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Original Article

Sulforaphane protects against LPS-induced liver injury in mice by antagonizing oxidative stress and apoptosis through AMPK activation

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ABSTRACT

Objectives: Given the adverse effect of liver injury on a multitude of body functions, it is vital to understand its underlying mechanism and how to overcome it. In this study, lipopolysaccharide (LPS) was used to induce liver injury, while sulforaphane (SFN), a natural phytochemical, was used as the antagonist to overcome the deleterious effect.

Methods: Twenty-four mice were divided into three groups: Control group (0.9% saline), LPS induction group (0.75 mg/kg), and SFN treatment (25 mg/kg) followed by LPS induction group (0.75 mg/kg), all with access to food and water *ad libitum*. Blood samples from retro-orbital sinus were used to measure liver function through two aminotransferases (i.e., alanine transaminase [ALT] and aspartate transaminase [AST]) whereas liver homogenate was used to measure glutathione (GSH), catalase (CAT), and superoxide dismutase (SOD) (antioxidant activity markers); caspase-3 (apoptosis marker); malondialdehyde (MDA) (lipid peroxidation marker); and NO. AMP-activated protein kinase (AMPK), a cellular energy homeostasis and lipid metabolism sensor, was also measured. Statistical analysis including normalization, analysis of variance, Kruskal–Wallis test, and significance of $P < 0.05$ were applied to all collected data.

Results: SFN treatment significantly attenuated all tests compared to the induced liver injury by LPS where significant reduction was observed in the levels of hepatic function markers (AST and ALT), lipid peroxidation marker (MDA) as well as apoptosis marker (caspase-3) whereas a marked increase was observed for antioxidant activity markers (SOD, CAT, and GSH) and AMPK.

Conclusion: These results indicate the protective effect of SFN as it re-instated the levels of antioxidation while decreasing the level of the biomarkers, which were significantly increased during liver injury induction by LPS.

Keywords: Liver injury, lipopolysaccharide, oxidative stress, protective effect, sulforaphane

Introduction

Due to the high morbidity and mortality rates, the liver injury represents a major threat to human health worldwide. Indeed, according to the Centers for Disease Control and Prevention in the United States, liver injury is the ninth leading cause of death,[1] resulting in around two million deaths per year globally.[2] As of 2023, there is no cure for liver injury, whereas the available treatments are only symptomatic.[3] The major hallmark of liver injury is the loss of hepatocyte function, which is characterized by inflammation, oxidative stress, and apoptosis.[4] Therefore, researchers have demonstrated that inhibiting inflammation, oxidative stress, and/or apoptosis could alleviate the development of liver injury.[5]

In recent years, increasing attention has been paid to nutraceuticals due to the potential for use as an alternative or complementary medication with regard to allopathic drugs.[6] Studies have demonstrated that naturally occurring plant phytochemicals show the potential to delay the progress of liver injury through different mechanisms, including neutralizing the detrimental effects of oxidative stress, retarding inflammation, and suppressing apoptosis.[7,8]

Sulforaphane (SFN) (1-isothiocyanato-4-(methylsulfinyl)-butane[SFN]), a natural isothiocyanate, is found in high concentrations within broccoli plants (*Brassica oleracea* var. *Italica*) and cruciferous vegetables.[9] SFN has been shown to exert anti-inflammatory,[10,11] anti-oxidative stress, and

anti-apoptotic properties in many tissues.[9,12,13] Moreover, the anti-oxidative capability of SFN in liver injury has been demonstrated in a number of previous animal studies.[14,15] For instance, intraperitoneal administration of SFN decreased the malonaldehyde (MDA) and reactive oxygen species (ROS) levels, increased the antioxidant enzyme glutathione (GSH) level, and increased the catalase (CAT) and superoxide dismutase (SOD) activities.[16-18] In addition, SFN pre-treatment has been shown to inhibit apoptosis in the liver through several different mechanisms including the reduction of apoptotic initiator poly (ADP-ribose) polymerase cleavage,[19] decreasing the serum level of the cytokine tumor necrosis factor-α,[20] increasing the activity of Na^+–K^+-ATPase and Ca^{2+}-ATPase[21] and decrease the levels of certain caspases known to mediate cell survival, such as caspase-1 and caspase-3.[13]

