



Effect of Thiol-reducing Agents and Antioxidants on Sulfasalazine-induced Hepatic Injury in Normotermic Recirculating Isolated Perfused Rat Liver

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Sulfasalazine is a widely administered drug against inflammatory-based disorders in human. However several cases of liver injury are associated with its administration. There is no stabilized safe protective agent against sulfasalazine-induced liver injury. Current investigation was designed to evaluate if N-acetylcysteine (NAC) and dithioeritol (DTT) as thiol reducing agents and/or vitamins C and E as antioxidants have any protective effects against sulfasalazine-induced hepatic injury in an *ex vivo* model of isolated rat liver. Rat liver was cannulated and perfused via portal vein in a closed recirculating system. Different concentrations of sulfasalazine and/or thiol reductants and antioxidants were administered and markers of organ injury were monitored at different time intervals. It was found that 5 mM of sulfasalazine caused marked liver injury as judged by rise in liver perfusate level of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) ($p < 0.05$). A significant amount of lipid peroxidation and hepatic glutathione depletion were detected in drug-treated livers, accompanied with significant histopathological changes of the organ. Administration of NAC (500 μ M), DTT (400 μ M), Vitamin C (200 μ M), or vitamin E (200 μ M) significantly alleviated sulfasalazine-induced hepatic injury in isolated perfused rat liver. The data obtained from current investigation indicate potential therapeutic properties of thiol reductants and antioxidants against sulfasalazine-induced liver injury.

Key words: Dithioeritol, Drug-induced liver injury (DILI), Glutathione, Hepatoprotection, N-acetyl cysteine, Oxidative stress

INTRODUCTION

Drug-induced liver injury (DILI) is a major clinical complication developed after administration of many pharmaceuticals (1-4). Sulfasalazine is widely used in inflammatory bowel disease and rheumatoid arthritis (5). However, hepatic injury due to sulfasalazine administration remains a clinical challenge. Several cases of sulfasalazine-induced liver injury are reported (6,7), which might even be fatal (8,9). The frequency of serious hepatic events associated with sul-

fasalazine administration is estimated at 0.4% of treated patients (10,11). The precise mechanism(s) of hepatic injury induced by sulfasalazine is not fully understood. Moreover, there is no effective protective agent against this complication in human.

Some investigations proposed the role of oxidative stress and its consequent events in sulfasalazine-induced liver injury (12,13). Impairment of cellular defense mechanisms against oxidative stress is also reported after sulfasalazine administration to animals (12,14). Hence, it seems reasonable that antioxidants and thiol reducing agents such as N-acetyl cysteine (NAC) could reverse and/or alleviate liver injury induced by sulfasalazine.

Isolated organs are useful models for examining xenobiotics-induced injury without the complication of many interacting factors which are difficult to control in other experimental models (15,16). Current study was designed to investigate the role of oxidative stress, in sulfasalazine-induced hepatic injury and finding a hepatoprotective agent with therapeutic potential against this complication. Different antioxidants and thiol-reducing agents were adminis-

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Abbreviations: DILI, Drug-induced liver injury; SSZ, Sulfasalazine; ROS, Reactive oxygen species.

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tered to evaluate if they are able to alleviate sulfasalazine-induced liver damage. Different concentrations of drug and hepatoprotective agents were perfused to liver and the biomarkers of hepatic injury including AST, ALT, and LDH were monitored in liver effluent buffer. Hepatic glutathione content and the amount of lipid peroxidation were assessed as markers of oxidative stress-induced tissue injury. Moreover, histopathological changes of the organ was evaluated at the end of each experiments.

MATERIAL AND METHODS

Chemicals. N-acetyl cysteine (NAC), and Dithioeritol were obtained from Acros (New Jersey, USA). Trichloroacetic acid (TCA), Ethylenediaminetetraacetic acid (EDTA), Vitamin E, and Vitamin C were obtained from Merck (Darmstadt, Germany). Kits for evaluating biomarkers of liver injury were obtained from Pars Azmun (Tehran, Iran). Thiobarbituric acid (TBA) was obtained from SERVA (Heidenberg, New York). All salts used for preparing buffer solutions were of analytical grade and obtained from Merck (Darmstadt, Germany).

