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Research Article

Sulfasalazine-Induced Hepatic Injury in an *Ex Vivo* Model of Isolated Perfused Rat Liver and the Protective Role of Taurine

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ABSTRACT

Background: Sulfasalazine is one of the most commonly administered drugs for the treatment of rheumatoid arthritis and inflammatory bowel disease in humans. On the other hand, acute liver failure led to liver transplantation and/or patient death might occur after sulfasalazine administration. There is no precise mechanism for hepatic injury induced by sulfasalazine and no specific hepatoprotective agent has been developed against this complication. Current investigation was designed to study oxidative stress as a proposed mechanism for sulfasalazine-induced liver injury and evaluate the role of taurine administration as a safe hepatoprotective agent. ***Methods:*** Rat liver was isolated after cannulation through portal vein and perfused with Krebs-Henseleit buffer. The liver was exposed to different concentrations of sulfasalazine and taurine. ***Results:*** Sulfasalazine (5 mM) administration caused significant hepatic injury as judged by elevation in liver perfusate level of LDH, AST, ALT, and potassium ion (K^+). Significant amounts of reactive oxygen species (ROS) and lipid peroxidation were detected in sulfasalazine treated livers. Furthermore, hepatic glutathione reservoirs were depleted. Histopathological examination of liver tissues confirmed the above mentioned biochemical data. Co-administration of taurine (5, 10 and 20 mM), significantly mitigated sulfasalazine-induced hepatic injury in isolated rat liver. ***Conclusion:*** The data obtained from current investigation indicate potential therapeutic properties of taurine against sulfasalazine-induced liver injury.

Introduction

Drug-induced liver injury (DILI) is a major cause of morbidity and mortality among patients.[1] Many pharmaceuticals are reported to have adverse effects on liver.[2-4] Sulfasalazine is widely administered against rheumatoid arthritis in humans. On the other hand, several cases of sulfasalazine-induced liver injury have been reported.[5,6] Sulfasalazine-induced liver injury might occur in a hepatic necrosis or sudden hepatic failure manner which finally leads to patients' death.[7,8] Despite intense research, there are still no specific therapies against different cases of drug-induced liver injury. Hence, finding safe hepatoprotective agents with therapeutic potential might have a great clinical value.

Taurine is the most abundant amino acid in human body which not corporates into protein structures.[9] Many physio/pharmacological properties including antioxidative and osmoregulatory effects are attributed to taurine.[9-11] Furthermore, this amino acid has been reported to protect vital organelles such as mitochondria against toxic insults.[12-14] Taurine has also been reported to act as a hepatoprotective compound against many xenobiotics, including several drugs.[15-19] Moreover, it has been shown that taurine serves as a hepatoprotective agent in different experimental models of liver disease.[20,21]

Although the precise mechanism of liver injury induced by sulfasalazine is not clear, several lines of evidence suggest that oxidative stress, at least in part, is responsible for the liver injury induced by this drug.[22,23] Hence, hepatoprotective agents with antioxidant capabilities might be effective against sulfasalazine-induced liver injury.

Current investigation was designed to evaluate oxidative stress as a proposed mechanism of liver injury induced by sulfasalazine and investigate the role of taurine administration in an *ex vivo* model of isolated perfused liver.

Materials and Methods

Chemicals

Hydroxymethyl aminomethane (Tris), trichloroacetic acid (TCA), phosphoric acid, and

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Ethylenediaminetetraacetic acid (EDTA), were purchased from Merck (Darmstadt, Germany). Taurine, Thiobarbituric acid (TBA), 2', 7'-Dichlorofluorescin diacetate (DCFDA), and 5, 5´-Dithionitrobenzoic acid (DTNB), were purchased from Sigma-Aldrich (St. Louis, MO). Kits for liver biochemistry assay (ALT, AST, and LDH) were purchased from Pars Azmun (Tehran, Iran). All salts used for preparing buffer solutions were of analytical grade and obtained from Merck (Darmstadt, Germany).