With SNF being the focus of a plethora of publications describing its efficacy in animal models (mainly mice and rats) as well as many clinical trials, its taxological profile was of utmost importance. SFN lethal median dose (LD_{50}) was determined to be around 212.67 mg/kg from a study on mice through intraperitoneal dose administration where they found that different doses <100 mg/kg had no significant effect on the seizure threshold whereas 200 mg/kg significantly decreased it with signs of severe toxicity including deep sedation, ataxia, ptosis, and tremors were noted several minutes post a dose of 300 mg/kg.[22] Most studies on the effect of SNF were chosen way lower than LD_{50} and proven to have protective, preventive, and ameliorating properties. In addition, SNF was considered safe and well-tolerated by patients in clinical trials with low doses such as 50–150 μmol orally per day for treating children with autism.[23,24] A recent review compared the dosage and means of administration of SNF in animals where they demonstrated that oral administration was found with a median effective dose of 175 μmol/kg body weight whereas intraperitoneal administration was 113 μmol/kg body weight,[25] given the latter a higher probability to elicit toxicity.

The estimated human equivalent dose (HED) is an equation used to help better extrapolate doses for human clinical trials from animal studies to better design them. It states that HED (mg/kg) can be calculated by multiplying the animal dose (mg/kg) with a constant ration depending on the animal species (i.e., 0.081 for mice and 0.162 for rats).[26] With such an equation, the LD_{50} of SNF is estimated to be 17.23 mg/kg, indicating this phytocompound is relatively safe for human consumption by diet.

Adenosine monophosphate (AMP)-activated protein kinase (AMPK) is a key player in the cellular energy regulatory pathways within multiple organs throughout the body.[27] AMPK may also play a major role as an oxidative stress sensor and redox regulator that is important in terms of maintaining intracellular homeostasis during various stress challenges.[28] Moreover, prior studies have shown that active AMPK exerts an anti-apoptotic effect in multiple types of cells as well as a suppressive effect on caspase-3.[29-31] As liver injury is mainly characterized by elevated levels of oxidative stress and apoptosis, the activation of AMPK signaling to boost the antioxidant capacity and suppress the apoptotic activity has been suggested as a potential therapeutic target in liver injury.

Given the high prevalence of liver disease worldwide and the lack of pre-clinical alternatives, animal models of liver injury are crucial to improving our understanding of the disease's pathogenesis as well as enabling the identification of therapeutic targets and testing of novel drugs.[32,33] Lipopolysaccharide (LPS), an endotoxin that is among the constituents of the outer membrane of Gram-negative bacteria,[34] can induce inflammation and oxidative stress, leading to liver injury.[35] In addition, LPS is relatively easy to administer and inexpensive to use when compared with other liver-injury-inducing agents.[36] Therefore, the LPS-induced liver injury model has become a widely used animal model due to closely mimicking the clinical symptoms of liver injury.

To the best of our knowledge, only a few studies have investigated the effect of SFN on LPS-induced liver injury. Thus, the present study aims to examine the possible protective effect of SFN against LPS-induced liver injury in mice by evaluating its anti-oxidative and anti-apoptotic properties and determining its possible AMPK-related mechanism of action.

