at –80 ℃ and used for determination of hepatic glutathione. The remainder was centrifuged at 10,000 *g* for 10 min. The mitochondrial pellet was washed twice with isolation buffer, and then resuspended in the same buffer solution. The freshly prepared mitochondria were used to determine the respiratory control ratio (RCR), ADP/O ratio and mitochondrial membrane potential. The submitochondrial particles were prepared by freezing and thawing the mitochondrial suspension.

Preparation of shark HSS

Shark HSS was prepared from the livers of immature sharks according to the procedures described by LaBrecque *et al.* [31,32] and Fleig *et al.* [13]. In brief, the livers were homogenized in ice-cold 0.9% sodium chloride at 35:100 (*W*/*V*) using an electric homogenizer. The homogenate

was incubated at 65 °C in a water bath for 15 min, and the insoluble material was removed by centrifugation at 27,000 *g* and 4 °C for 20 min. Six volumes of ice-cold 40% ethanol were added to the supernatant and stirred at 4 °C for 2 h. After centrifugation at 27,000 *g* and 4 °C for 20 min, the precipitate was dissolved in water. The insoluble material was precipitated by centrifugation at 27,000 *g* and 4 °C for 20 min, and the supernatant was collected, lyophilized and stored at –80 °C. The frozen shark HSS was used within 15 d of preparation.

Determination of the respiratory control and ADP/O ratios

The RCR and ADP/O ratio were determined polarographically using a Clark oxygen electrode (Hansatech, Pentney, UK) [33]. A mitochondrial suspension containing 2.5 mg of protein was added to 2.5 ml of reaction buffer containing 225 mM mannitol, 75 mM sucrose, 10 mM Tris-HCl, and 5 mM Tris-H_3PO_4. Oxygen consumption was measured in the absence (giving the state 4 activity) and presence (giving the state 3 activity) of 0.25 mM ADP. Glutamate (10 mM) and malate (10 mM) were used as the substrate for site I (NADH-linked respiration), and 20 mM succinate for site II ($FADH_2$-linked respiration). The RCR was expressed as the ratio of state 3 to state 4 respiration, while the ADP/O ratio was expressed as the ratio of ADP to oxygen atoms consumed during state 3 respiration.

Enzyme assay of mitochondrial electron transport system

The activities of NADH-cytochrome c reductase (NCCR), succinate-cytochrome c reductase (SCCR) and cytochrome c oxidase (CCO) were measured using the method described by Yang *et al*. [3]. For the determination of NCCR activity, 5 mg of submitochondrial particles was added to 1 ml of buffer containing 25 mM potassium phosphate (pH 7.4), 2.5 mg/ml bovine serum albumin, 5 mM $MgCl_2$, 10 mM ferricytochrome c and 2 mM KCN. The reaction was started by the addition of 25 mM NADH and followed by reduction of ferricytochrome c, and recorded at 25 °C at 550 nm for 3 min. For the determination of SCCR activity, 5 mg of submitochondrial particles was added to 1 ml of 50 mM potassium phosphate (pH 7.4), 20 mM succinate, 10 mM ferricytochrome c, 2 mg/ml rotenone and 2 mM KCN at room temperature. The change in absorbance at 550 nm was recorded at 25 °C for 5 min. For the determination of CCO activity, 5 mg of submitochondrial particles was added to 1 ml of 50 mM potassium phosphate (pH 7.4) and 10 mM ferricytochrome c. The change in absorbance at 550 nm was recorded at 25 °C for 5 min. The extinction coefficient of cytochrome c was 19 per mM·cm. Mitochondrial ATPase activity was assessed using an assay kit according to the manufacturer's instructions.

Mitochondrial antioxidant defenses

The activities of mitochondrial superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GRd), and hepatic and mitochondrial GSH levels were determined using an assay kit according to the manufacturer's instructions.

Measurement of lipid peroxidation products

The concentrations of malondialdehyde (MDA) in the liver and mitochondria, measured spectrophotometrically at 532 nm, were used to quantify the mitochondrial and homogenate lipid peroxidation (LPO) products using an assay kit according to the manufacturer's instructions.

Statistical analysis

The results were given in mean±SE. All observations were made on at least eight animals, and the assays were performed twice. One-way analysis of variance and the unpaired Student's *t*-test were used for the statistical analysis of the results. $P<0.05$ was considered to be significant.

