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Article

The effects of silymarin plus glutathione on the prevention of liver ischemia-reperfusion injury

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Liver ischemia-reperfusion (IR) injury is a major clinical trouble encountered in clinical practice. This study aimed to examine the therapeutic effects of silymarin (SM) plus glutathione (GSH) on hepatic IR injury using a rat model of liver IR. Fifty male rats were randomly divided into five groups, each consisting of 10 rats as follows: Sham, IR, SM-IR, GSH-IR and SM plus GSH-IR. All groups except sham were subjected to 30-min ischemia and 24-h reperfusion. The treated groups received 100 mg/kg of SM, GSH and a mixture of SM plus GSH, 60 min prior to the IR. After a period of 24 h, blood and liver samples were collected for biochemical and histopathological evaluations. Pretreatment with SM, GSH and SM plus GSH before hepatic IR significantly decreased IR-induced elevations of aminotransferases, and significantly reduced the histopathological damage scores of the liver in the late phase of IR injury. Moreover, SM plus GSH treatment prior to liver IR significantly suppressed inflammatory process and oxidative stress as demonstrated by attenuations in tumor necrosis factor-α, myeloperoxidase and the thiobarbituric acid-reactive substances. These findings suggest that administration of SM plus GSH prior to liver IR may protect the liver parenchyma from the effects of an IR injury.

Keywords: Silymarin. Glutathione. Liver ischemia-reperfusion. Hepatic injury.

INTRODUCTION

The liver is a substantial organ actively involved in many metabolic functions. Liver IR injury is a major complication in clinical practice that compromises liver function and can affect postoperative morbidity, mortality, recovery, and overall clinic outcomes including liver transplantation, liver resections, trauma, hemorrhagic shock and resuscitation (Papadopoulos *et al*., 2013; Saidi, Kenari, 2014; Nastos *et al*., 2014). Hepatic IR provokes parenchymal liver damage, with clinical manifestations associated with rising liver injury markers. The main cause of hepatic injury is an oxidative stress, which results from an excess production of reactive oxygen/nitrogen species in cells exposed to hypoxia/reoxygenation. This metabolic situation triggers subsequent reactions between these reactive species with nucleic acids, lipids, proteins and the other cellular components, which ultimately destroy molecular architecture in the cells. IR injury occurs not only in hepatocytes, Kupffer cells, and endothelial cells lining the vascular wall but also in testicular cells in relation to testicular torsion/detorsion (Moghimian *et al*., 2017; Alban *et al*., 2018; Ameli *et al*., 2018). Hepatic IR injury occurs considerably during liver surgery and remains a main cause of concern in the post-surgical period. In this regard, the therapeutic improvement of the approaches in liver surgery requires the use of novel pharmacologic agents aimed at controlling post-protective outcome in liver surgery distress.

SM, which is known as milk thistle that consisted of polyphenol and flavonoid compounds, is extracted from the plant S*ilybum marianum*. It holds many therapeutic substances including antioxidant, anti-inflammatory, immunomodulatory, hepatoprotective, gastroprotective,

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anti-bacterial, anti-viral, vasodilatory and antithrombotic properties (Demir *et al*., 2014; Hellerbrand *et al*., 2016). Overall, SM is safe at therapeutic doses in humans and only transient adverse effects such as gastrointestinal upsets, slight headache, nausea, urticaria, itching, mild flushing and heat sensation in different applications have been experienced in some studies (Soleimani *et al*., 2019). A reduced form of glutathione, an important thiol compound, is present in high concentrations in hepatocytes. It works in synergy with cellular enzymatic and non-enzymatic antioxidants to neutralize and scavenge free radical species, thus preventing or reducing oxidative stress (Vairetti *et al*., 2007; Lushchak, 2012). GSH participates not only in antioxidant defense systems but also it is consumed in many metabolic processes, such as by oxidation, conjugation, and hydrolysis in cellular metabolism (Lushchak, 2012). Yet, no report in the literature evaluated the beneficial effect of SM plus GSH therapy on hepatic injury in liver IR. Thus, the aim of this work was to examine whether pretreatment with SM, GSH and their combination could prevent hepatic IR injury in a rat liver IR model.

