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Original Research Communication

Selenium status in diet affects acetaminophen-induced hepatotoxicity via interruption of redox environment

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Abstract

Aims: Drug-induced liver injury, especially acetaminophen-induced liver injury, is a leading cause of liver failure worldwide. Mouse models were used to evaluate the effect of microelement selenium levels on the cellular redox environment and consequent hepatotoxicity of acetaminophen (APAP).

Results: APAP treatment affected mouse liver selenoprotein thioredoxin reductase (TrxR) activity and glutathione level in a dose- and time-dependent manner. Decrease of mouse liver TrxR activity and glutathione level was an early event, and occurred concurrently with liver damage. The decreases of ratio of GSH/GSSG, TrxR activity and the increase of protein *S*-glutathionylation were correlated with the APAP-induced hepatotoxicity. Moreover, in APAP-treated mice both mild deprivation or excess supplementation with selenium increased the severity of liver injury compared to that observed in mice with normal dietary selenium levels. An increase in the oxidation state of the TrxR-mediated system, including cytosolic thioredoxin1 (Trx1) and peroxiredoxin1/2, and mitochondrial Trx2 and Prx3, were found in the livers from mice reared on selenium-deficient and excess selenium-supplemented diets upon APAP-treatment.

Innovation: This work demonstrates that both Trx and GSH systems are susceptible to APAP toxicity *in vivo*, and that the thiol-dependent redox environment is a key factor in determining the extent of APAP-induced hepatotoxicity. Dietary selenium and selenoproteins play critical roles in protecting mice against APAP overdose.

Conclusion: APAP treatment in mice interrupts the function of the Trx and GSH system which are the main enzymatic antioxidant systems, in both cytosol and mitochondria. Dietary selenium deficiency and excess supplementation both increase the risk of APAP-induced hepatotoxicity.

Introduction

Drug-induced liver injury has proved to be a leading cause of market withdrawal of approved medicines (27). Acetaminophen (APAP) is used worldwide for the treatment of fever and pain but it is responsible for a large percentage of acute liver damage seen in patients (26). The hepatotoxicity of APAP was firstly described several decades ago and was recognized as a cause of acute liver damage in the 1980s (26). In 1989, liver injury was defined as occurring when serum alanine aminotransferase (ALT) levels were more than twice the upper limit of normal levels (36).

The mechanism of APAP-induced liver damage is indicated from the depletion of glutathione (GSH) levels and results from modification of mitochondrial proteins by the electrophilic APAP metabolite *N*-acetyl-*p*-benzoquinone imine (NAPQI), resulting in dose-dependent hepatotoxicity (21,28,52). At therapeutic doses, most APAP is excreted via conjugation with glucuronic acid or sulfuric acid, with the remainder metabolized by cytochrome P450 enzymes to produce the toxic NAPQI which reacts with reduced glutathione (GSH) by 1,4-conjugate addition to produce the GS-NAPQI adduct (43,47). Intentional or unintentional APAP overdose results in the formation of a larger amount of NAPQI, with consequent depletion of GSH and reaction with mitochondrial proteins (2,43). Mitochondrial dysfunction and liver damage occur as a consequence of these changes. A recent study showed that the methylation-controlled J protein (MCJ), a negative regulator of mitochondria, can be targeted during APAP-induced liver injury based on the observation that MCJ silencing could protect mice from APAP overdose-mediated liver damage (4). It has also been reported that APAP exposure can deplete GSH levels in mitochondria and promote reactive oxygen species (ROS) generation which activates the translocation of c-Jun N-terminal kinase (JNK) into mitochondria, thereby affecting mitochondrial function (14,20,41). In addition, injection of exogenous mitochondria into APAP-treated mice alleviates the associated liver injury (53). The above evidence indicates the pivotal role for mitochondria in APAP-induced liver injury.

In addition to intentional and unintentional APAP overdose, some other factors contribute to APAP toxicity including fasting, obesity, alcohol use, malnutrition, and even

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genetic variability. The involvement of alcohol in hepatotoxicity of APAP has been controversial for a long time. A weak association of APAP-induced hepatotoxicity with a low dose (4-10 g/d) alcohol intake was found in 1994 (61), while later studies revealed that chronic alcoholics were susceptible to therapeutic dose of APAP; simultaneous ingestion of APAP and alcohol attenuated hepatotoxicity as alcohol competes as a substrate for the primary metabolic enzyme CYP2E1, thereby reducing conversion of APAP into NAPQI (27,46). APAP-induced liver damage is routinely treated by administering the FDA-approved *N*-acetylcysteine, but this treatment is only effective in the early period following APAP poisoning (9,16,60).

The essential trace element selenium has been documented to be beneficial for life for a long time. The physiological significance of selenium has been primarily related to its presence in selenoproteins in the form of selenocysteine (Sec, Se-Cys, U) (30,39). In addition, the potent anticancer activity of selenium has been linked to the interaction of selenium compounds with selenoproteins (11,12,18). Twenty five selenoprotein genes have been identified in human cells and Sec is present in selenoproteins including thioredoxin reductases (TrxRs), glutathione peroxidases (GPxs) and iodothyronine deiodinases (DIOs) (24,30,39). TrxRs and GPxs are critical members of the thioredoxin (Trx) and glutathione (GSH) systems, respectively. Thioredoxin and GSH systems are two major thiol/selenol dependent antioxidant system with overlapping function. They are also involved in *in vivo* redox equilibrium regulation, protecting the cells from oxidative stress (8,32,51,58,59). Decreased TrxR (17,55) and GPx (48) activity were found in selenium-deficient animal model. The replacement of Sec with cysteine in the C-terminus of TrxR occurs in selenium-deficient rats, resulting in reduced catalytic activity (33). Pretreatment with a single dose of selenite via intraperitoneal injection exhibited protective effects against APAP-induced hepatotoxicity in the rat three decades ago (50), which may be due to the pro-oxidant effects of selenite to cause the increase of glutathione level. However, the regulation of TrxR activity by dietary selenium, and consequently the roles of TrxR-mediated pathways in APAP-induced hepatotoxicity have not been well investigated.

Since Sec has higher nucleophilicity than cysteine (Cys), it is expected to be highly reactive towards electrophilic agents including APAP metabolite NAPQI (22,37). Indeed,

additional studies provide proof of the association between APAP-induced hepatotoxicity and the inhibition of cytosolic TrxR1 and mitochondrial TrxR2 activities (40). However, upregulated TrxR1 and GSH level induced by nuclear factor-E2-related factor 2 (Nrf2) activation may protect the liver from the APAP damage (19). Therefore, the changes of the redox environment regulated by Trx systems upon the treatment with APAP are not well understood. In this study, we investigate the role of the thiol-redox pathways regulated by Trx and GSH systems in APAP-induced liver injury. Particularly, we studied the effects of the selenium deficiency or excess on APAP-caused liver damage and the resulting changes in the related cellular redox environment.

Results

Effects of treatment with APAP on GSH level and TrxR activity in mouse liver

APAP overdose can induce acute liver damage and a dose-dependent hepatotoxicity of APAP has been indicated in previous studies (25,44). Here, mice were exposed to 100, 300, or 500 mg/kg APAP and survival was measured for the following 3 days. Death only occurred in the first 24 h following APAP exposure in a dose-dependent manner, with 1 mouse dying in the 300 mg/kg APAP group and 4 mice dying in the 500 mg/kg APAP group (Fig. 1A). We subsequently treated mice with various APAP doses (100, 300, 500 mg/kg; 10 mice in each group) and investigated APAP toxicity after 1h, 6 h and 24 h. The results showed that APAP toxicity occurred during 24 h following APAP treatment, consistent with the above. In the group treated with 500 mg/kg APAP, 6 out of 10 mice died, whilst no mice died in the other groups. We analyzed serum alanine transaminase (ALT) activity, liver TrxR activity GSH levels in the surviving mice. There was no significant change in ALT activity in surviving mice between groups 1 h and 6 h after APAP treatment. In contrast, a dramatic elevation in ALT activity was observed after 24 h in the groups treated with 300 and 500 mg/kg APAP (Fig. 1B).