Results

Serum enzyme activities

Serum AST and ALT were used as indices of TAA-induced hepatotoxicity. Both AST and ALT activities were dramatically increased at all stages of the second TAA administration, and peaked at 24 h in the TAA group compared with the control group ($P<0.05$). The administration of TAA plus shark HSS significantly reduced serum AST and ALT activities compared with the TAA group at 12 h to 48 h ($P<0.05$) (**Fig. 1**). No differences were observed between the AST and ALT activities of the shark HSS and control groups.

Mitochondrial substrate oxidation

Decreased mitochondrial oxidation of both glutamate-malate and succinate in the presence of ADP (state 3) was observed at all stages examined after the second TAA administration, with significant changes in the ADP/O ratio at all stages for glutamate-malate and only at 24 h for succinate. These effects led to weakened mitochondrial control ratios [**Fig. 2(B–D)**] compared with the control

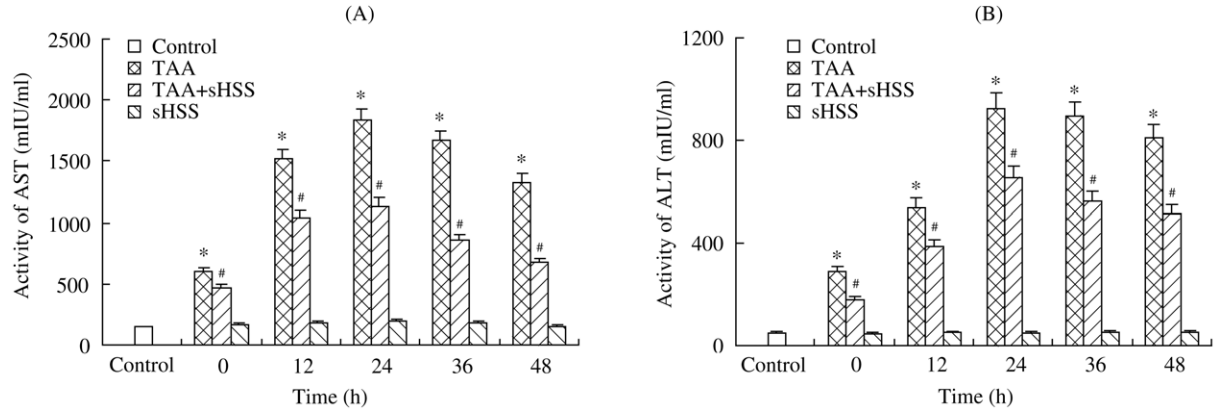

Fig. 1 Effects of TAA and shark HSS on serum AST and ALT

(A) Serum AST. (B) Serum ALT. The results are presented as mean±SE (n=8). *P<0.05 compared with control group; #P<0.05 compared with TAA group.