MATERIAL AND METHODS

This work was approved by Ondokuz Mayıs University Ethics Committee amid the use of animals and care (ethics code: 3, 26 Oct.2016; the research code: pyo.tip.1904.17.004). Animals were obtained from the Experimental Animal Research and Application Center, in Samsun, Turkey. Then, the rats were housed in stainless steel cages in a thermally controlled room (22-24 °C and 12 hr light/dark cycle).Fifty male Sprague-Dawley rats (300-350 g) were let to acclimatize to the local environment for one week on a rodent standard laboratory diet prior to the experimental study. Animals had free access to food and water ad libitum. After an overnight fast, rats were subjected to surgical processing.

Experimental protocols and treatment groups

The rats were divided into five groups (n= 10) as follows: Sham, IR, SM-IR, GSH-IR, and SM plus GSH-IR. Each rat was anesthetized with an intraperitoneal injection of 3 mg/kg xylazine HCL (Rompun®, Bayer) and 80 mg/kg ketamine HCL (Ketalar®, Pfizer) for anesthetic sedation in the animals. Using sterile materials and techniques, a midline laparotomy was created under anesthesia in rats. Organ structures in the portal triad (portal vein, bile duct, and hepatic artery) to the left and median liver lobes were exposed and then occluded by using a nontraumatic microvascular clamp in order to perform hepatic ischemia for 30 minutes. The microvascular clamp was cautiously removed after 30 minutes, and the abdomen was immediately closed with a continuous 4-0 silk suture, followed by a 24-hour reperfusion period. In the sham group, the same volume of isotonic saline containing 10% of DMSO was injected intraperitoneally (i.p.) to the rats under anesthesia, and the rats were subjected to laparotomy as well as a display of the portal triad without hepatic ischemia. In the IR group, rats were subjected to clinical ischemia and reperfusion as stated above. SM (Solgar Co., Milk Thistle Standardized capsule) and reduced form of glutathione (Sigma Co., GSH) were respectively dissolved in isotonic saline supplemented with 10% of DSMO. SM-IR group received SM (100 mg/kg bw., i.p.); GSH-IR group received GSH (100 mg/kg bw., i.p.); and also their combination (SM plus GSH) was applied for SM plus GSH-IR group, 60 min prior to liver IR. Body temperature of the rats was kept close to 37 °C with the aid of a heating lamp. All rats were given an antibiotic (cefuroxime, 20 mg/kg/day) against a probably bacterial infection through i.m injection after surgical procedures. After a 24-hour period, the animals were anesthetized with ketamine and xylazine and the abdomen was reopened for collection of liver specimens and blood. Blood samples were drawn from the heart using an injector and gathered in tubes. Then, the rats were sacrificed by exsanguination. Serum was separated using a bench centrifuge at 3,000 g, collected in glass tubes and stored at -80 °C for subsequent biochemical analyses. The livers were rapidly removed, washed in isotonic saline solution and fixed in 10% neutral buffered formalin for histopathological examinations.

Biochemical analyses

Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were estimated to assess

the extent of hepatocellular injury using an automated chemical analyzer (Cobas 6000 C501). Myeloperoxidase (MPO) and tumor necrosis factor-alpha (TNF-α) levels in serum samples were determined using rat Elisa Kits (YL Biont, Cat No: YLA 0046RA; YLA 0118RA, respectively). Serum lipid peroxidation level was measured spectrophotometrically in terms of the thiobarbituric acid-reactive substances (TBARS) using malondialdehyde as standard (Singh, Padmavathi, Rao, 2000).