APAP treatment affected GSH levels and TrxR activities in mouse liver extracts in a dose- and time-dependent manner. Treatment with all APAP doses caused a rapid decrease in GSH level and TrxR activity at 1h post treatment (Fig. 1C and D), with GSH level showing significantly recovery at 6 and 24 h post-treatment. It is notable that both TrxR activity and

GSH levels were significantly enhanced compared to controls at 24 h post-treatment in the 100 mg/kg group, but not in the 300 and 500 mg/kg groups (Fig. 1C and D). Liver GSH levels had returned to normal levels after 6 h at all doses and were also at normal levels in the 300 and 500 mg/kg groups at 24 h, with the enhanced levels noted above in the 100 mg/kg group (Fig. 1C). Relative TrxR activity was significantly reduced in the 300 and 500 mg/kg groups after 6 hours and TrxR activity was still significantly reduced in the 500 mg/kg group compared to saline controls after 24 h, showing long-lived effects at higher APAP doses (Fig. 1D). We therefore speculated that treatment of mice with low APAP doses activates a compensatory mechanism in the liver resulting in increased TrxR activity and GSH levels at 24 h post-treatment. In dead mice, treatment with 500 mg/kg APAP caused a dramatic decrease in both TrxR activity and GSH levels after 24 h.

Although total GSH levels in all groups recovered after 24 h, the ratio of GSH/GSSG was lower in the livers of mice treated with 300 and 500 mg/kg APAP compared to the control group treated with saline (Fig. 2A), indicating higher oxidative stress levels in these two groups. Under oxidative stress conditions, glutathionylation/deglutathionylation of proteins catalyzed by glutaredoxin (Grx) is a marker of cellular redox environment (57). Therefore, we investigated the protein glutathionylation level in liver samples and found a dose-dependent increase 24 h after APAP exposure (Fig. 2B), indicating an increase of cellular oxidative environment. We also analyzed cytosolic and mitochondrial Trx protein expression in the livers of mice treated with APAP after 24 h. There was no significant difference in the levels of Trx2, TrxR2, Prx1 and Prx2 between these groups but a significant upregulation of TrxR1 expression was observed in the 300 mg/kg ($p = <0.01$) and 500 mg/kg ($p = <0.05$) groups and significant ($p = <0.05$) decrease in Trx1 and Prx3 expression was observed in the 500 mg/kg APAP group (Fig. S1).

Effects of selenium status on APAP-induced liver injury

The above results showed that APAP treatment caused a dramatic change in TrxR activity and GSH levels. Since selenoproteins TrxRs and GPxs are the major components in the Trx and GSH antioxidant systems, an understanding is required of the roles of the Trx and GSH systems in mediating the redox environment in APAP-induced liver injury.

Consequently, the effects of selenium status on APAP-induced liver injury were investigated. Mild selenium-deficient (SD) and selenium-enriched (SE) mouse models were established by feeding the weanling mice with mild selenium-deficient or -enriched diets for twelve weeks. There was no significant difference in the selenium levels in serum between groups (Fig. 3A), but liver selenium level in mice fed with a mild selenium-deficient diet was significantly (p = < 0.05) lower than that in mice fed with selenium-normal (SN) or selenium-enriched (SE) diets (Fig. 3B). Subsequently, mice fed with these diets were treated with 300 mg/kg APAP. This resulted in a significant increase in AST and ALT levels in all the groups (Fig. S2). Compared to the group fed with a diet containing normal selenium levels, the two groups of mice fed with selenium- deficient and -enriched diets showed higher sensitivity to APAP at 24 h after treatment, as revealed by histological analysis and survival rates (Fig. 4). Treatment with 300 mg/kg APAP did not cause death in mice fed with a selenium-normal diet (Fig. 4A). In contrast, feeding mice with a selenium-deficient or selenium-enriched diet predisposed them to increased liver damage. Three and one mice died upon the treatment with 300 mg/kg APAP in the selenium-deficient and enriched groups, respectively. Liver damage levels were assessed by HE staining and semi-quantified using scores on pathological sections. The scores range from 0 to 4 with increasing values indicating higher levels of liver damage. Results revealed that mildly selenium-deficient mice were the most sensitive to APAP-induced liver injury (Table S1) and displayed disappearing hepatic sinusoids and disorganized hepatocytes upon histological analysis. Excess selenium in the diet did not result in protection of the liver from damage caused by APAP treatment, but instead enhanced liver damage (Fig. 4A and B).

Correlation of TrxR activity with mice susceptibility to APAP

To further explore the relationship between dietary selenium, TrxR activity and APAP-induced liver damage, we measured TrxR activity and GSH levels in the livers of surviving mice. Changes in liver TrxR activity appeared to be dependent on the selenium dose when exposed to APAP. Selenium deficiency decreased liver TrxR activity (Fig. 5A) in proportion with the decrease in the amount of selenium within the liver (Fig. 3), but had little effect on GSH levels in whole cells (Fig. 5B). Interestingly, mitochondrial TrxR activity did not

differ amongst the three groups (Fig. 5C). At 24 h post-treatment with APAP, total TrxR activity had decreased significantly both in selenium-deficient and selenium-enriched groups, which was significantly different from the result obtained for the selenium-normal group. Furthermore, compared to the selenium-deficient group, TrxR activity and GSH levels in selenium-normal and selenium-rich mice were higher (Fig. 5A and B), which correlated with the extent of liver damage as detected by HE staining (Fig. 4B). Surprisingly, treatment with APAP increased mitochondrial GSH amount in selenium-deficient and selenium-enriched groups, thus making it higher than in the selenium-normal group. However, mitochondrial TrxR activity was only decreased in selenium-enriched mice following APAP treatment. This result is consistent with a model in which the reduction of overall TrxR activity (Fig. 1C) can be primarily attributed to the decrease in cytosolic TrxR1 activity. TrxR1 and TrxR2 display differences in substrate- and inhibitor-specificity although both selenoenzymes are generally thought to be similar in structure and properties (42). Indeed, cytosolic TrxR1 has reacts more rapidly with NAPQI than mitochondrial TrxR2 as judged by the observation that maximum inhibition of TrxR1 activity occurred after 1 h whilst maximum inhibition of TrxR2 activity occurred after 6 h following APAP exposure (22).

Elevated oxidation of cytosolic and mitochondrial Trx system in both selenium-deficient and selenium-enriched mice

The cytosolic Trx system is involved in ROS scavenging by transferring electrons to peroxiredoxin (Prx1/2). Therefore, decreased TrxR activity could cause ROS accumulation in selenium-deficient mice, consequently increasing susceptibility to oxidative stress. To test this hypothesis, we detected the expression levels of various enzymes by Western Blotting (Fig. 6). Results showed no significant change in Trx1, TrxR1 or Prx1 levels between the three groups with or without APAP treatment. In contrast, Prx2 protein levels were significantly increased in selenium-deficient group following APAP challenge (Fig. 6).