Materials and Methods

Chemicals

The LPS (*Escherichia coli*, O111: B4) used in this study was purchased from *in vivo* Gen (San Diego, California, United States). A stock solution of 5 mg/mL (weight/volume [w/v]) of LPS was prepared by dissolving 5 mg of powdered LPS in 1 mL of endotoxin-free water. The SFN was obtained from Santa Cruz Biotechnology (Dallas, Texas, United States). It was dissolved in 3% dimethyl sulfoxide (DMSO) prepared in normal saline. The sucrose, ethylenediaminetetraacetic acid (EDTA), and 3-morpholinopropane-1-sulfonic acid (MOPS) were purchased from SolarbioLife Sciences (Beijing, China). The ethanol was purchased from Thermo Fisher Scientific (Waltham, Massachusetts, United States), whereas the sodium hydroxide (NaOH) was purchased from MyBioSource (San Diego, California, United States).

Animals

The 24 Swiss albino male mice (SWR/J) (18–25 g) used in this study were obtained from the Animal House Unit of King Fahad Medical Research Center (KFMRC), King Abdulaziz University, Jeddah, Saudi Arabia. Three to five mice were housed per cage, and the mice were maintained under a 12-h light/dark cycle at approximately room temperature (23 ± 2°C) and humidity (65%). All the mice had access to food and water *ad libitum*. Moreover, the mice were treated in accordance with the guidelines of the Animal Unit Committee of KFMRC. All

the experiments were performed according to the guidelines of the Biomedical Ethics Research Committee (Reference No. 603–20) of King Abdulaziz University. They also accorded with the rules and regulations of the Animal Care and Use Committee of KFMRC, which complied with the "System of Ethics of Research on Living Creatures" guidelines prepared by the King Abdulaziz City for Science and Technology and approved by Royal Decree No. M/59 dated August 24, 2010.

Experimental design

The mice were randomly divided into three groups (eight mice per group): the control group (0.9% saline vehicle), the LPS induction group (0.75 mg/kg), and the SFN treatment (25 mg/kg) followed by LPS induction (0.75 mg/kg) group. The total duration of the study was 2 weeks [Figure 1]. During the 1st week, the mice in the control and LPS groups were injected daily with intraperitoneal (IP) normal saline, whereas the mice in the SFN group were injected with IP SFN. Disease induction was performed in the 2nd week, with the mice in each group receiving two IP injections daily: Saline + 3% DMSO in the control group, 0.75 mg/kg of LPS + 3% DMSO in the LPS group, and 25 mg/kg of SFN + 0.75 mg/kg of LPS in the SFN+LPS group.

Determination of liver function

Blood samples were drawn from the retro-orbital sinus of mice in all the groups before euthanasia. The samples were centrifuged at 3000 g for 10 min after the pallet was discarded. The levels of two aminotransferases known to be markers of hepatic function – namely, serum aspartate transaminase (AST) and alanine transaminase (ALT) – were determined using enzyme-linked immunosorbent assay (ELISA) kits (MyBioSource, San Diego, California, United States) according to the manufacturer's protocols. The results were read at 450 nm using a microplate reader (BioTekInstruments, Winooski, Vermont, United States).

Preparation of liver homogenate

The livers obtained from all the groups were weighed, chopped into small pieces, and had lysis buffer added to the tissues. The lysis buffer was prepared by dissolving 17.1 g of sucrose (0.25 M) in 100 mL of distilled water, then 2 mL of EDTA (1 mM), 10 ml of MOPS (5 mM), and 0.2 mL of ethanol (0.1% [v/v]) were, respectively, added, with the pH adjusted to 7.2 using NaOH (1M). The mixture was then homogenized using an ultrasonicator (BioLogics, Cary, North Carolina, United States). Following complete homogenization, the homogenate was centrifuged in a cold centrifuge (4°C) at 5000 g for 5 min. Aliquots were then prepared and stored at −80°C.

Measurement of hepatic antioxidant activity

In the liver homogenate, the SOD, CAT, and GSH activity levels were measured using a colorimetric assay kit (SolarbioLife Sciences, Beijing, China) according to the manufacturer's protocol. In brief, the CAT activity was measured using the rate of the decrease in the H_2O_2, the SOD activity was determined using xanthine oxidase methods, and the GSH activity was measured based on its reaction with 5,5'-dithiobis-2-nitrobenzoic acid to form a product that could be detected spectrophotometrically.