Histopathological study

The liver tissues were fixed in 10% neutral formaldehyde solution, embeddedin paraffin, cut into 4-µm sections, and stained with H&E (hematoxylin and eosin). The histological tissue sections obtained from the liver were examined with an optical microscope (Olympus BX51; Olympus Optical Co., Japan) by an expert pathologist who blinded to study design. The liver pathological lesions in the study groups were scored from 0 to 4 for sinusoidal congestion, vacuolization of hepatocyte cytoplasm, and hepatocyte necrosis as defined by Suzuki *et al.*, (1991).

Statistical analysis

IBM Statistics 22.00 software was used for the statistical analyses. For the normality assumption, data were initially analyzed by one-way analysis of variance. Afterwards, pairwise comparisons were performed using the Tukey multiple comparisons test. The Kruskal-Wallis analysis of variance was used to estimate the statistical significance of differences in the experimental groups in order to appraise the severity of the pathological lesions. The Mann-Whitney U test with Bonferroni's correction was used for comparisons between the groups. P values below 0.05 were statistically considered significant.

RESULTS

Effects of SM&GSH on liver function, inflammation and oxidative stress after liver IR

Hepatocellular damage was assessed by measuring serum ALT and AST values, which were significantly elevated after 24 h of IR injury in IR group compared to the sham group ($p< 0.001$). However, serum ALT and AST activities were lower in response to hepatic IR injury in rats treated with SM and GSH when compared to the IR group, and the difference was statistically significant ($p< 0.001$) as shown in Figure 1. Inflammatory marker TNF-α was significantly increased in rats of IR group as compared to sham ($p< 0.001$). Administration of SM and GSH attenuated elevations in TNF-α level in the groups SM-IR, GSH-IR, and SM plus GSH-IR as compared to the IR group ($p< 0.001$). Furthermore, hepatic IR injury triggered oxidative stress as indicated by augmentation of serum MPO ($p= 0.002$) and TBARS ($p< 0.05$) levels in rats of IR group when compared to sham rats. Administration of SM and GSH before hepatic IR alleviated oxidative stress by decreasing MPO and TBARS values in the SM and GSH groups when compared to the IR group ($p< 0.05$), (Table I).

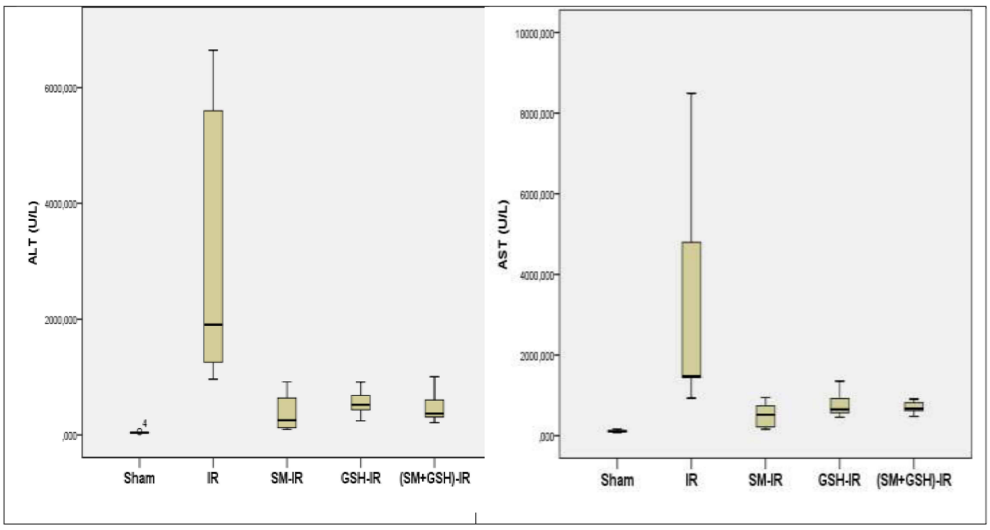

FIGURE 1 - Effects of SM (100 mg/kg bw., i.p.), GSH (100 mg/kg bw., i.p.), and their combination (SM plus GSH) pretreatment on hepatocellular damage after hepatic IR injury. Serum samples were obtained at 24 h in the experimental groups sham, IR, SM-IR, GSH-IR, and a mixture of SM plus GSH-IR for measuring alanine transaminase (ALT) and aspartate transaminase (AST) activity. ALT and AST activities were statistically lower in the SM-IR, GSH-IR and SM plus GSH-IR groups when compared to the IR group ($p< 0.001$).