Isolated perfused liver

Isolated perfused liver (IPL) is an appropriate experimental tool to study the pharmacological response of this organ in the absence of extrahepatic influences.[24,25] This model is widely employed to investigating the mechanisms of liver injury induced by different xenobiotics.[25,26] In this method liver is cannulated via portal vein and perfused with hemoglobin- and albumin-free Krebs-Henseleit buffer.[25] Hence, the organ will be in contact with the accurate concentrations of the xenobiotics.

In current investigation, rat liver was exposed to different concentrations of sulfasalazine and taurine and the perfusate level of LDH, AST, ALT, and K^+ were monitored. Furthermore, liver tissue ROS formation, lipid peroxidation and glutathione content were assessed. After all, liver histopathological changes were evaluated to investigate if taurine has any protective properties against sulfasalazine-induced liver injury.

Liver isolation and perfusion

Male Sprague-Dawley rats (200-300 g) were purchased from the Laboratory Animal Breeding Center, Shiraz University of Medical Sciences, Shiraz, Iran. Animals were housed in plastic cages over hardwood bedding and allowed free access to food and tap water. The experimental protocols followed the ethical guidelines of the Faculty of Pharmacy, Shiraz University of Medical Sciences, for the care and use of laboratory animals. After anesthetizing the animals with pentobarbital (60 mg/kg, i.p.), livers were cannulated via the portal vein with hemoglobin- and albumin-free Krebs-Henseleit buffer (pH 7.4, 37°C) gassed with carbogen (95% O_2, 5% CO_2).[25] The perfusate was pumped through livers with a peristaltic pump at a flow rate of 3.0-3.5 ml/min/g liver weight, in a re-circulating mode.[25] Total volume of circulating buffer was 200 mL in all experiments.

Study procedures

Isolated perfused rat liver was exposed to different concentrations of sulfasalazine and taurine. Hepatic injury was determined at scheduled time intervals to evaluate the effects of various concentrations of sulfasalazine on rat liver. In current study, the injurious concentration of the drug is reported as the concentration, which leads to significant rise in all assessed biomarkers of liver injury after 120 minutes of organ perfusion. Treatments for sulfasalazine concentration-response in isolated perfused rat liver were: A) Krebs-Henseleit buffer perfused livers (Control group) (n=4); B) Sulfasalazine 250 µM (n=4); C) Sulfasalazine 500 µM (n=4); D) Sulfasalazine 1 mM (n=4); E) Sulfasalazine 5 mM (n=4). Taurine-treated livers were also divided into the three following groups: A) Sulfasalazine 5 mM + Taurine 5 mM (n=4); B) Sulfasalazine 5 mM + Taurine 10 mM (n=4); C) Sulfasalazine 5 mM + Taurine 20 mM (n=4).

Samples were taken from liver perfusate at different time points and assessed for biomarkers of liver injury. At the end of each experiment (180 minutes), liver samples were tested for ROS formation, lipid peroxidation, glutathione content and histopathological changes. Spectrophotometric scanning of the perfusate solutions confirmed that there was no physicochemical interaction between taurine and sulfasalazine in current system. Taurine caused no sign of liver injury when it was administered alone at the mentioned concentrations. All chemicals were dissolved in Krebs-Henseleit buffer.

Perfusate biochemistry and electrolytes

Mindray BS-200® auto analyzer and Pars Azmun® standard kits were used to measure ALT, AST, and LDH in liver perfusate at different time points.[27,28] Perfusate K^+ level was analyzed by an IL943- Flame Photometer (Sysmedlabs®).

Reactive oxygen species (ROS) assay

Liver tissue (500 mg) was homogenized in 5 mL of ice-cold Tris-HCl buffer (40 mM, pH=7.4). The resulted tissue homogenate (100 µL) was mixed with Tris-HCl buffer (1 mL) and 5 µL of 2′, 7′-dichlorofluorescein diacetate (1µM). The mixture was incubated for 30 minutes in 37ºC in a Gyromax® incubator shaker. Finally, the fluorescence intensity of the samples were assessed using a multifunctional microplate reader (FLUOstar Omega®) with $\lambda_{excitation}$=485 nm and $\lambda_{emission}$=525 nm.[29,30]