Measurement of hepatic MDA and NO content

In the liver homogenate, the MDA and NO contents were measured using a colorimetric assay kit (Solarbio Life Sciences, Beijing, China) according to the manufacturer's protocol. Briefly put, the MDA content was measured using the thiobarbituric acid method, while the NO content measurement was performed based on the product of the NO_2 and diazonium sulfonamide reaction (diazo compounds) under acidic conditions, where the compounds could further couple with naphthyl vinyl diamine to form a product that could be spectrophotometrically detected.

Determination of caspase-3 activity

The hepatic activity of caspase-3 was assessed using liver homogenate through an active caspase-3 ELISA kit (MyBioSource, San Diego, California, United States) according to the manufacturer's instructions. 100 µL of each sample, standard and blank was added to a well plate in duplicate followed by 100 µL of phosphate-buffered saline (pH 7.0–7.2) added to the blank control well and 10 mL of balance solution added into only the sample wells and

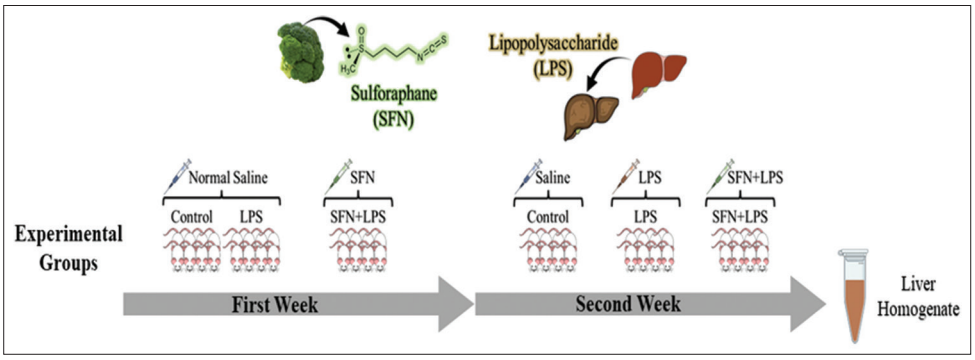

Figure 1: Illustration of the experimental design

mixed. After that, 50 µL of conjugate was added to each well, incubated for 1 h at 37°C, washed 5 times with diluted wash solution, and 50 mL of substrate A and B were added to each well and incubated at 37°C for 20 min. Finally, 50 mL of the stop solution was added to each well to stop the reaction. The absorbance was read at 450 nm using a microplate reader purchased from BioTek Instruments (Winooski, Vermont, United States).

Determination of AMPK activity

The hepatic AMPK activity was determined using a phosphorylated AMPK ELISA kit purchased from MyBioSource (San Diego, California, United States) according to the manufacturer's instructions. The absorbance was read at 450 nm using a microplate reader purchased from BioTek Instruments (Winooski, Vermont, United States).

Statistical analysis

All the data were expressed as the mean ± standard error of the mean and statistically analyzed using GraphPad Prism 9.1.2 software. Normality was evaluated by means of the Kolmogorov–Smirnov test. The one-way analysis of variance followed by the *post hoc* Tukey's test was used for comparisons between the groups in terms of all the results except for the non-normally distributed variables, for which the Kruskal–Wallis test followed by Dunn's test was used. The differences between the groups were considered statistically significant if $P < 0.05$.

Results

Serum aminotransferase concentrations

The results demonstrated a significant ($P < 0.0001$) increase in the AST and ALT concentrations in the LPS group when compared with the control group [Figure 2]. By contrast, SFN administration significantly ($P < 0.0001$) reduced the concentrations of AST and ALT when compared LPS group [Figure 2].