Liver Pathological Findings

Histopathological examinations from rats' liver specimens after 30 min of ischemia and 24 h of reperfusion revealed significantly larger damage in the IR group compared to sham group as depicted in Figures 2&3 ($p< 0.001$). Notably; SM, GSH, and SM plus GSH administration prior to hepatic IR injury significantly attenuated total pathological damage scores in the treatment groups SM, GSH, and a mixture of SM plus GSH as compared to IR group ($p< 0.001$, $p= 0.013$, $P= 0.002$, respectively). Total pathologic damage scores of the liver on hepatic injury induced by IR were lower in SM plus GSH-IR group than in GSH-IR and SM-IR groups (Figure 3).

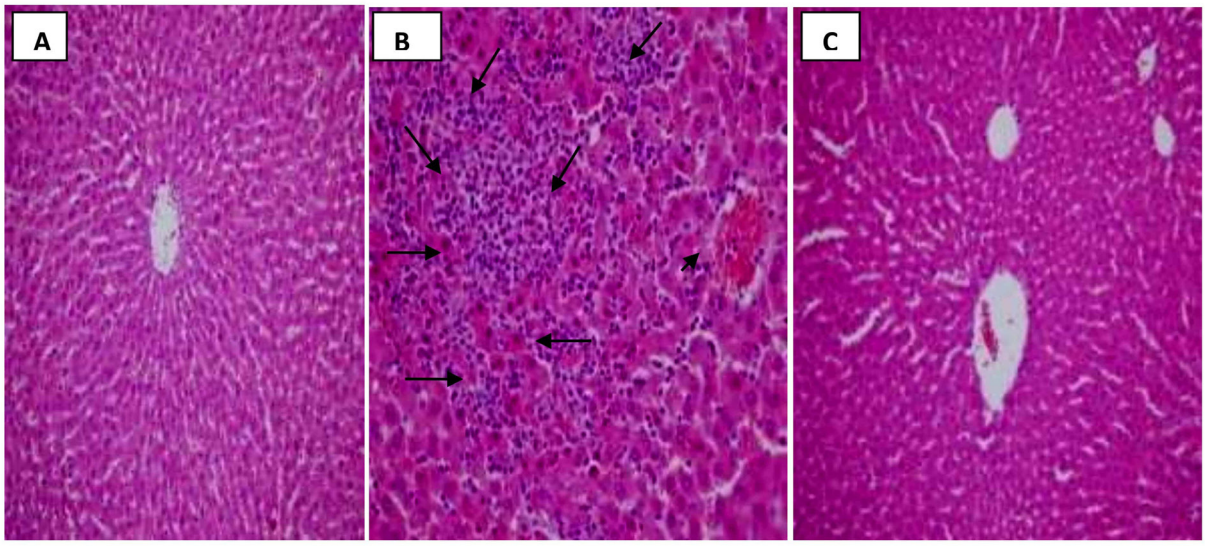

FIGURE 2 - (A-C) - Effects of SM plus GSH on the histopathological changes in liver tissue comparing sham, IR and SM plus GSH groups. Representative images of the liver pathological findings: **A**. Normal liver parenchyma histology but the presence of a little bit sinusoidal congestion within the liver parenchyma in the sham group, H&E X200. **B**. Wide infiltration of inflammatory cells (mostly neutrophils, [arrows]), hepatocyte necrosis and overt congestion [arrowhead] at 24 h of IR in the IR group, H&E X400. **C**. Slight congestion and sinusoidal dilatation in the liver tissue treated with a mixture of SM (100 mg/kg) plus GSH (100 mg/kg), 60 min prior to liver IR. H&E X200.