Lipid peroxidation assessment

Level of lipid peroxidation in liver tissue was assessed by measuring thiobarbituric acid reactive substances (TBARS) in different experimental groups. The reaction mixture consist of thiobarbituric acid (0.6%, w/v), phosphoric acid (1% w/v, pH=2), and 0.5 mL of tissue homogenate. The mixture was shaken, and then heated in boiling water (100 °C) for 45 minutes. After the incubation period, mixture was cooled, and then 2 mL of n-butanol was added and vigorously shaken. Samples were centrifuged (3000 g for 5 minutes) and the absorbance of developed color in n-butanol phase was read at 532 nm using an Ultrospec 2000®UV spectrophotometer. [31]

Hepatic glutathione content

Liver glutathione content was assessed by a method described by Sedlak *et al*.[32] Briefly, samples of liver (200 mg) were homogenized in 8 mL of ice-cooled EDTA (20 mM). Five milliliters of liver homogenate were mixed with 4 mL of distilled water and 1 mL of trichloroacetic acid (50% w/v). The mixture was shaken and centrifuged (10000 *g*, 4°C) for 25 minutes. Afterwards, 2 mL of the supernatant was mixed with 4mL of Tris buffer (pH=8.9), and 100 µl of DTNB (0.01 M in methanol). The absorbance of developed color was measured in 412 nm using an Ultrospec 2000® UV spectrophotometer.[31]

Liver histopathological evaluation

Samples of liver were fixed in buffered formalin solution (0.4% sodium phosphate monobasic, NaH_2PO_4, 0.64% sodium phosphate dibasic, Na_2HPO_4, and 10% formaldehyde in distilled water). Paraffin-embedded sections of liver were prepared and stained with haematoxylin and eosin (H&E) before light microscope viewing.

Statistical Analysis

All data are expressed as the Mean±SEM. Comparison of data sets was performed by the one way analysis of variance (ANOVA) with Tukey's test as the *post hoc* test. Differences were considered significant when $p<0.05$.

Results

Different concentrations of sulfasalazine were perfused to rat liver (Table 1 & 2). It was found that sulfasalazine (5 mM) administration resulted in a significant increase of LDH, K^+, AST, and ALT leakage from rat liver as compared to control group (Tables 1 & 2). The 5 mM concentration of sulfasalazine was considered as the injurious dose of the drug in current system, and used for further experiments.

Table 1. Liver perfusate K^+ and LDH content after administration of different concentrations of sulfasalazine.

	Perfusate K^+ content (mmol/l)			**Perfusate LDH level (U/l)**		
Treatment	Time (Hour):			Time (Hour):		
	1	**2**	**3**	**1**	**2**	**3**
Control (Only buffer)	5.34±0.52	4.73±0.3	6.46±0.6	4±2	9±4	21±4.6
+ Sulfasalazine 250 µM	4.01±0.6	5.7±0.44	5.95±0.6	18±2.44	26±6	87±36
+ Sulfasalazine 500 µM	4.38±0.47	4.98±0.4	4.65±0.27	18±3.5	34±12	110±8 *
+ Sulfasalazine 1 mM	6.23±0.24	5.4±0.32	5.45±0.18	15±5	24±9	137±27 *
+ Sulfasalazine 5 mM	8.84±0.8 *	10.24±0.51*	11.2±0.3 *	138±59 *	387±114*	1146±223*

Samples of liver perfusate (500 µl) were taken at different time intervals (every 1 hour) and assessed for the biomarkers of liver injury.
Data are presented as Mean±SEM for at least four independent experiments.
*Indicates significantly higher values as compared with control livers (Only buffer) ($P<0.05$).

Table 2. Effect of different concentrations of sulfasalazine on ALT and AST level of liver perfusate.

	Perfusate AST level (U/l)			**Perfusate ALT level (U/l)**		
Treatment	Time (Hour):			Time (Hour):		
	1	**2**	**3**	**1**	**2**	**3**
Control (Only buffer)	14±4	34±3.75	39±7.8	7±1.4	25±6	36±11
+ Sulfasalazine 250 µM	18±2.45	37±8	78±21	7±3	20±4	69±25
+ Sulfasalazine 500 µM	18±3.5	57±23	97±18	18±3	64±28	87±12
+ Sulfasalazine 1 mM	19±8	46±17	109±25*	27±6	45±17	51±22
+ Sulfasalazine 5 mM	276±69*	426±46*	690±89*	119±9*	208±16*	518±75*

Samples of liver perfusate (500 µl) were taken at different time intervals (every 1 hour) and assessed for the biomarkers of liver injury.
Data are given as Mean±SEM for at least four independent experiments.
*Indicates significantly higher values when compared to control (only buffer) livers ($P<0.05$).