Furthermore, the redox states of Trx1and Prx1/2 were investigated by redox Western blotting following the fixing of protein free thiols in tissue lysates with iodoacetamide (IAM) (Fig. 7). In non-reducing gels, thiols in the sulfhydral form of these redoxins (Trx1,

Prx1/2) are alkylated by IAM, and hence proteins appear as monomers in lower molecular weight positions. When the redoxins are oxidized, they form disulfides with other proteins or themselves, thus appearing at higher molecular weight positions or as fuzzy bands. Addition of DTT to samples before analysis converts the disulfide form of these proteins into the monomeric sulfhydral forms. Compared to the group fed with a selenium-normal diet, selenium-deficiency and -enriched groups did not have a dramatic change in the redox status of mice liver redoxins, indicating that the majority of upstream TrxR1 was working correctly and the cellular redox environment was well-controlled. Treatment with APAP resulted in oxidation of redoxins in all groups. Compared to the group fed with normal selenium diet, selenium-deficient and -enriched groups had more oxidized redoxins, indicating that the Trx-mediated redox environment was more severely disrupted in these groups, particularly in the selenium-deficient group (Fig. 7). This was consistent with the liver histology results, which showed that APAP induced the severest liver damage in selenium-deficient mice.

Accumulating evidence demonstrated the correlation between APAP-induced hepatotoxicity and the reaction of toxic metabolite NAPQI with mitochondrial proteins causing mitochondria dysfunction (44,49). Thus, we investigated the expression of TrxR2, Trx2, and Prx3 which are primarily present in mitochondria. There were no significant changes in the levels of these proteins between these three groups (Fig. 8). Furthermore, we examined their redox states following the treatment with APAP. Treatment with APAP caused oxidation of Trx2 and Prx3 in all groups. Compared with the selenium normal group, more Trx2 and Prx3 were oxidized in the SD and SE groups (Fig. 9), particularly in the selenium-deficient (SD) group. This result was similar to the observation made on cytosolic proteins (Fig. 6 and 7). The above results demonstrated that APAP treatment affects redox status of Trx system in both the cytosol and mitochondria. Selenium-deficiency or excess supplementation did not induce a dramatic change in the redox environment, but disrupted the redox environment upon APAP treatment.

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Discussion

Selenium functioning as a component of selenoproteins plays a critical role in human health and disease (6,38,39). Thus its deficiency can contribute to the progression of various diseases, notably Keshan disease which is prevalent in selenium-deficient regions in China. In addition, selenium-deficiency can sensitize cells to oxidative stress (6,38), an effect that is also induced by APAP toxicity. It is reasonable to postulate decreased antioxidant capacity in selenium-deficient mice based on the fact that selenium deficiency could lead to decreased activity of some selenoproteins, such as the important antioxidant enzymes TrxRs and GPxs. Interestingly, selenium deficiency caused decreased TrxR activity, while selenium excess did not result in TrxR activity elevation. Selenium supplementation increased TrxR activity compared to low amounts, but plateaued at normal levels. Thus, it is reasonable that we did not detect increased TrxR activity in the livers of mice fed on a selenium-enriched diet. Conversely, a more severe liver injury occurred in SE mice compared selenium-normal mice. Selenium compounds, typically sodium selenite (5,13), at a high concentration have been used to kill tumor cells. Selenite is reduced by NADPH and TrxR in mammals to produce hydrogen selenide which has high reactivity with oxygen resulting in the generation of ROS. Selenomethionine (SeMet) is the major selenium component of the diet in this study. It is metabolized in a different way but ultimately produces metabolite hydrogen selenide, with consequent increases in oxidative stress (29). Increased ROS levels in SE mice without APAP treatment was indicated by the higher basal oxidation of Prx1 and Prx3 compared to SN mice. Consequently, SE mice showed an increased sensitivity to APAP-induced liver injury.

Many factors including fasting, alcohol and malnutrition *etc.* may affect APAP-induced liver injury, but the effects of dietary selenium status on APAP toxicity have been rarely studied (54). This study demonstrates that both selenium deficiency and excess aggravated APAP-induced hepatotoxicity via amplifying the oxidative stress induced by APAP, as evidenced by the higher oxidation status of redoxins in selenium-deficient and selenium-enriched mice. The redox state of proteins in the cultured cells has been well-studied and has been shown to be an important factor in determine enzyme activity and the biological response. For example, the redox state of Trx1 is a marker of the cellular

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redox environment and determines cell death or survival, with increased Trx1 oxidation resulting in more cell death (31). However, the redox state of proteins in the tissues is rarely investigated. In this study we examined the redox status of redoxins in liver tissue using redox Western Blotting by detecting the intermolecular disulfides by fixing protein free thiols with IAM. The possibility that selenium deficiency induced loss of antioxidant enzymes (GPx and TrxR) activity, contributing to a decrease of Trx system-mediated antioxidant pathways activity, was investigated. Thus the higher sensitivity of selenium-deficient mice to APAP exposure explains the different responses of mice to APAP challenge. Although non-selenoprotein antioxidant enzymes such as heme oxygenase-1 (HO-1) and NAD(P)H quinone oxidoreductase (NQOR) are upregulated in selenium-deficient mice by activation of nuclear factor-E2-related factor 2 (Nrf2), this oxidation state is maintained at a high level which makes the liver of mice raised on SE diets more likely to reach the toxic threshold when exposed to the same dose of APAP (7).

In this study, we found that selenium status affected hepatotoxicity induced by APAP and selenium-deficient mice showed the most severe liver damage, as judged by histological analysis. However, serum transaminases activity in selenium-deficient and selenium-enriched group did not increase significantly compared to the selenium-normal group. This inconsistency may be explained by the results of Jan *et al*. (22). In the early stages of APAP challenge, TrxR1 and TrxR2 activity are efficiently inhibited with return to normal levels or above by 24 h post-treatment. Serum transaminase activity kept increasing up to 18 h whilst at 24 h post-APAP treatment both AST and ALT activity had decreased, but histological analysis indicated increased liver injury at 24 h. Based on these results, we speculate that Nrf2 was activated to enhance antioxidant systems activity and decrease necrotic cell death in the latter stages of APAP-induced hepatotoxicity, but these compensating effects did not halt the progress of liver damage. In response to oxidative stress induced by exogenous stimulus, Nrf2 translocates from the cytosol to the nucleus to upregulate the transcription of redox-related genes such as *Txnrd1*, *Hmox1* and *Nqo1*. This adaptive activation of Nrf2 may protect cells from death and maintain cellular functions (15). Prx2 was previously shown to have anti-oxidative activity and scavenge ROS (62). A second study showed that Prx2 cooperated with Nrf2 to defend against oxidative stress,

which is of importance for cell survival (34). Therefore, it is not surprising that Prx2 expression largely upregulated in selenium-deficient mice when exposed to APAP.

Conclusion

Our results demonstrate that mild selenium-deficient mice are vulnerable to APAP-induced hepatotoxicity. Furthermore, oxidation of both the mitochondrial and cytosolic Trx systems are involved in this process. This indicates an overall increase in oxidative stress in the liver of mice fed on both selenium-deficient and selenium-enriched diets following exposure to APAP. Taken together, we conclude that selenium-deficiency lowered the activity of selenoenzyme-containing antioxidant systems, thus disrupting the intracellular redox balance. Decreased TrxR activity resulting from selenium deficiency was further exacerbated by APAP metabolite NAPQI, which contributed to a more extreme disruption of the redox environment.

Selenium exerts its beneficial effect or toxic effects largely depending on its dose (23). High or low selenium levels may decrease the capability of liver cells to defend against oxidative stress induced by exogenous stimuli. However, there is a rather limited dose range for the beneficial biological functions of selenium because of the narrow therapeutic window between selenium-deficiency and selenium overdose (10,56). This emphasized the importance of safe administration of selenium.

Innovation

This study indicates that selenium could be a major factor in affecting the extent of APAP-induced hepatotoxicity and thus provides a new insight into the mechanism by which both selenium deficiency and selenium overdose could enhance serious liver damage. In addition, this study emphasizes the importance of the overall Trx system oxidation state, comprising the mitochondrial and cytosolic Trx systems.