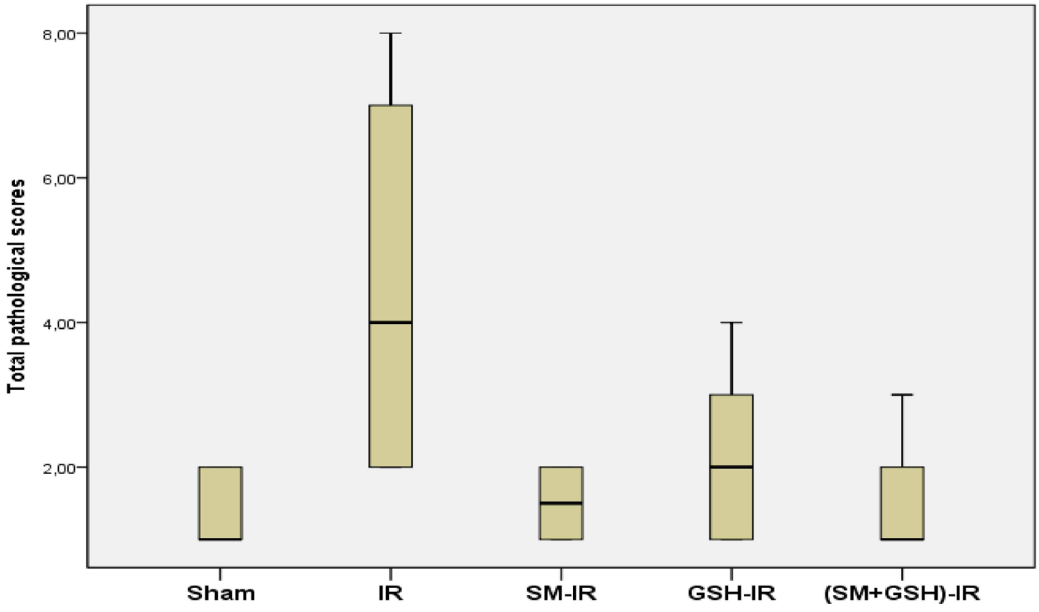

FIGURE 3 - The effects of SM (100 mg/kg bw., i.p.) and GSH (100 mg/kg bw., i.p.) on histopathological hepatic damage scores after 30-min hepatic ischemia and 24-h reperfusion. Animals that were treated with SM, GSH and their combination before hepatic IR injury had the lowest degree of total pathological findings in the liver tissue according to IR group rats. Also, total pathologic injuries induced by the liver IR were lower in SM plus GSH-IR group than in GSH-IR and SM-IR groups.

DISCUSSION

The results of current study mainly showed that pretreatment with SM and GSH could alleviate hepatic IR injury in a rat liver IR model. The hepatic damage induced by IR is associated with biochemical findings, e.g. that ALT, AST, TNF-α, MPO and TBARS levels in serum reduce in connection with administration of SM, GSH and a mixture of SM plus GSH prior to liver IR (Table I, Figure 1). In addition, it is noteworthy that SM plus GSH treatment before hepatic IR significantly decreased the liver histopathological damage scores on hepatic injury induced by IR (Figure 2&3).