Sulfasalazine (5 mM) administration increased biochemical biomarkers of hepatic injury, including K^+, LDH, AST, and ALT in liver effluent (Table 2 &3). Moreover, a significant amount of ROS (Figure 1) accompanied with lipid peroxidation (Figure 2), and hepatic glutathione depletion (Figure 3) was detected in sulfasalazine-treated livers. Furthermore, significant liver histopathological changes, including tissue necrosis, sinusoidal dilation, and hepatocellular vacuolization were observed in drug-treated group (Figure 4).

Different concentrations of taurine (5, 10 and 20 mM), were administered to evaluate its protective properties against sulfasalazine-induced liver injury. It was found that, taurine effectively alleviated sulfasalazine-induced liver injury as evidenced by a significant decrease in leakage of hepatic injury markers (ALT, AST, LDH and K^+) in perfusate buffer (Tables 3 & 4).

Table 3. Effect of taurine on liver perfusate LDH and K^+ level after sulfasalazine administration.

	Perfusate K^+ content (mmol/l)			**Perfusate LDH level (U/l)**		
	Time (Hour):			Time (Hour):		
Treatment	**1**	**2**	**3**	**1**	**2**	**3**
Control (Only buffer)	4.78±0.3	5.63±0.41	6.1±0.2	3.66±2	6±2.3	14.6±4
+ Sulfasalazine 5 mM	9.3±0.54 *	11±0.35 *	12±0.43 *	154±38 *	342±121 *	1232±198 *
+ Taurine 5 mM	7.6±0.3[a]	9.4±0.1	9.7±1	71.2±8[a]	483±133	842±272
+ Taurine 10 mM	6.17±0.1[a]	6.6±0.4[a]	7.7±0.4[a]	25±6.4[a]	142.5±62[a]	244±38[a]
+ Taurine 20 mM	7.1±0.3[a]	7.8±0.3[a]	8.8±0.7[a]	53±4.66[a]	119±35[a]	661±46[a]

Samples of liver perfusate (500 µl) were taken at different time intervals (every 1 hour) and assessed for the biomarkers of liver injury.
Data are obtained from four independent experiments and represented as Mean±SEM.
*Indicate significantly higher values as compared with control (Only buffer) group (P<0.05).
[a] Indicates significantly lower values as compared with sulfasalazine only-treated livers (P<0.05).

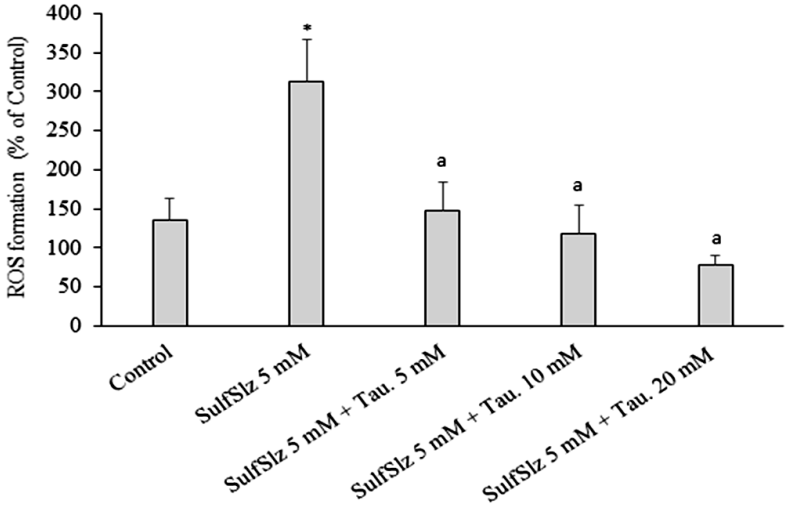

Figure 1. Reactive oxygen species (ROS) formation in isolated perfused rat liver after sulfasalzine administration. SulfSlz: Sulfasalazine; Tau.: Taurine.
Data are geiven as Mean±SEM for four independent experiments.
*Indicates significantly higher value as compared with control livers (P<0.05).
[a] Shows significantly lower value as compared with sulfasalazine-treated livers (P<0.05).