MDA and NO contents

LPS administration resulted in a significant ($P < 0.0001$) increase in the MDA and NO contents when compared with the control group [Figure 3]. By contrast, SFN administration caused a significant ($P < 0.0001$) decrease in the MDA content when compared with the LPS group, although no significant difference in the NO content was observed [$P = 0.0706$; Figure 3].

Hepatic antioxidant enzyme activity

Figure 4 illustrates the findings concerning the antioxidant enzymes. Here, LPS administration caused a significant ($P < 0.0001$) decrease in the SOD and CAT activities and the concentration of GSH when compared with the control group. By contrast, SFN treatment significantly ($P < 0.0001$) increased the SOD and CAT activities and the GSH concentration when compared with the LPS group.

Hepatic caspase-3 activity

LPS administration significantly ($P < 0.0001$) increased the caspase-3 activity when compared with the control group, whereas SFN treatment significantly ($P < 0.0001$) decreased the caspase-3 activity [Figure 5].

Hepatic AMPK activity

The findings revealed that LPS administration significantly ($P < 0.0001$) decreased the AMPK activity when compared with the control group, whereas SFN treatment significantly ($P < 0.0001$) increased the AMPK activity [Figure 6].

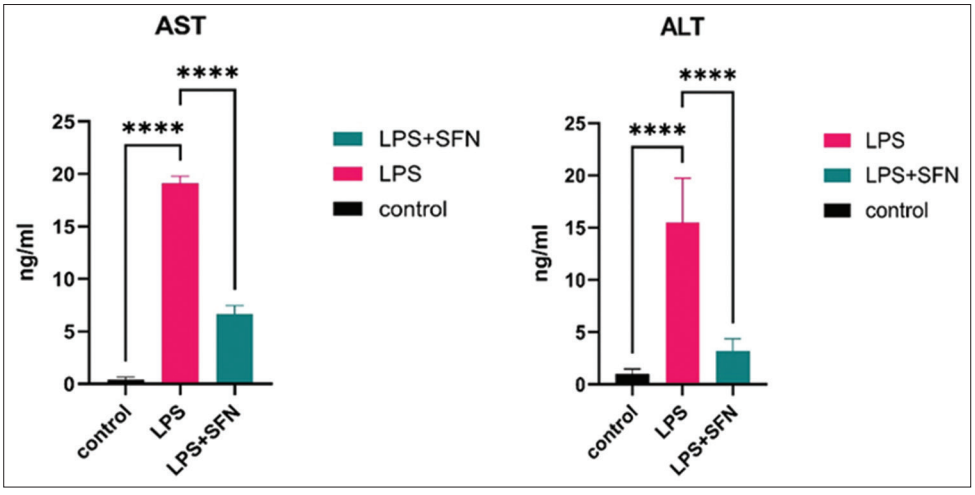

Figure 2: Serum aminotransferase concentrations. The LPS endotoxin caused an increase in the AST and ALT concentrations, whereas SFN administration significantly decreased the aminotransferase levels. ****Represents $P<0.0001$. LPS: lipopolysaccharide, SFN: Sulforaphane, AST: Serum aspartate transaminase, ALT: Alanine transaminase