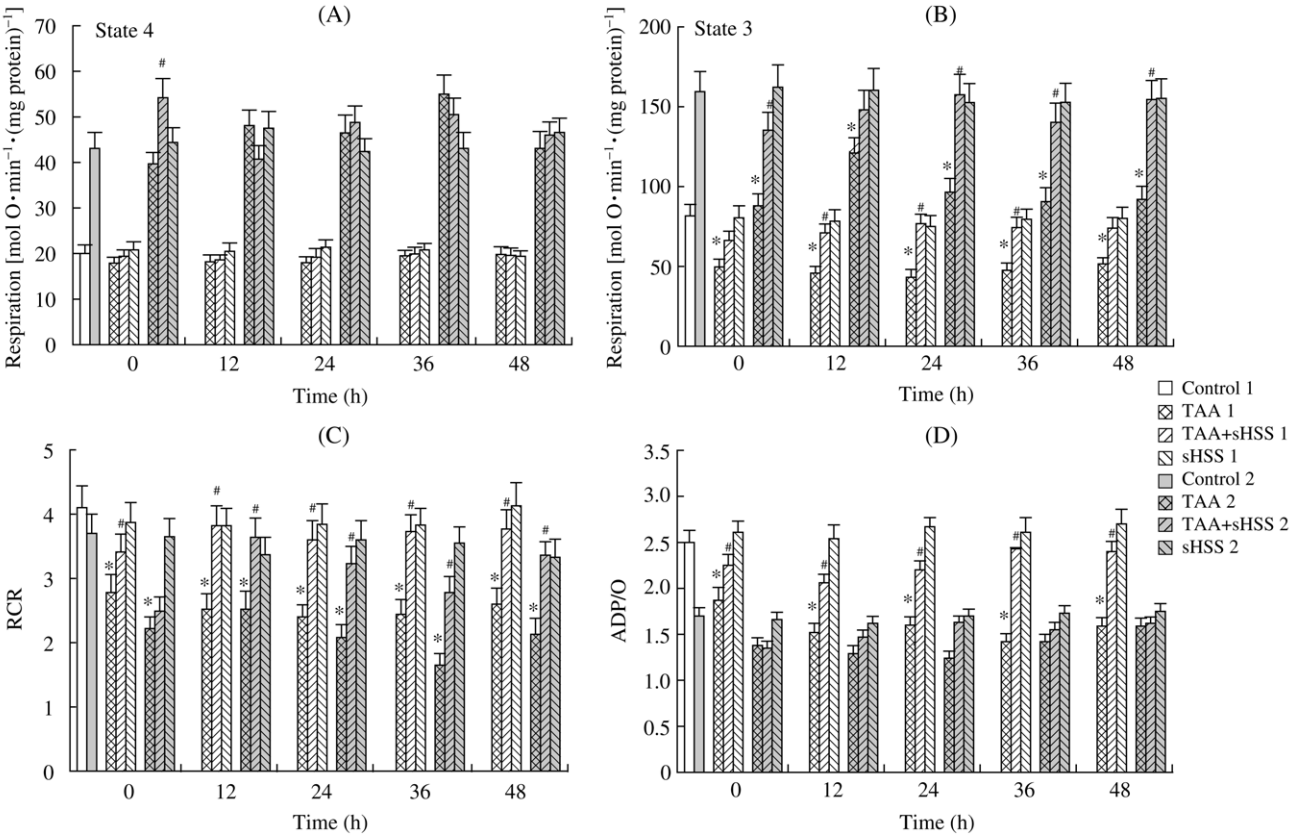

Fig. 2 Effects of TAA and shark HSS on mitochondrial oxidation of site I and site II substrates

(A) State 4 respiration. (B) State 3 respiration. (C) RCR. (D) ADP/O ratio. The results are presented as mean±SE (n=8). The state 4 respiration, state 3 respiration, RCR and ADP/O ratio were measured using glutamate-malate (site I) and succinate (site II) as the substrates. The RCR was derived from the following equation: RCR=state 3/state 4. *P<0.05 compared with control group; #P<0.05 compared with TAA group. 1, site I; 2, site II.

group (P<0.05). The administration of shark HSS elicited an increase in oxidation of ADP (state 3) at 12 h to 36 h for glutamate-malate, and at 0 h and 24 h to 48 h for succinate, resulting in an increased RCR at all stages for glutamate-malate and at 12 h to 48 h for substrates [**Fig. 2(C)**] (P<0.05).