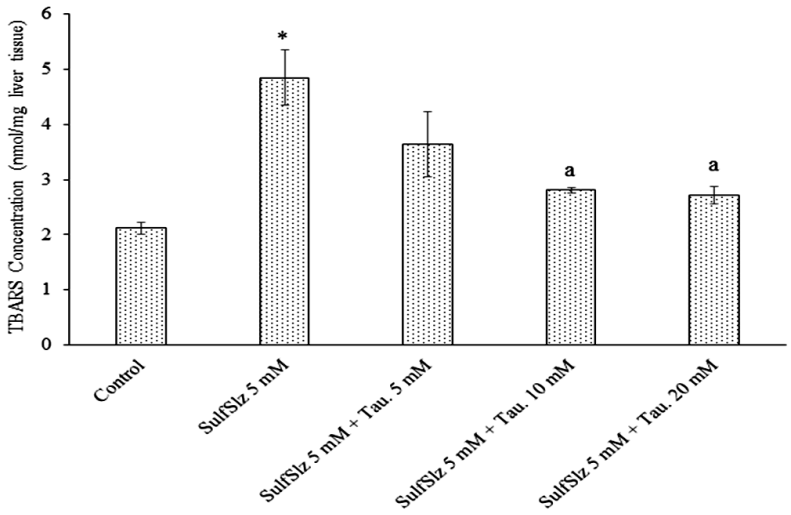

Figure 2. Sulfasalazine-induced lipid peroxidation in isolated perfused rat liver.
Data are given as Mean±SEM for four independent experiments. Lipid peroxidation was evaluated after 180 minutes of liver perfusion. SulfSlz: Sulfasalazine; Tau.: Taurine.
*Indicates significantly higher values than control group (P<0.05).
[a] Indicates significantly lower values as compared with sulfasalazine only-treated livers (P<0.05).

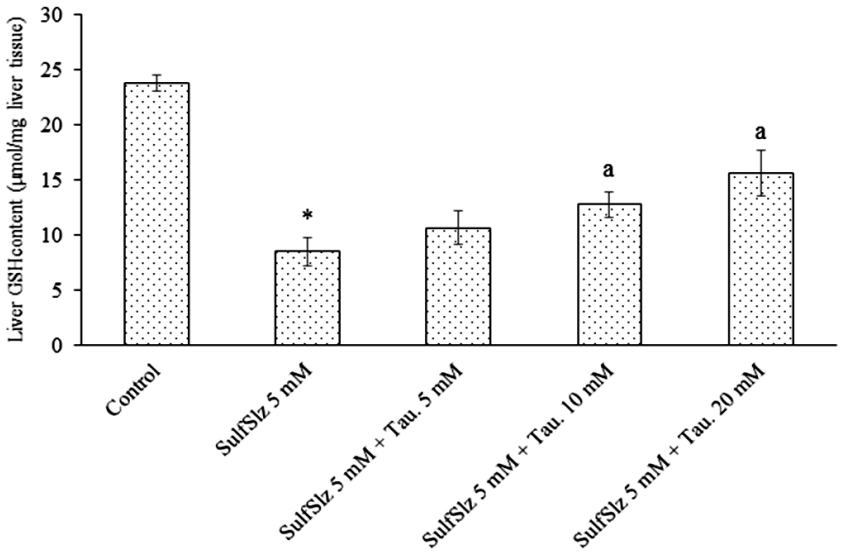

Figure 3. Liver glutathione content in sulfasalazine and/or taurine-treated groups. Data are represented as Mean±SEM for four independent experiments as assesssed after 180 of liver perfusion. SulfSlz: Sulfasalazine; Tau.: Taurine.
*Indicates significantly lower than control group (P <0.05).
[a] Indicates significantly higher as compared with sulfasalazine-treated livers (P<0.05).