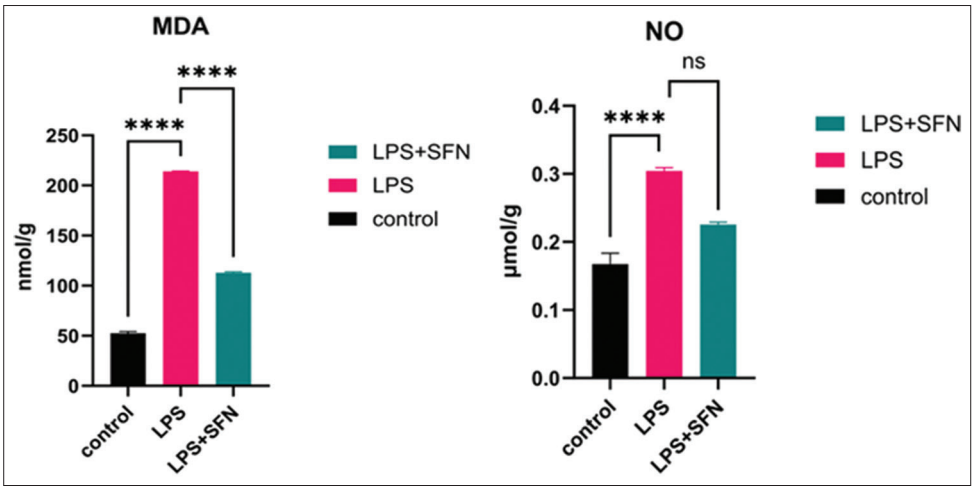

Figure 3: Malondialdehyde and nitric oxide contents. LPS administration increased the MDA and NO contents, whereas SFN decreased the measured MDA content but caused no difference in the NO content. ****Represents *P*<0.0001. LPS: Lipopolysaccharide, SFN: Sulforaphane, MDA: Malondialdehyde, NO: Nitric oxide

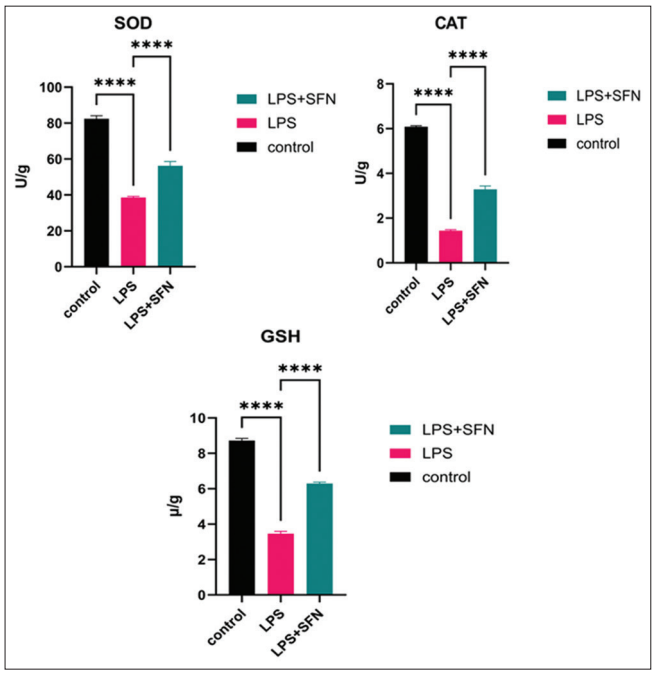

Figure 4: Antioxidant enzyme activities and concentrations. LPS administration decreased the antioxidant enzymes, whereas SFN treatment increased the measured antioxidant enzymes. ****Represents *P*<0.0001. LPS: Lipopolysaccharide, SFN: Sulforaphane, SOD: Superoxide dismutase, CAT: Catalase, GSH: Glutathione

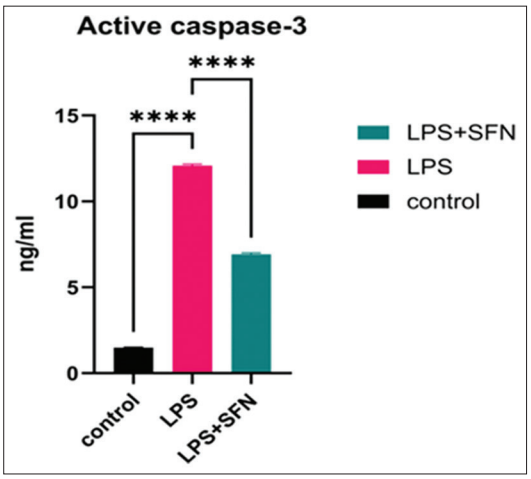

Figure 5: Caspase-3 activities. LPS administration increased the caspase-3 activity, whereas SFN treatment decreased it. ****Represents *P*<0.0001. LPS: Lipopolysaccharide, SFN: Sulforaphane