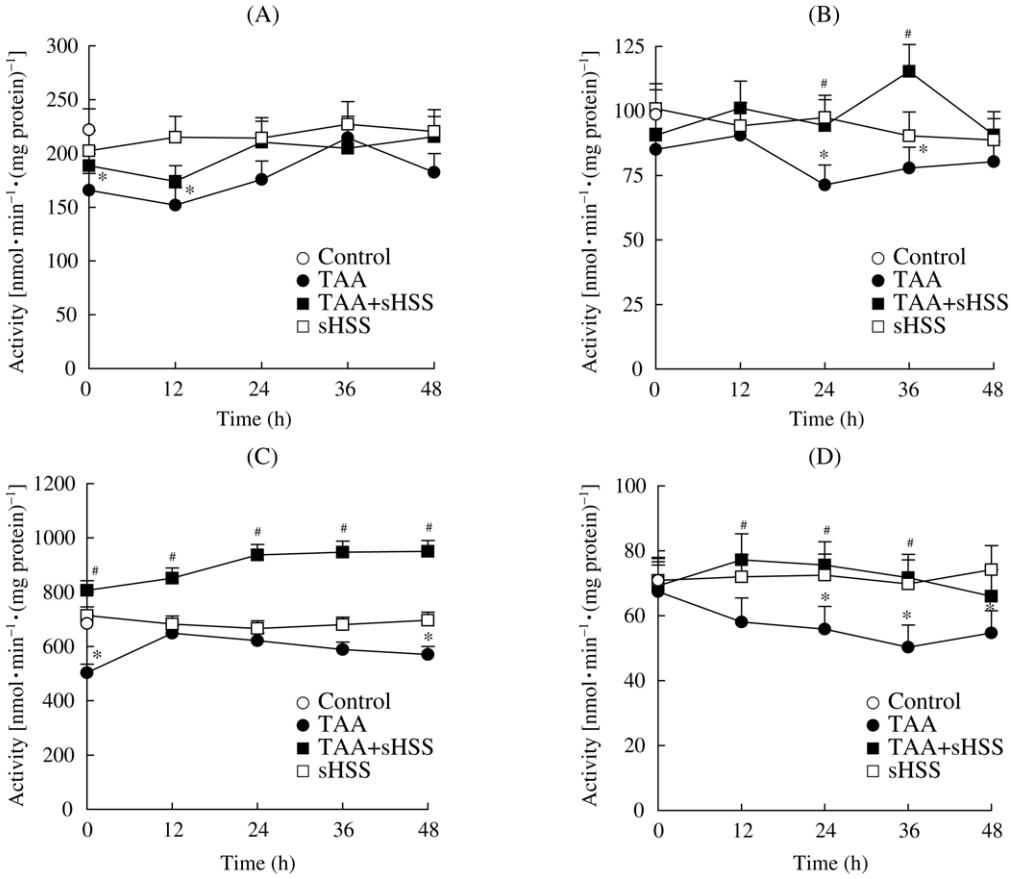

Fig. 3 Effects of TAA and shark HSS on NCCR, SCCR, CCO and ATPase activities

(A) NCCR activities. (B) SCCR activities. (C) CCO activities. (D) ATPase activities. The results are presented as mean±SE (n=8). *P<0.05 compared with control group; #P<0.05 compared with TAA group.

Enzyme activities of the mitochondrial electron transport system

Mitochondria isolated from the TAA group showed significant decreases in NCCR activity (only at 12 h), SCCR activity (at 24 h to 36 h), CCO activity (at 0 h and 48 h) and ATPase activity (at 12 h to 48 h) compared with the control group (**Fig. 3**) (P<0.05). Significant enhancement of SCCR activity (at 24 h and 36 h), CCO activity (at all stages) and ATPase activity (at 12 h to 36 h) was observed in mitochondria from the TAA plus shark HSS group compared with those from the TAA group (**Fig. 3**) (P<0.05).

Mitochondrial antioxidant capacity

Within the mitochondria, the activities of antioxidant enzymes, including SOD, GPx and GRd, as well as mitochondrial GSH levels were assayed. Mitochondrial GRd activities at all stages examined were significantly reduced in the TAA group compared to the control group [**Fig. 4(B)**] (P<0.05). However, mitochondrial GPx and SOD activities in the TAA group had no significant difference from those in the control group for the duration of the study [**Fig. 4(A, C)**]. But the administration of shark HSS prior to the second dose of TAA enhanced mitochondrial GPx activity (all stages), GRd activity (at 12 h to 48 h) and SOD activity (at 0 h and 24 h to 48 h) compared with the corresponding activities in the TAA group (**Fig. 4**) (P<0.05). In all TAA-treated rats, the total hepatic GSH level was not significantly different from that of the control rats, although the mitochondrial GSH level was significantly lower at all stages examined. The hepatic GSH level in the TAA plus shark HSS group at 0 h to 12 h and the mitochondrial GSH level at 12 h and 36 h to 48 h were enhanced markedly, compared with those from the TAA group (**Fig. 5**) (P<0.05).