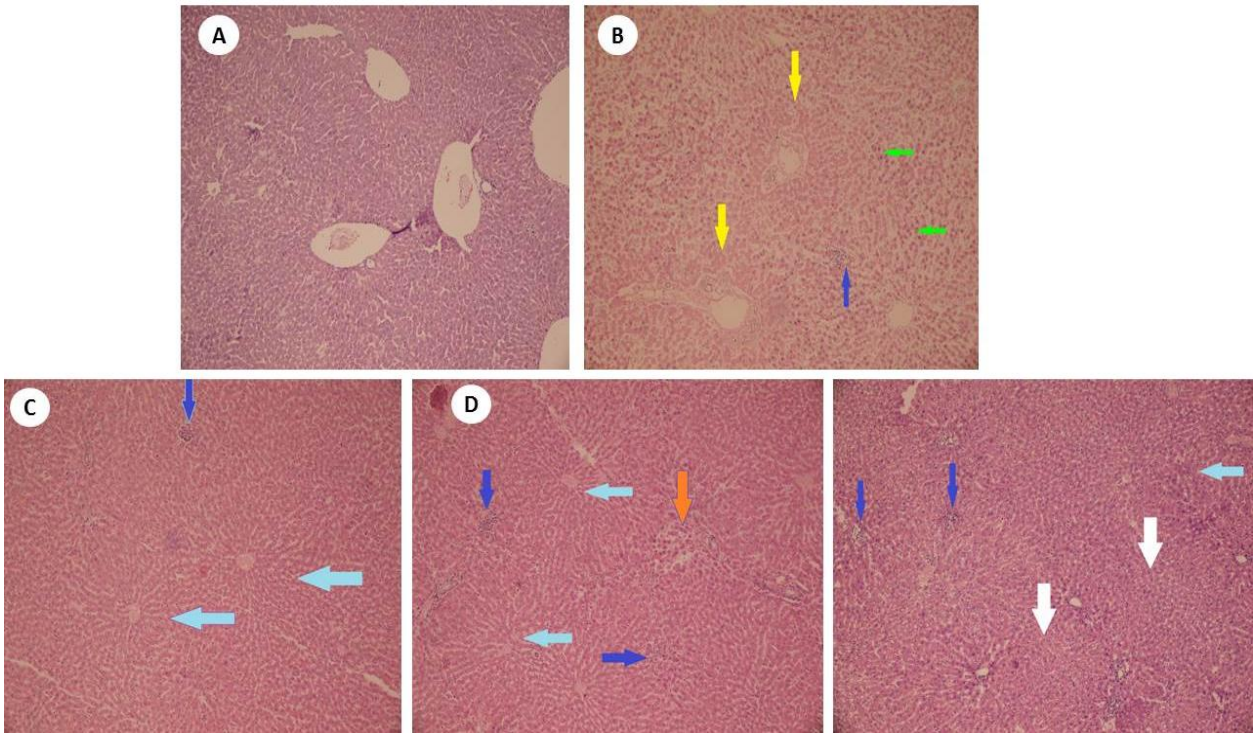

Figure 4. Liver histopathological changes after sulfasalazine administration in isolated rat liver. **A**: control group perfused with Krebs-Henseleit buffer for 180 minutes. No significant pathological change except for a mild sinusoidal dilation was detected in this group. **B**: Sulfasalazine (5 mM) was perfused to isolated rat liver for 180 minutes. Sinusoidal space dilation, hepatocellular necrosis and vacuolization were observed in this group. **C, D & E**: rat livers were perfused with sulfasalazine (5 mM) and Taurine (5, 10 and 20 mM respectively). Sinusoidal dilation and hepatocellular vacuolization were detected (C-E) but, no sign of cell necrosis observed in these groups. Guide for marked lesions: Yellow: Necrosis; Green and Orange: Apoptosis; Deep Blue: Inflammation; Pale Blue: Sinusoidal congestion; White: Hydropic changes.