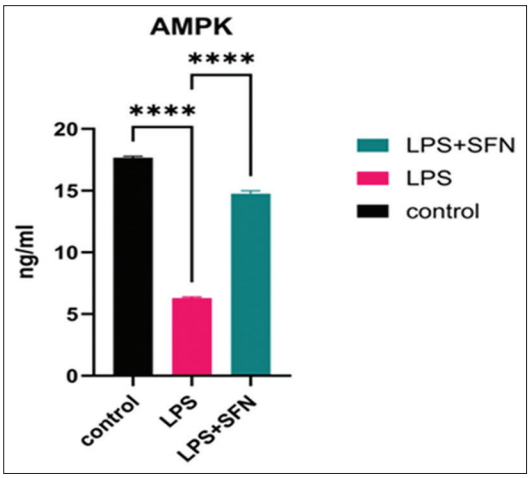

Figure 6: AMPK activities. LPS administration decreased the AMPK activity, whereas SFN treatment increased the measured AMPK activity. ****Represents *P*<0.0001. LPS: Lipopolysaccharide, SFN: Sulforaphane, AMPK: AMP-Activated protein kinase

Discussion

Prevention methods against liver injury (be it hepatitis or cirrhosis) mainly focus on vaccines, early screening and detection of the disease, reducing obesity, reducing alcohol consumption level, and monitoring risk factors related to age, medical history, or hygiene[37-40] but there is a lack of methods for healthy individuals to help protecting and preventing the disease. Therefore, finding natural products that can offer potential protective approaches is a very important

task. Therefore, this study sought to investigate the possible protective mechanisms of SFN pre-treatment in relation to LPS-induced liver injury in mice. Our results demonstrate a clear pattern regarding the effects of LPS and SFN, whereby SFN administration for 2 weeks significantly protected against LPS-induced liver injury. Moreover, our data demonstrate the anti-oxidative and anti-apoptotic effect of SFN as a possible protective mechanism against LPS-induced liver injury that occurs through AMPK activation.

The two studied aminotransferase enzymes (AST and ALT) are intracellular enzymes (which occur inside cells, not high in blood serum), meaning that their elevation in the blood represents cellular damage.[41] They are predominantly found in the liver and so are routinely used as indicators of abnormal hepatic physiology (i.e., liver injury) because they serve as sensitive biomarkers in the serum.[42] One of the main effects of hepatic injury induced through LPS is the elevation of the AST and ALT enzymes.[43] In our study, the results show that the LPS group had increased AST and ALT activities when compared with the control group, whereas IP injection of SFN for 14 days significantly decreased the AST and ALT activities, indicating SFN to exert a protective and ameliorating effect against LPS in the liver. These results accord with those of several previous studies.[14,20,44-46]

Excessive ROS production that results in oxidative stress is believed to be an early event in the progression of liver injury.[47] Enzymes such as CAT and SOD play a pivotal role in ROS regulation and protection from tissue damage.[48] Moreover, antioxidant compounds such as GSH play an essential role in cell defense against oxidative stress through modulating the physiological levels of ROS. Furthermore, MDA and NO (a lipid peroxidation end product) act as indicators of cell membrane damage and lack of antioxidant defense.[49] One of the major pathological mechanisms of LPS in relation to liver injury entails disturbing the cellular antioxidant defenses and causing the release of mitochondrial ROS.[50] In this study, LPS administration induced oxidative stress in mice liver, as indicated by the increased levels of MDA and NO, the reduced level of GSH, and the reduced CAT and SOD activities when compared with the control group. Similar results have been broadly reported by several prior studies.[3,51-53] By contrast, IP injection of SFN increased the hepatic antioxidants' effect and reduced the level of oxidative stress, as evidenced by the remarkably mitigated MDA and NO levels, the significantly restored CAT and SOD activities, and the significantly restored GSH levels. These results indicate that SFN's hepatoprotective effect could be attributed to its antioxidant potential. Data concerning SFN's hepatoprotective efficacy against oxidative damage using an LPS-induced liver injury model are limited, with only a few previous studies having discussed the exact mechanism involved.[14,17,20,44,45]