TABLE I - Changes in TNF-α, MPO and TBARS levels in the study groups

Groups (n=10)	Parameters		
	TNF-α (ng/L)	MPO (ng/ml)	TBARS (µmol/L)
Sham	76.12 (50.61-98.79)	13.40 (10.50-17.73)	2.19 (0.94-3.76)
IR	160.10 (89.68-251.50)	21.06 (14.11-37.10)	2.56 (2.19-4.57)
SM-IR	56.21 (32.05-185.50)	17.45 (10.00-27.56)	0.86 (0.50-1.08)
GSH-IR	69.70 (54.12-102.70)	13.61 (11.51-22.37)	0.94 (0.66-1.21)
SM plus GSH-IR	77.65 (57.93-106.45)	13.62 (10.42-37.12)	0.87 (0.54-1.01)

Data are expressed as median (min-max) for parameters. IR, ischemia-reperfusion; SM, silymarin; GSH, reduced form of glutathione; TNF-α, tumor necrosis factor-alpha; MPO, myeloperoxidase; TBARS, the thiobarbituric acid-reactive substances. The treated groups were received SM (100 mg/kg bw., i.p.), GSH (100 mg/kg bw., i.p.) and their combination (SM plus GSH), 60 min prior to liver IR.

During liver surgery, either for intrahepatic liver lesion or for liver transplantation, a period of ischemia or total vascular exclusion can occur. In addition, oxidative stress, Kupffer cell activation, inflammation, immune cell activation, infiltration of inflammatory cells, pH paradox, mitochondrial permeability transition, hepatocellular damage, apoptosis and cell necrosis frequently happen in hepatic IR (Papadopoulos *et al.*,2013; Datta, Fuller, Davidson, 2013; Saidi, Kenari,2014; Nastos *et al.*,2014; Cursio, Colosetti, Guggenheim, 2015). The release of reactive oxygen/nitrogen species appears to compass two phases in ischemic-reperfused tissues. The first phase occurs immediately after reperfusion and extends for a few hours (~30 min–4 h) interested in a typical oxidative stress situation. In this phase, there is an increase in mitochondrial production of reactive oxygen species (ROS) with subsequent dysfunction and failure of oxidative phosphorylation in hepatocytes, endothelial cells, and likely activated Kupffer cells. The second phase, which starts ~4–6 h after the onset of reperfusion, extends for hours or days depending on the maintenance of the oxidative stress insults. This phase is associated to the appearance of irreversible tissue damage and inflammation, with neutrophils infiltration and macrophages recruitment. Activated neutrophils in turn engage myeloperoxidase, resulting in excess ROS, and also an intracellular source of ROS formation is especially xanthine oxidase and NADPH oxidase machinery (Alban *et al.*, 2018; Shokoohi *et al.*, 2018). Similar phases/processes can be seen in testicles by torsion/detorsion. A number of studies have shown that the overproduction of reactive free radicals in the liver induced by IR and in testicles affected by varicocele causes DNA detriments, lipid and protein damage, resulting in cellular injury and apoptosis (Alban *et al.*, 2018; Shokoohi *et al.*, 2020).

The extent of hepatocellular damage in liver IR injury is normally measured by elevation of serum ALT and AST enzyme levels which are released into the bloodstream when the liver is inflamed or injured. In the present study, we evaluated the protective effects of SM and GSH on hepatic IR injury. Serum ALT and AST values were markedly higher in the IR group compared to the sham group 24 h after liver IR injury. However, ALT and AST activities were significantly lower in the groups treated with SM, GSH and SM plus GSH prior to hepatic IR when compared to IR group (Figure 1). Previous studies have reported that pretreatment with SM significantly reduced plasma ALT and AST levels in the initial phase of liver injury after IR in comparison with untreated hepatic IR rats (Oliveira *et al*., 2001; Younis, Shaheen, Mahmoud, 2016). A research data declared that the GSH treatment prior to hepatic IR surgery significantly lowered the serum ALT and AST activities (Suyavaran *et al*., 2015). Thus, the administration of SM and GSH before IR seems to be hepatoprotective against liver IR injury in rats.