Materials and methods

Animals were obtained from Ensiwerer company (Chongqing, China) and RIPA lysis buffer was obtained from Beyotime Biotechnology (P0013B, Shanghai, China). Selenium-deficient (0.09 mg/kg), selenium-normal (0.2 mg/kg) and selenium-rich (1 mg/kg) diets

were manufactured by Medicience company (Jiangsu, China). Acetaminophen (APAP, 11969B) and iodoacetamide (IAM, 24054A) were purchased from Adamas-beta (Shanghai, China). Omni-PAGE gels were purchased from EpiZyme (Shanghai, China). All the antibodies used in this study were from proteintech (Hubei, China) except anti-Trx1, anti-TrxR1, anti-Trx2 and anti-TrxR2 antibodies which were from IMCO (Stockholm, Sweden). 5,5'-Dithiobis-(2-nitrobenzoic acid) (DTNB, BS038) and nicotinamide adenine dinucleotide phosphate (NADPH, MG025) were from Biosharp (Anhui, China). Glutathione reductase (GR, G8810) was purchased from Solarbio (Beijing, China). Aurothioglucose (ATG, 32672321) was purchased from Wako (Osaka, Japan). Sep-Pak C18 cartridges (WAT023590) were purchased from Waters (Massachusetts, USA). All other reagents were obtained from the Sigma-Aldrich Chemical Co. or Fisher Scientific Ltd and were used without purification.

Animal treatments

The mice experiments carried out in this study were approved by the local Animal Ethics Committee. To investigate the susceptibility of KM mice to APAP, mice were exposed to 100, 300 or 500 mg/kg APAP and survival recorded for 3 days. In order to explore the effects of dose and time on APAP-induced hepatotoxicity, mice fed with a normal selenium level diet were administered 100, 300 or 500 mg/kg APAP by intragastric injection, and sacrificed at either 1h, 6 h or 24 h post-administration for further analysis.

In the experiment to investigate the effects of dietary selenium status on APAP hepatotoxicity, 3-week old weanling KM mice were divided into three groups including mild selenium-deficient (SD), selenium-normal (SN) and selenium-enriched (SE) group (20 mice per group) and fed with enough water and diets with the different levels of selenium (SD: 0.09 mg/kg, SN:0.2 mg/kg, and SE: 1 mg/kg), with light for 12 h and dark for 12 h. In this study, mild selenium deficiency was used to mimic a more realistic situation. The food and water consumed were recorded per 2 days. After 3 months, 20 mice in each group fed with different selenium level diets were divided into 2 groups (10 mice per group) and treated with 300 mg/kg APAP or saline respectively, and sacrificed after 24 h. The serum was collected for aspartate aminotransferase (AST) and alanine aminotransferase (ALT)

activity detection, and liver tissue for enzyme activity measurements and Western Blot analysis.

Detection of serum ALT and AST activity

ALT and AST activity in serum were detected using automatic biochemistry analyzer (BIOBASE, BK400) following the standard procedure set by manufacturer after 10-fold dilution with saline. The activity values were recorded and calculated to get the activity before dilution.

Liver histology

Liver tissues were cut into several pieces, one was kept in 4% (w/v) paraformaldehyde for hematoxylin and eosin staining. Briefly, after dehydration, embedding, slicing and dewaxing, liver tissues were processed to nuclear staining with hematoxylin and cytosolic staining with eosin to observe liver cellular morphology.

GSH/GSSG detection

The ratio of GSH/GSSG in liver was detected using the method described by Calabrese et al.(1,35) with minor modifications. About 10 mg fresh liver tissue was lysed using an auto-homogenizer in 1 mL of solution containing 10 mM DTNB (dissolved in buffer 1 containing 100 mM PBS and 5 mM EDTA, pH 7.5) or 10 mM NEM (dissolved in buffer 2 containing 100 mM PBS and 5 mM EDTA, pH 6.8). After centrifugation at 12,000 g, 10 min at 4 ℃, the protein concentration of supernatant was measured by Bradford assay using BSA standard protein solution (2 mg/mL) as reference. For GSSG detection, the sample containing NEM was passed through the Sep-Pak C18 cartridge to remove NEM and eluted with buffer 1. The 50 μ L collected sample was added to a cuvette containing 50 µL mixture of 50 nmol DTNB and 0.1 U glutathione reductase in buffer 1. After 1 min incubation, 44 nmol NADPH in 100 µL buffer 1 was added to initiate the reaction. The change of absorbance at 412 nm was recorded using a microplate reader (Bio Tek, USA) for 5 min at 1 min intervals. After 50-fold dilution, the sample with DTNB was processed using the same procedure as for GSSG detection to measure the total GSH level. The GSSG and

total GSH amounts were calculated using a standard curve which was prepared according to the same procedure for sample detection.

Protein extraction for redox state detection

The method of redox state analysis of target protein was modified from the method to detect the protein redox state in the cultured cells (45). Briefly, 100 mg fresh liver tissue was rinsed with cold saline twice, then cut into small pieces and moved into 1ml radio immunoprecipitation assay (RIPA) lysis buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 mM IAM dissolved in 1 M Tris-HCl buffer (pH 8.3). Homogenization was processed using electric homogenizer (Servicebio, China) with the setting of 120 Hz for 2 minutes in pre-cold sample mould. Lysis was centrifugated at 4℃, 13,000 g for 10 min and supernatant was collected. Protein concentration was determined by BCA assay using BSA as reference and lysis was diluted to certain concentration with saline for redox state analysis. The samples for protein expression analysis were prepared using similar method but without IAM addition.

Mitochondria isolation

Mitochondria was isolated with classical gradient centrifugation method with small modification (63). Briefly, 100 mg liver tissue rinsed was cut into small pieces in plate and washed with cold saline twice, then removed to glass homogenizer. 1 ml mitochondria isolation buffer (containing 210 mM mannitol, 70 mM sucrose, 1 mM EDTA, 10 mM Hepes–NaOH, pH 7.5, and 1mM PMSF was added immediately before use) followed by homogenizing with 10 strokes to disrupt cells. The lysis was centrifuged at 4℃, 1000 g for 10 min to remove cell debris and supernatant was collected for further mitochondria isolation. Mitochondria was obtained by centrifuging at 13,000 g, 4℃ for 10 min and was subsequently resuspended in 200 μl lysis buffer. BCA assay was used to measure mitochondrial protein concentration, and mitochondrial GSH content and TrxR activity were detected.

TrxR activity detection

TrxR activities in liver whole cell and mitochondria extracts were detected using a modified DTNB assay (3). Briefly, 25 μg protein was incubated with reaction mixture containing 100 μM NADPH for 2 min at room temperature, then 300 nM ATG was added in reference wells to inhibit TrxR activity. After incubation for another 5 min at room temperature, the mixture of 100 μM NADPH and 2 mM DTNB was added to start the DTNB reduction reaction. The increase of absorbance at 412 nm was recorded by microplate reader (Bio Tek, USA) for 5 min at 51s-interval. Relative TrxR activity was represented by the increase of absorbance at 412 nm in 1 min and TrxR activity of sample was calculated by subtracting the value of reference wells from sample wells. TrxR activity of each sample was from the average of triplicate.

GSH amount detection

GSH contents in liver whole cell and mitochondrial extracts were measure with DTNB assay described above. 5 μg liver protein was incubated with reaction mixture containing 100 μM NADPH and 50 nM GR for 5 min at room temperature in sample wells, while the GR was absent in reference wells. After addition of mixture containing 100 μM NADPH and 2 mM DTNB, the increase of absorbance at 412 nm was immediately recorded by microplate reader for 5 min at 51s-interval. Relative GSH content was showed with slope which represented the increased value of absorbance at 412 nm in 1 min. Similarly, the value of reference wells was subtracted from sample wells. All samples were measured in triplicate.