Lipid peroxidation

Administration of TAA induced an increase in MDA level in the liver at all time points examined and in mitochondria at 0 h and 48 h in TAA group compared with control group. In TAA plus sHSS group, hepatic MDA levels at all time

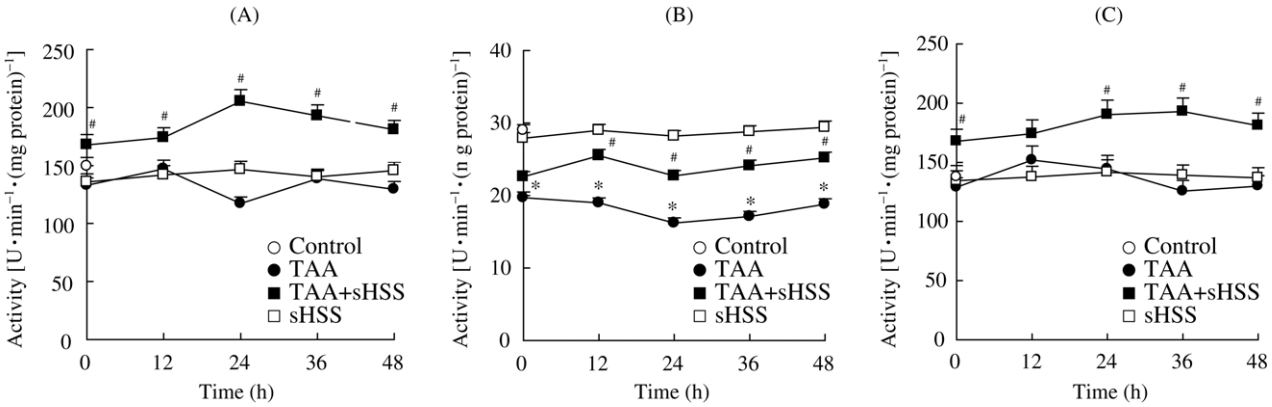

Fig. 4 Effects of TAA and crude shark HSS on the activities of mitochondrial GPx, GRd and SOD in the liver

(A) Mitochondrial glutathione peroxidase (GPx). (B) Glutathione reductase (GRd). (C) Glutathione superoxide dismutase (SOD). The results are presented as mean±SE (n=8). Submitochondrial particles were used to determine the mitochondrial GSH level as well as GPx, GRd and SOD activities. *P<0.05 compared with control group; #P<0.05 compared with TAA group.

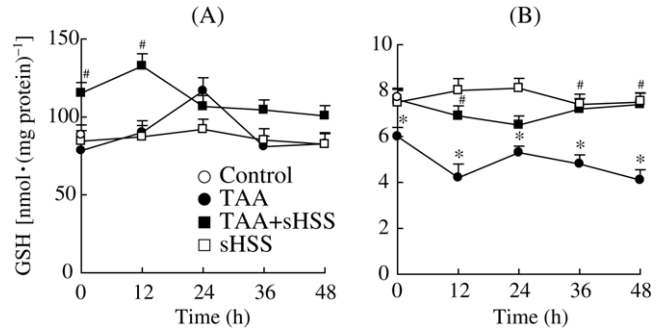

Fig. 5 Effects of TAA and crude shark HSS on hepatic and mitochondrial GSH levels

(A) Hepatic GSH level. (B) Mitochondrial GSH level. The results are presented as mean±SE (n=8). Liver homogenates were used to determine the hepatic GSH level. Submitochondrial particles were used to determine the mitochondrial GSH level. *P<0.05 compared with control group; #P<0.05 compared with TAA group.

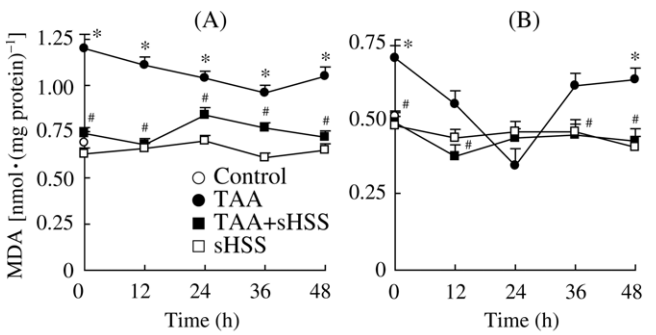

Fig. 6 Effects of TAA and shark HSS on hepatic (A) and mitochondrial (B) MDA levels

(A) Hepatic MDA level. (B) Mitochondrial MDA level. The results are presented as mean±SE (n=8). Liver homogenates were used to determine the hepatic thiobarbituric acid (TBARS) level. *P<0.05 compared with control group; #P<0.05 compared with TAA group.

points examined and mitochondrial MDA levels at 0 h to 12 h and 36 h to 48 h were significantly lower compared with TAA group (**Fig. 6**) (P<0.05).