ROS formation and lipid peroxidation were also lower in the presence of taurine as compared to sulfasalazine-treated livers (Figure 1 and 2). Moreover, taurine also prevented the decrease in hepatic glutathione reservoirs induced by sulfasalazine (Figure 3). After all, it was found that sulfasalazine-induced liver histopathological lesions were mitigated by different concentrations of taurine (Figure 4). There was no sign of liver necrosis when taurine was administered to sulfasalazine-treated livers (Figure 4).

Table 4. Effect of taurine on perfusate AST and ALT level after sulfasalazine administration to isolated rat liver.

	Perfusate AST level (U/l)			Perfusate ALT level (U/l)		
	Time (Hour):			Time (Hour):		
Treatment	1	2	3	1	2	3
Control (Only buffer)	9.54±4.13	25.33±6.4	44.17±8.75	4.5±1.2	34.3±3.8	39±7.8
Table 4 Continued.						
+ Sulfasalazine 5 mM	301±86 *	397±55 *	544±92 *	101±16*	186±21*	492±23*
+ Taurine 5 mM	87±12.4 [a]	326±54.2	616±81.2	66.6±13.5 [a]	203±76	566±39
+ Taurine 10 mM	91.4±21.3 [a]	141±15.6 [a]	163±26 [a]	30.8±14.2 [a]	77.5±18 [a]	172.5±54 [a]
+ Taurine 20 mM	83±15 [a]	150±30.4 [a]	485±71 [a]	34±6.33 [a]	111±24 [a]	147.2±31 [a]

Samples of liver perfusate (500 µl) were taken at different time intervals (every 1 hour) and assessed for the biomarkers of liver injury.
Data are given as Mean±SEM for four livers in each experiment.
* Shows significant difference as compared with control (Only buffer group) (P<0.05).
[a] Indicates significantly lower values than sulfasalazine only-treated group (P<0.05).

Discussion

Sulfasalazine (5 mM) caused liver injury in isolated perfused rat liver system as revealed by increase in the liver perfusate level of LDH, AST, ALT and K^+. Furthermore, a significant amount of ROS formation accompanied with lipid peroxidation, liver glutathione depletion and histopathological changes of the organ were detected after sulfasalazine administration. Different concentration of taurine (5, 10, and 20 mM) effectively alleviated sulfasalazine-induced liver injury.

Although further investigation about the mechanism(s) of liver injury induced by sulfasalazine is still needed, some studies indicate the role of oxidative stress and its consequent events in sulfasalazine-induced hepatic and/or other organs injury.[22,23,33] Sulfasalazine is cleaved to sulfapyridine and mesalazine by bacterial azoreductases in large intestine. Sulfapyridine is almost completely absorbed compared with about 20–30% absorption for mesalazine. Approximately 10-30% of the parent drug is also absorbed from the small intestine.[34] It is not clear whether the whole molecule of sulfasalazine and/or its intestinal metabolites are responsible for the oxidative stress induction and finally the liver injury induced by this drug. More investigations on the fate of sulfasalazine and its intestinal metabolites in liver might shed light on the mechanisms of liver injury induced by this drug. ROS formation (Figure 1) and lipid peroxidation (Figure 2) in addition to hepatic glutathione depletion (Figure 3) in current investigation, might indicate the role of oxidative stress in sulfasalazine-induced liver injury. Defect in enzymatic cellular defense mechanism is reported after sulfasalazine administration to animals.[22] Therefore, at least in part, oxidative stress seems to be a mechanism underlying liver injury induced by sulfasalazine. Oxidative stress has been recognized as a pivotal mechanism contributing to the toxicity of many xenobiotics, including several drugs.[35-37] Since glutathione is utilized as the primary reducing agent in cell, its depletion might ensue cell death. Taurine might provide protection against sulfasalazine through its potential antioxidative properties (Figure 5). Moreover, this amino acid is a good membrane stabilizer and might prevent defects in cell membrane induced by drugs such as sulfasalazine (Figure 5).[23]