Western blot analysis

Lysis buffer was supplemented with 10 mM IAM before homogenization for analysis the redox state of target proteins. Lysate was diluted with loading buffer without reductant and loaded onto 15% Omni-PAGE gel purchased from EpiZyme (Shanghai, China), then proteins were separated at 120 V for 60 min. After that, proteins were transferred to PVDF membrane in Tris-glycine buffer in 20% (v/v) methanol. For protein expression levels analysis, samples without IAM were processed to similar procedure but 50 mM DTT was added in loading buffer. After membrane blocking with 10% skim milk

solution for 2 h at room temperature, anti-Prx1 (1:1000 dilution), anti-Prx2 (1:1000 dilution), anti-Prx3 (1:5000 dilution), anti-Trx1 (1:1000 dilution), anti-TrxR1(1:1000 dilution), anti-Trx2 (1:1000 dilution), anti-TrxR2 (1:1000 dilution) and anti-GAPDH (1:10000 dilution) antibodies were incubated with membrane for 1 h at room temperature. Subsequent incubation with secondary antibody (Horseradish peroxidase-conjugated affinipure goat anti-rabbit IgG(H+L), 1:5000 dilution) for 2 h at room temperature was performed. Finally, chemiluminescence was measured using the appropriate apparatus (Clinx, China).

Data analysis

Data were shown as mean ± standard error of the mean. Data was firstly processed to outlier analysis using SPSS, then to column statistics and test for homogeneity of variance by GraphPad Prism 7.0 software. The difference among groups were analyzed by one-way ANOVA or two-way ANOVA, and the difference between two groups was analyzed by subsequent *post hoc* test. $P<0.05$ indicated the significant difference statistically.

Acknowledgments

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The authors would like to notice the passing away of our coauthor, colleague, and collaborator Arne Holmgren, a redox pioneer in research on thioredoxin and glutaredoxin. Arne will be greatly missed by all who had the privilege of working with him.

Author Disclosure Statement

No competing financial interests exist.

List of Abbreviations

ALT = alanine transaminase

ANOVA = analysis of variance

APAP = acetaminophen

AST = aspartate transaminase

ATG = Aurothioglucose

DIOs = iodothyronine deiodinases

DTNB = 5,5'-Dithiobis-(2-nitrobenzoic acid)

DTT = dithiothreitol

GPxs = glutathione peroxidases

Grx = glutaredoxin

GR = glutathione reductase

GSH = glutathione

HE = hematoxylin and eosin

HO-1 = heme oxygenase1

IAM = iodoacetamide

JNK = c-Jun N-terminal kinase

MCJ = methylation-controlled J protein

Msr = methionine sulfoxide reductases

NAPQI = *N*-acetyl-*p*-benzoquinone imine

NADPH = nicotinamide adenine dinucleotide phosphate

NAC = *N*-acetylcysteine

NEM = *N*- ethylmaleimide

NQOR = NAD(P)H quinone oxidoreductase

Nrf2 = nuclear factor-E2-related factor 2

Prx = peroxiredoxin

PMSF = phenylmethylsulfonyl fluoride

ROS = reactive oxygen species

Sec = selenocysteine

SD= selenium-deficient

SN = selenium-normal

SE = selenium-enriched

SeMet = selenomethionine

SDS = sodium dodecyl sulfate

TrxR = thioredoxin reductase

Trx = thioredoxin

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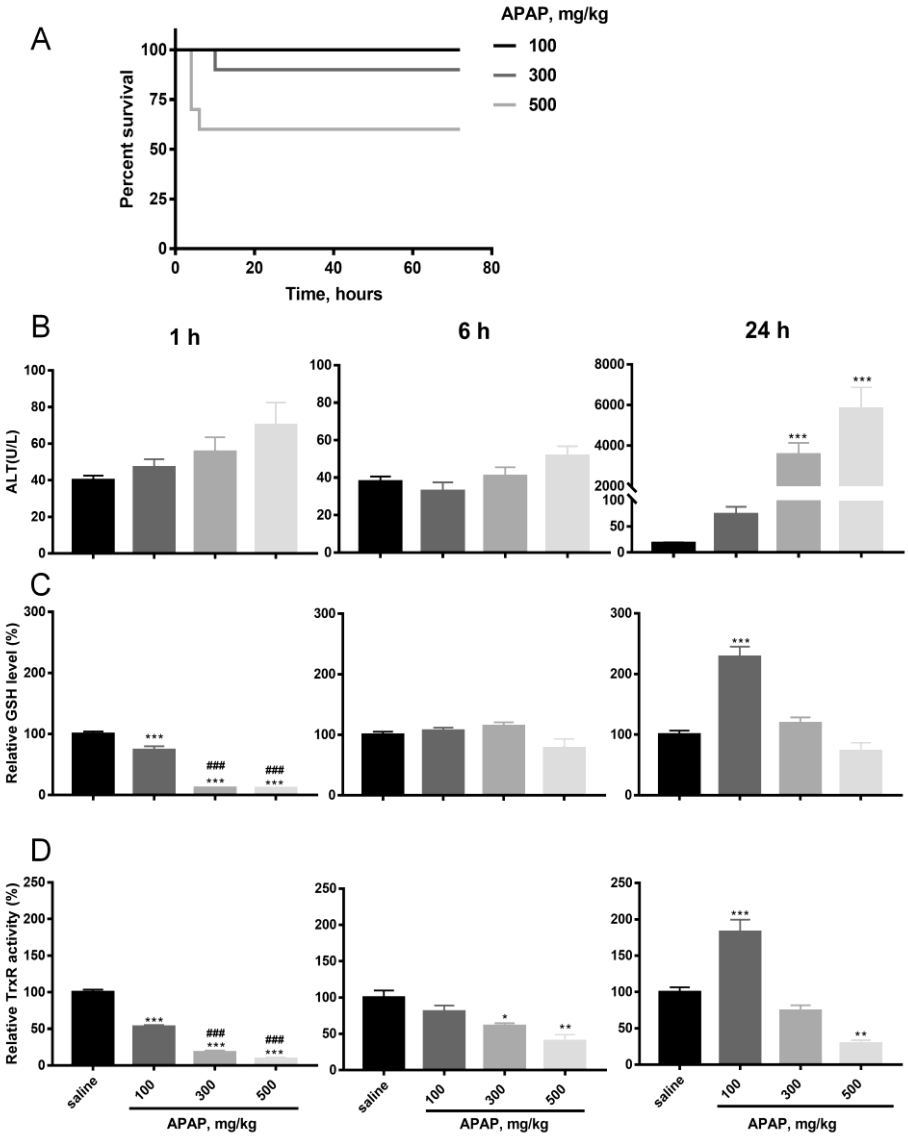

FIG.1. Effects of treatment with APAP on GSH level and TrxR activity in mouse liver extract. (A) Survival of mice treated with various doses of APAP (n=10); (B) Alanine transaminase (ALT) activity in serum of mice treated with the indicated doses of APAP at 1 h, 6 h or 24 h post-treatment. (C) Relative GSH levels in liver extracts from mice treated with APAP. GSH levels are normalized relative to the average in the saline control group (100%). (D) Thioredoxin reductase (TrxR) activity in liver extracts from mice treated with 100, 300 or 500 mg/kg APAP. TrxR activity was normalized to the average in the saline control group (100%). The difference of ALT activity, relative TrxR activity and GSH level was analyzed using two-way ANOVA followed by *post-hoc* test. Data are preseted as mean ± SEM. n=4-10. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