Activation of the executioner enzyme (caspase-3) and promotion of hepatic apoptosis are typical pathological features of liver injury.[54,55] Therefore, intervention in hepatic apoptosis has been suggested as an approach to *alleviating liver injury*. The results of our study demonstrate that caspase-3 was elevated in the LPS group and significantly reduced in the group treated with SFN, indicating that the anti-apoptotic effect of SFN might be an effective hepatoprotective strategy in liver injury. Our results support the findings of certain previous studies that revealed the endotoxin LPS to induce the expression of apoptotic markers such as caspase-3 in the liver and, therefore, contribute to liver injury,[35,56] whereas SFN attenuates the deleterious effect of apoptosis.[13]

The underlying mechanism by which SFN improves hepatic injury is not yet fully understood. However, prior studies have reported that the activation of AMPK has a potential role in modulating oxidative stress[57-60] and inhibiting apoptosis in different cell types, including hepatocytes.[61-63] Conversely, other studies have shown that SFN can fight different disorders through regulating the AMPK signaling pathway.[64,65] Thus, the present study investigated whether SFN can protect against liver injury through AMPK activation mediating its anti-apoptotic and anti-oxidative effects.

Our results concerning AMPK activity reveal opposing patterns regarding the situation post-LPS and post-SFN treatment, with the AMPK activity being reduced following the LPS-induced liver injury but significantly increased following the SFN treatment. This finding indicates, for the 1st time, the effectiveness of SFN as a *potent activator* of AMPK.

Conclusion

The present study found that 2 weeks of SFN treatment protected against LPS-induced liver injury in mice by increasing its anti-oxidative and anti-apoptosis ability through the *activation of AMPK*. Taken together, the results suggest that SFN might be an effective prophylactic agent for the treatment of liver injury.

Limitations and future studies

This study had a number of limitations that need to be discussed and further explored. First, the study did not involve histological or pathological examinations of the liver. Moreover, the anti-inflammatory property of SFN was not examined in this study. Therefore, it will be significant to continue examining SFN's prophylactic effect and its underlying mechanism in liver injury by addressing these two limitations. In addition, elucidation of the mechanism by which SFN activates the AMPK signaling pathway and induces its anti-oxidative and anti-apoptosis properties may provide the opportunity to develop preventive strategies for liver injury in the future.

Ethics Approval and Consent to Participate

The authors certify that the mice used in the experimental design were treated in accordance with the guidelines of the Animal Unit Committee of KFMRC. All the experiments were performed according to the guidelines of the Biomedical Ethics Research Committee (Reference No. 603–20) of King Abdulaziz University. They also accorded with the rules and regulations of the Animal Care and Use Committee of KFMRC, which complied with the "System of Ethics of Research on Living Creatures" guidelines prepared by the King Abdulaziz City for Science and Technology and approved by Royal Decree No. M/59 dated August 24, 2010. Jointly, the authors approved the publication of this manuscript.

Availability of Data and Material

The data supporting the findings of this study are available within the article. More detailed data used to support the findings of the current study are available from the corresponding author upon reasonable request.

Competing Interests

All the authors declared that there was no conflict and/or competing interests.

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Authors' Contributions

Rasha A. Mansouri; conceptualization, data curation, formal analysis, methodology, and supervision. Huda F. Alshaibi; investigation, methodology, and project administration. May M. Alqurashi; writing, reviewing, and proofreading. Maimoonah M. Shaikh; conceptualization, formal analysis, funding acquisition, investigation, methodology, validation, and writing the original draft. Khulud A. Bahaidrah; investigation, methodology, and resources. Noor A. Alzahrani; investigation, methodology, and resources.

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