Oxidative stress is characterized by an excessive free radical production in relation to tissue injury and inflammation in response to IR injury appear to play a substantial role in the initial phase (~2-3 h after reperfusion) and the late phase (from 6 to 48 h after hepatic reperfusion) of liver IR (Teoh, Farrell, 2003; Glantzounis *et al*., 2005). TBARS that indicate lipid peroxidation induced by oxidative stress and increased serum MPO level after IR suggests activation of an inflammatory response and oxidative stress. TNF-α, one of the main inflammatory mediators of hepatic IR injury, is known to have deleterious effects on the liver parenchyma (Suyavaran *et al*., 2015; Zhang *et al*., 2015). Findings from previous studies have also shown that hepatic IR can cause oxidative stress and inflammation to liver tissue (Oliveira *et al*., 2001; Xue *et al*., 2008; Suyavaran *et al*., 2015; Younis, Shaheen, Mahmoud, 2016). SM (100 mg/kg/day, i.p.) therapy for seven days attenuated the oxidative and intestinal damage induced by mesenteric IR (Demir *et al*., 2014). Treatment by SM (100 mg/kg, i.v.) 15 minutes before reperfusion declined the serum levels of nitrite and TNF-α, while the values of IL-10 were risen (Younis, Shaheen, Mahmoud, 2016). GSH (200 mg/kg bw., i.p.) treatment, 30 minutes prior to liver IR significantly lowered the hepatic TBARS level and TNF-α level in both serum and liver (Suyavaran *et al*., 2015). As our results revealed, significantly high levels of serum TBARS, MPO and TNF-α in rats subjected to hepatic IR injury without SM and GSH treatment were observed. However, TNF-α, MPO and TBARS values were decreased in animals treated with SM and GSH prior to IR when compared to IR group rats (Table I). Moreover, the severity and extent of the liver pathological insults were observed to be higher in the IR group compared to the sham group. Based on these pathological findings, pretreatment with SM and GSH appeared to decrease the pathological insults on the hepatic parenchyma caused by IR injury (Figure 2). Therefore, pretreatment of SM plus GSH appears to have a beneficial effect on liver IR injury. In these regards, beneficial impacts of SM and GSH are currently reported during IR (Suyavaran *et al*.,2015;Akbari-Kordkheyli *et al*., 2019).

As reported initially, SM alone possesses many valuable effects such as proper scavenging of free radicals and chelating free Cu and Fe, impeding free radical formation by inhibiting free radical-producing enzymes, ensuring the integrity of mitochondria or the cell in cellular stress conditions, keeping an optimal redox balance in the cell, decreasing inflammatory responses by inhibiting NF-κB pathways in liver toxicity and various liver diseases included hepatic IR injury in animal and human studies (Wellington, Jarvis, 2001; Abenavoli *et al*., 2010; Surai, 2015; Wang *et al*., 2018). SM is also known as a potent pharmacological agent with anti-oxidant and anti-inflammatory properties as well as raising patient serum levels of GSH (Wellington, Jarvis, 2001; Akbari-Kordkheyli *et al*., 2019; Heidarian, Nouri, 2019). GSH is to protect the liver against oxidative stress included the detoxification of endogenously produced toxicants by rather different mechanisms (Bilzer *et al*., 2002; Vairetti *et al*., 2007; Suyavaran *et al*., 2015; Kantah *et al*., 2016). The limitations of this study, is the firstly, through the studied dose of SM plus GSH therapy was chosen according to likely physiological dose, it is noteworthy that the results of the current study are limited due to using a single dose of SM plus GSH, and further examination of several doses is needed to ratify

the current conclusion. Secondly, this study lacks the evaluation of inflammatory response, oxidative stress, apoptosis and cell death that are the major causes of IR injury in liver tissues.

Consequently, we noticed the therapeutic effects of SM plus GSH administration in a rat model of liver IR. Our findings demonstrated that the application of SM plus GSH prior to surgery could protect effectively the liver against oxidative stress, inflammation and hepatic IR-induced injuries confirmed by histopathological observations of liver tissues. Additional studies are needed in order to test the hypothesis that SM plus GSH therapy before liver IR is able, in the experimental arena or clinical practice, to attenuate the hepatic damage from IR injury.

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CONFLICT OF INTEREST

Authors declare no conflicts of interest.

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