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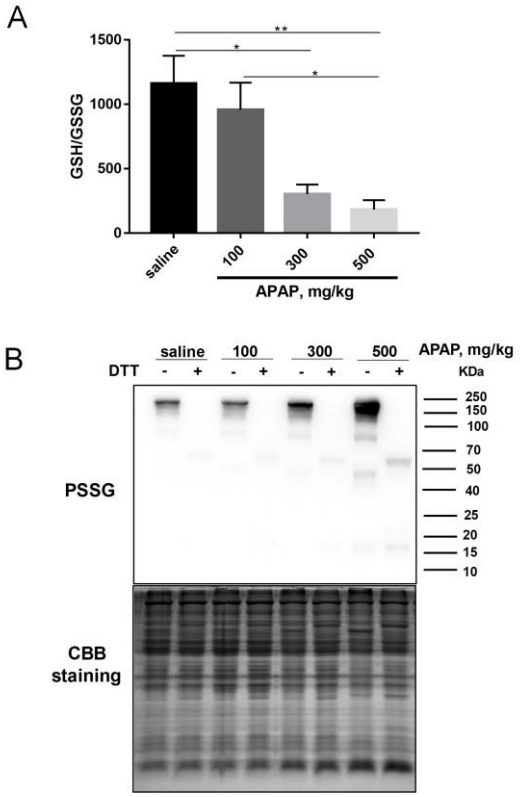

FIG.2. APAP induced dose-dependent decrease in the GSH/GSSG ratio and increase in protein glutathionylation within mice livers. (A) APAP-induced dose-dependent decrease in the GSH/GSSG ratio. The difference among groups was analyzed using one-way ANOVA followed by *post-hoc* analysis. Data are presented as mean ± SEM, n=6. *, $p<0.05$; **, $p<0.01$. (B) APAP-induced dose-dependent increase in protein glutathionylation. The experiment was performed in triplicate and one representative result is shown. The Coomassie Brilliant Blue (CBB) stained gel was used as a loading control.

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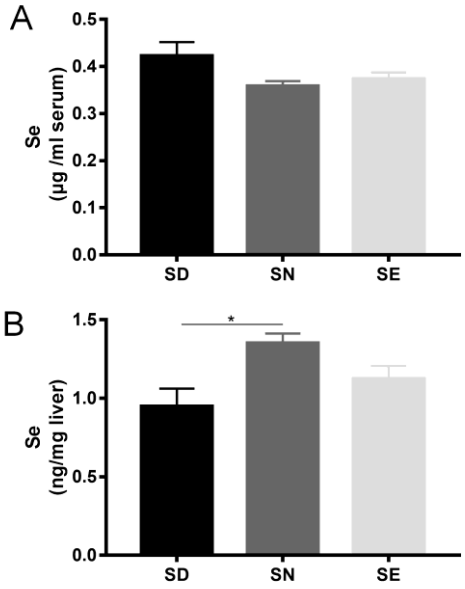

FIG.3. Selenium levels in serum and liver in the mice fed with diet containing various amount of selenium. Mice serum or liver tissue was digested with mixed acids of nitric acid and perchloric acid and selenium content in collected digestion solution was determined by ICP-MS. Selenium level in serum (A) and liver tissue (B) were calculated according to standard curve. The difference among groups was analyzed by one-way ANOVA followed by post-hoc test. Values are mean ± SEM, n=4-5. *, p<0.05.

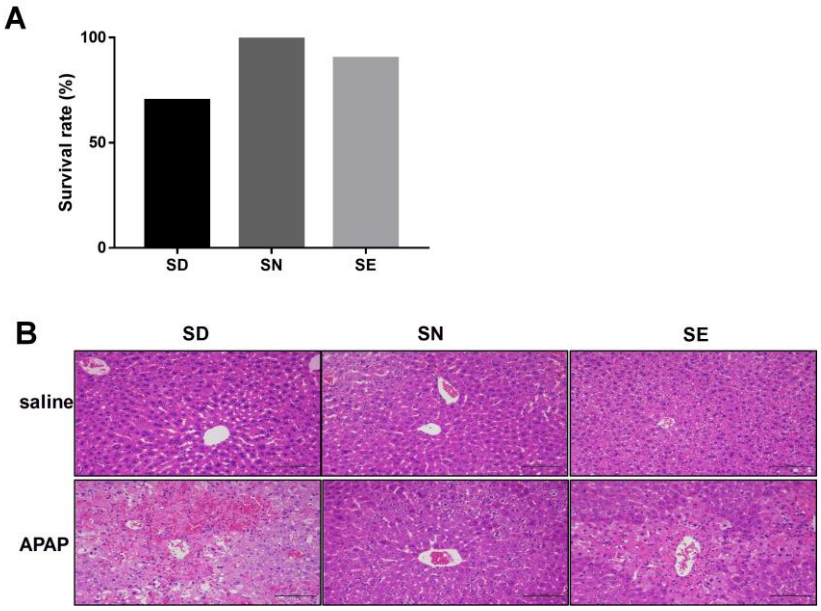

FIG.4. Selenium status affects the hepatotoxicity of acetaminophen. (A) After 300 mg/kg APAP challenge, the number of mice died within 24 h was recorded and 3 and 1 mice died in selenium-deficient group and selenium-enriched groups, respectively (n=10); (B) Liver tissues were collected for hematoxylin-eosin staining (magnification x 200), n=3. A representative example is shown.

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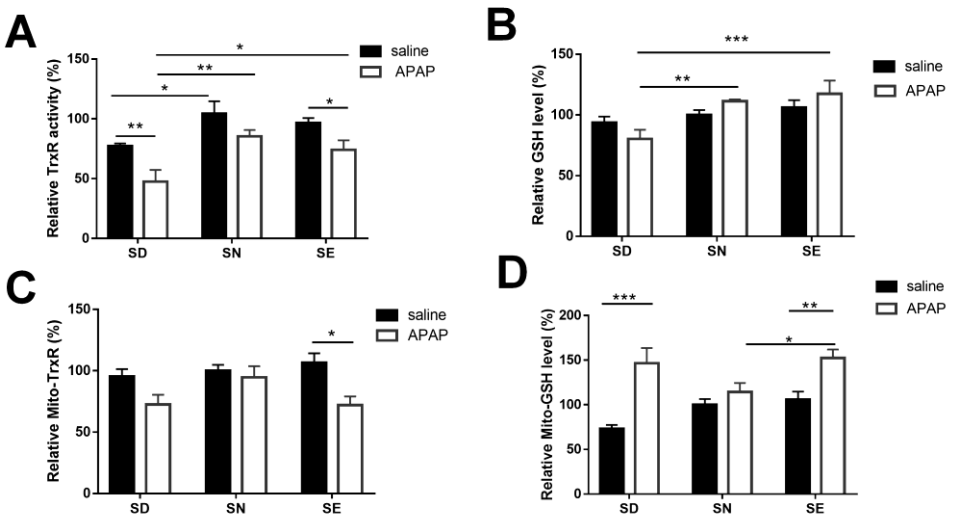

FIG. 5. Selenium status affects TrxR activity and GSH levels upon APAP treatment. (A) Total TrxR activity; and (B) GSH levels were measured using the DTNB assay. Mitochondria were separated following the mitochondria separation kit protocol and mitochondrial proteins were obtained using mitochondria lysis buffer; (C) mitochondrial TrxR activity; and (D) mitochondrial GSH amount as measured using the DTNB assay. Each sample was analyzed in triplicate. Differences were analyzed using two-way ANOVA followed by *post-hoc* analysis. Data are presented as mean ± SEM, n=7-10. *, $p<0.05$; **, $p<0.01$; *** $p<0.001$.