Discussion

The present results show that shark HSS can protect the liver from TAA-induced acute injury. Administration of TAA induces important alterations in the respiratory function and antioxidant capacity of liver mitochondria, suggesting that shark HSS can partially prevent acute injury to the liver in TAA-treated rats by stimulating some mitochondrial pathways in liver to increase the energy charge and by maintaining a normal mitochondrial function in the animals administered with hepatotoxin.

After the second TAA treatment, a significant decrease in substrate oxidation was induced in the mitochondria, especially when respiration was stimulated by the addition of ADP [**Fig. 2(B)**]. This means that the oxidative phosphorylating capacity of mitochondria was damaged, as shown by the low RCR [**Fig. 2(C)**] and the diminished rate of ATP synthesis [**Fig. 2(B,D)**]. It is known that the mitochondrial respiration ratio is dependent on the availability of ADP in the assay medium [34]. The respiration ratio is very low in the absence of ADP, but it increases several times with the addition of an appropriate amount of ADP. The mitochondria from the TAA plus shark

HSS group had a significantly higher O_2 consumption rate (state 3 respiration) and RCR compared with those from the TAA group. Previous report [35] had shown that HSS isolated from rats can increase mitochondrial respiratory activity, and that the protective effect induced by HSS is correlated with restoration of mitochondrial respiratory activity induced by CCl_4. These results are consistent with our findings. Energy required for all cellular processes is supplied by mitochondria through the process of oxidative phosphorylation, and a series of enzyme complexes are involved in this process that is coupled with oxidation of the electron donors. After TAA treatment, the NCCR and SCCR activities were reduced, suggesting that NADH-linked and $FADH_2$-linked oxidations may be impaired, and these coincided with a significantly lower substrate oxidation of both sites I and II [**Fig. 2(B)**]. These results show that TAA-induced damage to liver mitochondria is not site-specific. Therefore, the decrease in NAD^+-linked and succinate-supported respiration may be the result of impairment to the activities of the respiratory chain enzymes. Shark HSS administration induced a significant increase in respiratory enzyme activities compared with that in the TAA group, suggesting that the stimulatory effect of shark HSS on the oxidative phosphorylation system might be mediated by an activation or induction of different enzyme complexes involved in this process. These results comply with the fact that electron flow through the respiratory chain also regulates the oxidative phosphorylation process, specifically the irreversible reaction catalyzed by cytochrome c oxidase [36].

The mitochondrial respiratory chain is one of the major sources of detrimental free radicals in the human body [37]. Free-radical production comes from the reaction of mitochondrial electron carriers, such as ubiquinol, with oxygen to form superoxides [38]. Mitochondria have their own antioxidant defenses to protect against oxidative damage, including Mn-SOD to dismutate superoxides into hydrogen peroxide, GPx to detoxify peroxides and GRd to reduce oxidized glutathione to maintain the mitochondrial GSH pool in a reduced state [39]. MDA has been used as the primary indicator of oxidative stress. Other indicators, such as Cu,Zn-SOD and Mn-SOD, have been used as secondary markers of oxidative stress and correlate significantly with MDA [40]. The mitochondrial GSH level in the livers of rats treated with TAA was found to be significantly lower than that of rats in the control group, and a decrease in mitochondrial GSH level has been associated with impairment of GRd activity [41]. The mitochondrial GSH level and the activities of mitochondrial GPx, GRd and SOD in the TAA plus shark HSS group were significantly higher than those in the TAA group, indicating that shark HSS can improve mitochondrial antioxidant defenses. Lipid peroxidation is the result of an imbalance between oxidants and antioxidants or an impairment in the transport of GSH from cytosol to mitochondria. An increase in oxygen free radicals has been shown to occur during liver regeneration after partial hepatectomy [42]. In the mitochondria isolated from TAA-treated rats, the increase in ROS may contribute to the increase of mitochondria MDA level. Lipid peroxidation occurred in the whole liver of rats treated with TAA, as reported previously [43–45]. Administration of shark HSS could induce a decrease in hepatic MDA level and suppress hepatic lipid peroxidation (**Fig. 6**).

The present study has shown that acute liver injury induced by TAA is a result of an impairment to the mitochondrial respiratory function and antioxidant capacity and hepatic lipid peroxidation, which can be partially prevented by the administration of shark HSS. This suggests that shark HSS may be used for the treatment of clinical liver disease.

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Edited by
Ming-Hua XU