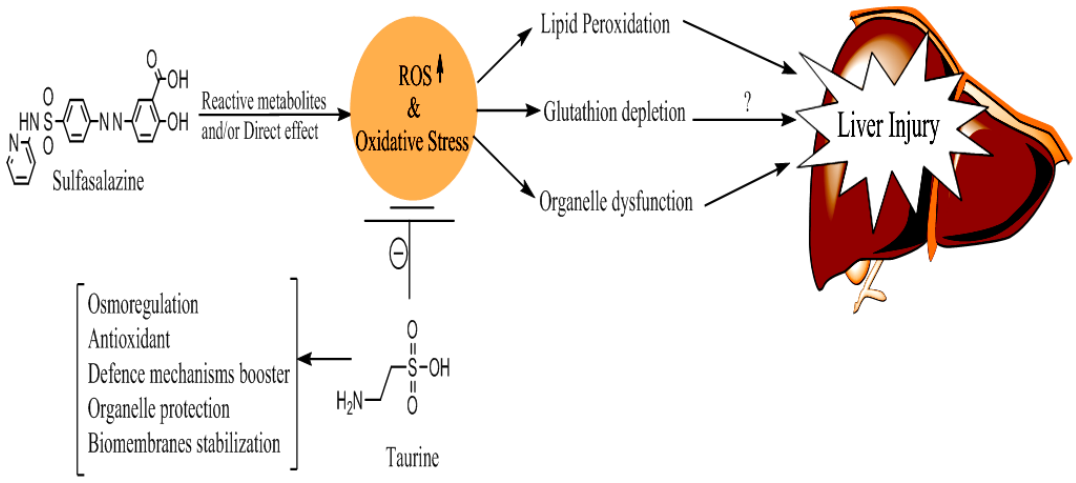

Figure 5. The proposed mechanisms for hepatoprotective effects of taurine against sulfasalazine-induced liver injury. ROS: reactive oxygen species.

Taurine effectively ameliorated rise in perfusate K^+ ion in current study (Table 3). As potassium is the most abundant intracellular ion, it might be concluded that taurine administration prevented cell swelling and rupture induced by sulfasalazine. After all, taurine prevented a decrease in hepatic glutathione content which might be another mechanism involved in the protective properties of this amino acid in current investigation (Figure 5). Different concentrations of taurine (5, 10 and 20 mM) alleviated sulfasalazine-induced liver injury dose dependently. Taurine (5 mM) prevented elevation in perfusate level of ALT, AST, LDH and K^+ at first hour of liver perfusion (Tables 1-4) but had no significant effect on lipid peroxidation (Figure 2), and/or hepatic glutathione content (Figure 3). Other taurine concentrations (10 mM and 20 mM) effectively alleviated all investigated biomarkers of liver injury in current investigation.

Although no specific therapeutic option has been developed against sulfasalazine-induced liver injury, some investigations mentioned the beneficial role of corticosteroids and/or N-acetylcysteine in human cases of sulfasalazine-induced hepatic damage.[38-40] However, some serious problems such as critical dependence upon corticosteroid therapy has been developed.[40] Hence, finding new and safe therapeutic options against drug-induced liver injury has great clinical value.

Taurine is reported to act as a hepatoprotective compound against drug-induced liver injury.[15-19] Moreover, it has been shown that this amino acid serves as a hepatoprotective agent in different experimental models of liver disease.[20,21,41] Taurine is present in different animal tissue at high concentrations.[42] Furthermore, it has been shown that this amino acid caused no significant adverse effect in human even in very high doses.[43-45] Therefore, taurine might be a potential safe hepatoprotective agent against sulfasalazine-induced liver injury. On the other hand, it has been shown that taurine might act as an anti-inflammatory agent which blocks cytokine release.[46,47] Since different cytokines have a central role in diseases such as rheumatoid arthritis, which sulfasalazine is administered for,[48] taurine might be capable of being administered as a supplemental agent in combination with sulfasalazine therapy. Taurine safety even at very high doses,[49] makes it a suitable agent for chronic administration in humans. Obviously, recommending hepatoprotectants as prophylactic and/or therapeutic options need more investigations in different experimental models and finally in clinical trials. Hence, further investigations are required to endorse the hepatoprotective effects of taurine against sulfasalazine-induced liver injury in other experimental models and finally in clinical situations.

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Conflict of interests

The author claims that there is no conflict of interest.

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