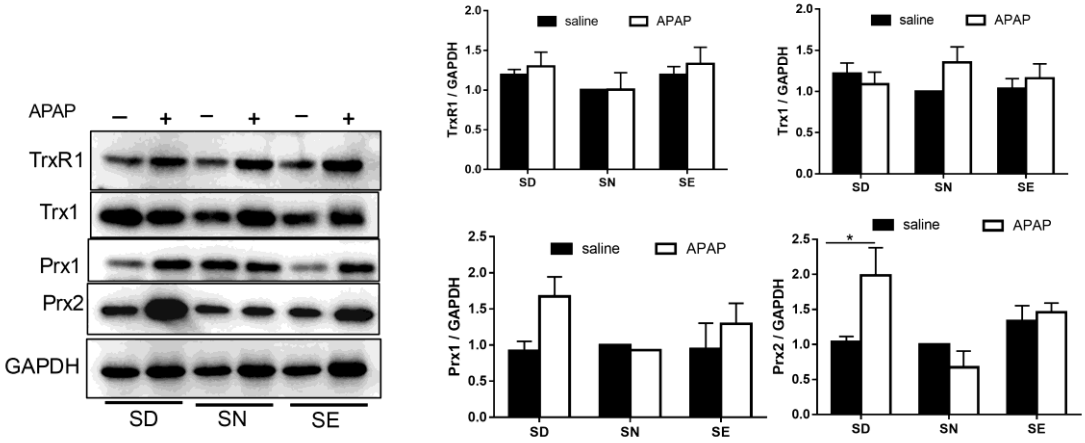

FIG.6. Effects of selenium status on cytosolic protein expression upon APAP treatment. TrxR1, thioredoxin1 (Trx1), peroxiredoxin1 (Prx1) and Prx2 protein levels were analyzed by Western Blotting and quantified using Image J software with GAPDH used as a loading control. Data were normalized by comparing the protein levels of targeted group to that in selenium-normal mice treated with saline (= 1). The experiment was performed with four repeats and one representative result is shown.

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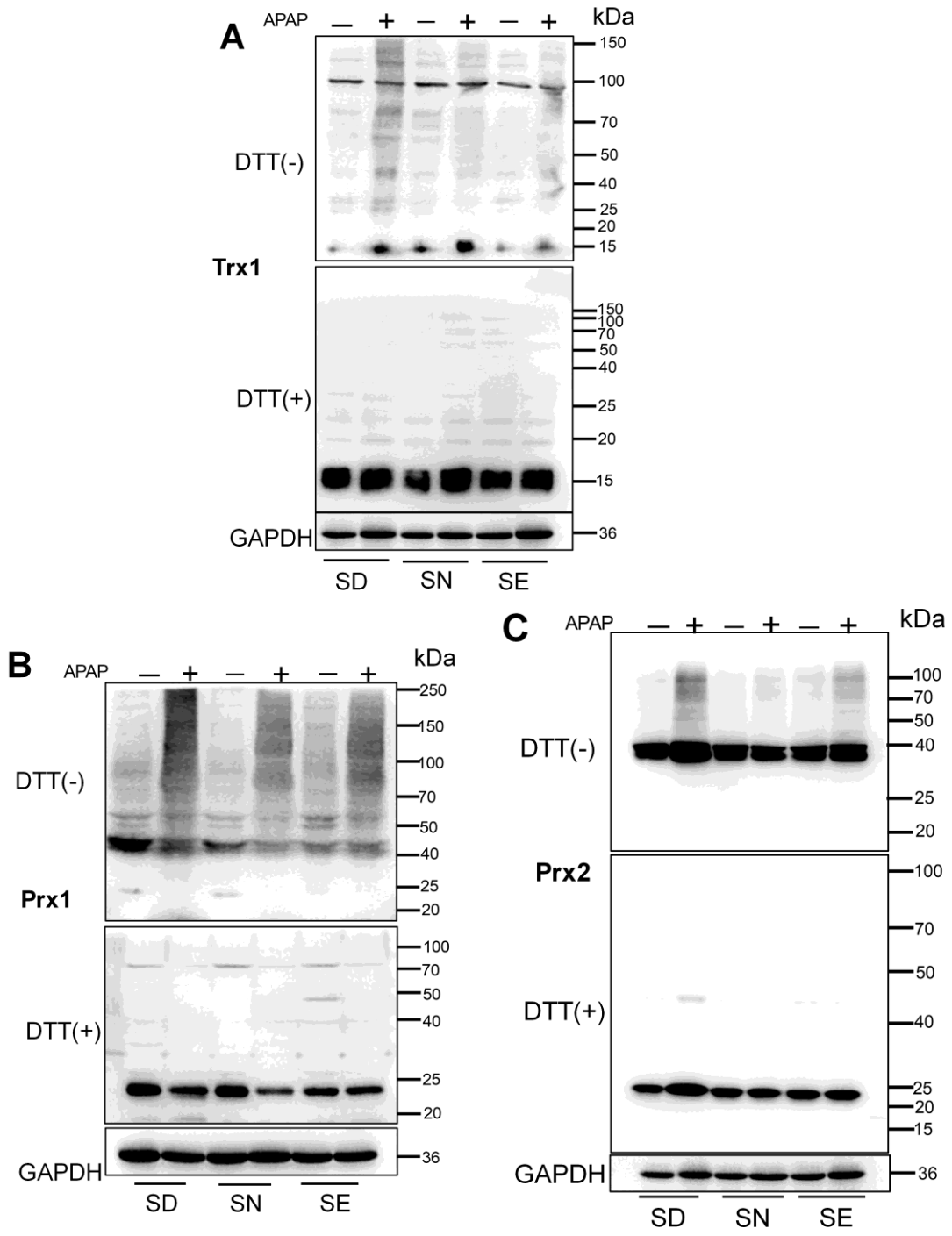

FIG.7. Selenium status affects the redox state of cytosolic Trx1, Prx1 and Prx2 upon APAP treatment. Redox status of (A) Trx1; (B) Prx1; and (C) Prx2. Each experiment was performed in triplicate and one representative result is shown. GAPDH is used as the loading control.

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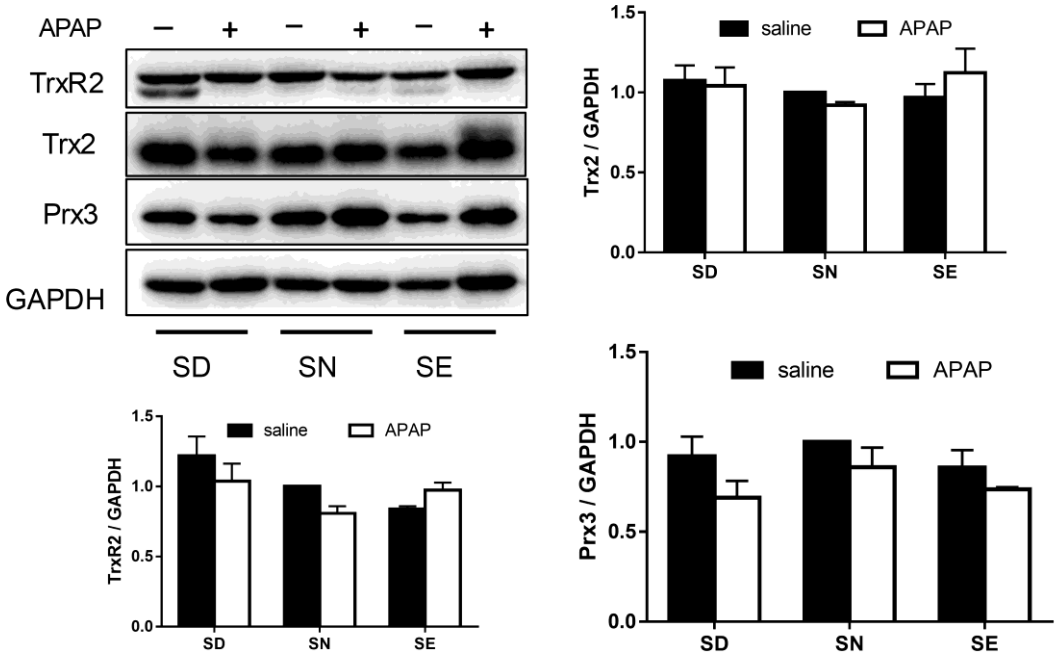

FIG.8. Effect of selenium status on mitochondrial protein expression upon APAP treatment. TrxR2, Trx2 and Prx3 protein levels were analyzed by Western Blotting and levels quantified using Image J software with GAPDH as the loading control. Data are normalized by comparing protein levels in the targeted group to that in selenium-normal mice treated with saline (=1). The experiment was performed with four repeats and one representative result is shown.

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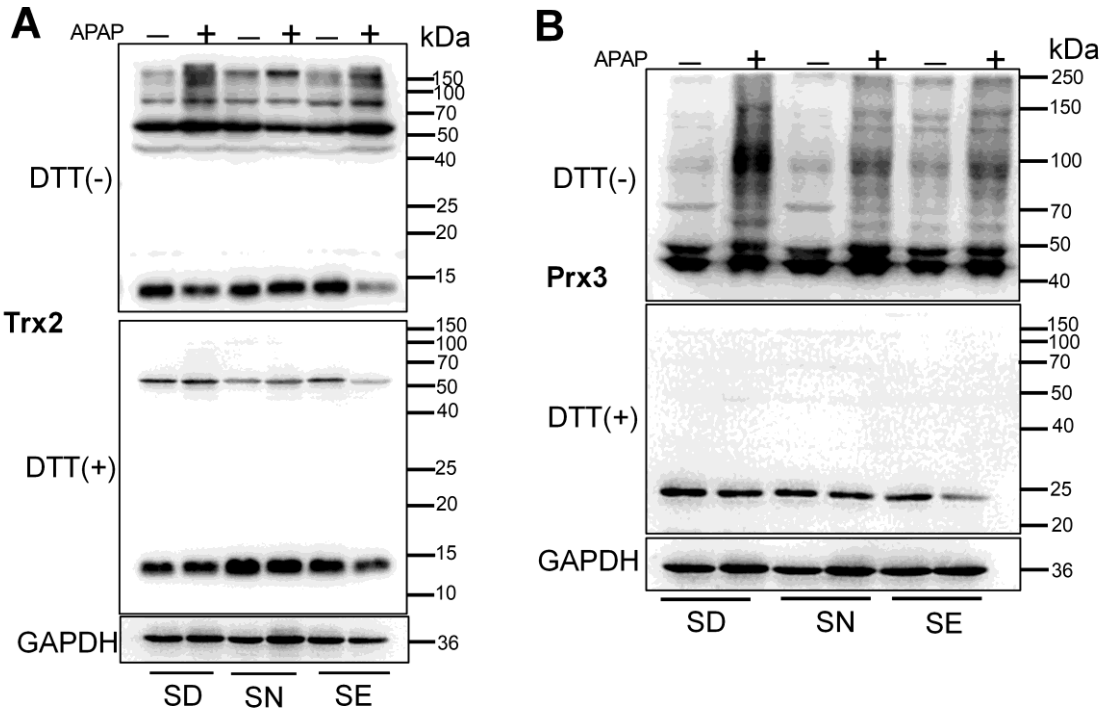

FIG. 9. Selenium status affects the redox state of mitochondrial Trx2 and Prx3 upon APAP treatment. Redox status for (A) Trx2; and (B) Prx3. The experiment was performed in triplicate and one representative result is shown. GAPDH is used as the loading control.

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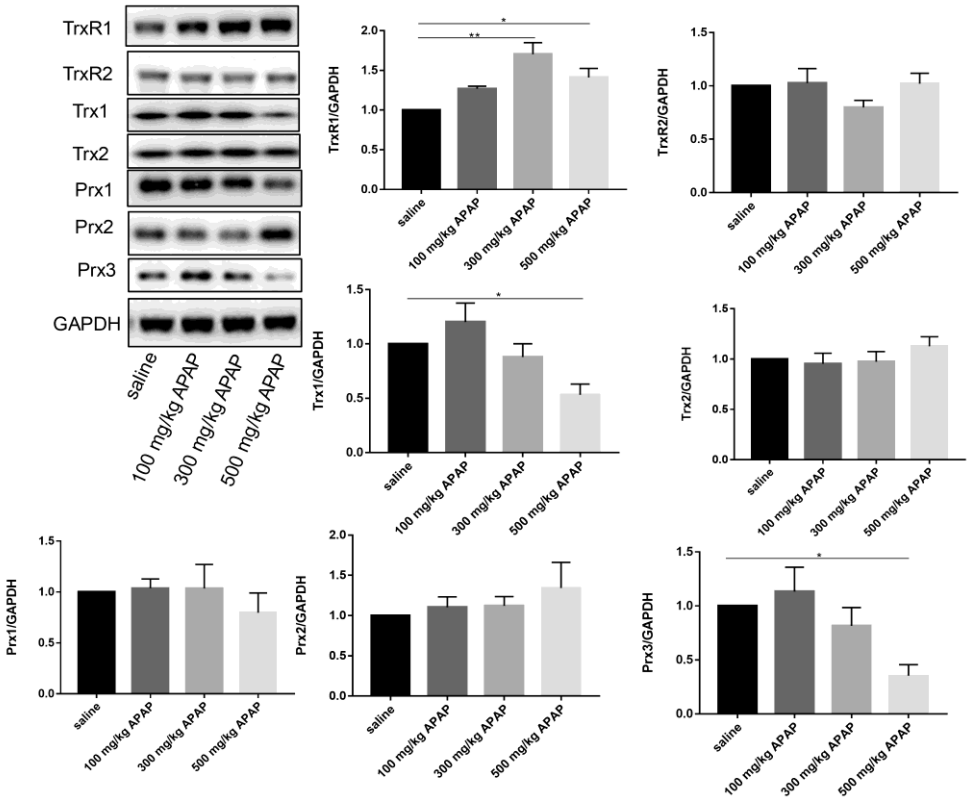

FIG.S1. Effects of APAP on protein expression of key enzymes in liver. After 24 h treatment with saline, 100 mg/kg, 300 mg/kg and 500 mg/kg APAP, mice were sacrificed and liver tissue was collected for further analysis. Protein levels of key enzymes including thioredoxin reductase (TrxR1/2), thioredoxin (Trx1/2) and peroxiredoxin (Prx1/2/3) were analyzed by western blot and quantified by software Image J using GAPDH as loading control. Data were normalized by setting the protein levels in mice treated with saline as 1. The experiment was performed in sextuplicate and a representative example was shown. Data are presented as mean ± SEM. *, $p<0.05$; **, $p<0.01$.

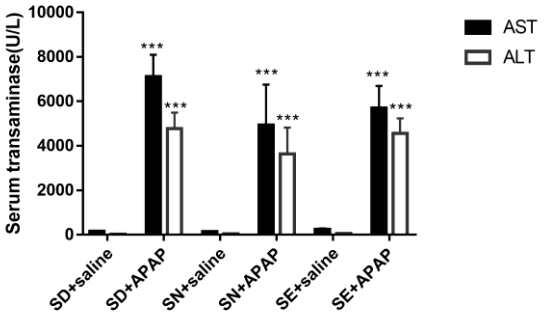

FIG.S2. Serum transaminases activity in mice treated with APAP. The serum in mice exposed to 300 mg/kg acetaminophen for 24 h were collected for aspartate aminotransferase and alanine transaminase activity determination using the kits. The differences between groups were analyzed by one-way ANOVA followed by *post-hoc* analysis. Data was shown as mean ± SEM, n=7-10. ***, $p<0.001$ vs respective control.

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This paper has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

Table S1. Pathological morphology assessment on liver tissue with or without APAP exposure.

Groups	necrosis	inflammation
SD + saline	0	0
	0	0
	0	0
SD + APAP	4	0
	4	0
	4	0
SN + saline	0	1
	0	0
	0	1
SN + APAP	0	0
	0	1
	1	0
SE + saline	0	0
	0	0
	0	0
SE + APAP	3	1
	1	0
	4	0

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