Isolated perfused rat liver preparation. Male Sprague-Dawley rats (200–300 g) were purchased from Laboratory Animal Breeding Center, Shiraz University of Medical Sciences and allowed free access to food and tap water. The animals were handled and used according to the animal handling protocol approved by the ethics committee of Faculty of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran (Reg. no. 93-01-36-7612). After anesthetizing the animals with pentobarbital (50 mg/kg, i.p.), the livers were cannulated and perfused via portal vein (16,17) with hemoglobin- and albumin-free Krebs-Henseleit buffer (pH = 7.4, 37°C) gassed with carbogen (95% O₂, 5% CO₂). The perfusate was pumped through the livers with a peristaltic pump at a constant flow rate of 3 mL/min/g liver weight, in a re-circulating mode. The perfusate buffer volume was 200 mL in all experiments.

Study procedure. Isolated rat liver was exposed to different concentrations of sulfasalazine and other postulated hepatoprotective agents for 180 mins of perfusion. Hepatic injury was determined at scheduled time intervals during the experiment to evaluate the effects of various concentration of sulfasalazine on liver. The injurious concentration of the drug is reported as a concentration value, which lead to a significant rise in all assessed biomarkers of liver injury after 120 mins of organ perfusion. Samples were taken from liver perfusate at different times and assessed for biomarkers of liver injury. At the end of each experiment (180 mins), liver samples were used for lipid peroxidation, glutathione content and histopathological tests. Spectrophotometric scanning of the perfusate solutions revealed no physiochemical

interaction between sulfasalazine and other protective agents in current system. Thiol reducing agents and antioxidants caused no sign of liver injury when they were administered alone at the mentioned concentrations.

Perfusate biochemistry. Mindray Random Access BS-200[®] auto analyzer (Mindray, Shenzhen, China) and Pars Azmun[®] standard kits (Pars Azmun, Tehran, Iran) were used to measure ALT, AST, and LDH activities in liver perfusate at different time points (18).

Lipid peroxidation in liver tissue. Level of lipid peroxidation was assessed by measuring thiobarbituric acid reactive substances (TBARS) in different experimental groups. Briefly, reaction mixture consists of trichloroacetic acid (15%, w/v), thiobarbituric acid (0.375%, w/v), HCl (0.25 N), and 0.2 mL of tissue homogenate. The mixture was shaken and heated in boiling water (100°C) for 45 mins. When the reaction mixture was cooled, 2 mL of n-butanol was added and vigorously shaken. Samples were centrifuged (3000 g for 5 mins) and the absorbance of developed color in n-butanol phase was read at 532 nm using an Ultrospec 2000[®] UV spectrophotometer (Pharmacia Biotech, Uppsala, Sweden) (19).

Hepatic glutathione content. The liver GSH levels were assessed by determining non-protein sulphhydryl contents with the Ellman reagent (20,21). Briefly, samples of liver (200 mg) were homogenized in ice-cooled EDTA (20 mM). Five milliliter of liver homogenate was mixed with 4 mL of distilled water and 1 mL of trichloroacetic acid (50% w/v). The mixture was centrifuged (10000 g, 4°C, for 25 mins). Two mL of the supernatant was mixed with 4 mL of Tris buffer (pH = 8.9), and 100 µL of DTNB (0.01 M in methanol) was added to the samples. The absorbance of developed color was measured in 412 nm using an Ultrospec 2000[®] UV spectrophotometer.

Liver histopathological evaluation. Samples of liver were fixed in neutral buffered formalin solution (0.4% sodium phosphate monobasic, NaH₂PO₄, 0.64% sodium phosphate dibasic, Na₂HPO₄, 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 were performed by the one way analysis of variance (ANOVA) with Tukey's as the *post hoc* test. Differences were considered significant when $p < 0.05$.

RESULTS

Isolated rat liver was exposed to different concentrations

Table 1. Concentration-response of the isolated rat liver to sulfasalazine

Treatment	Perfusate ALT (U/L)	Perfusate AST (U/L)	Perfusate LDH (U/L)
Control (only buffer)	21 ± 14	34 ± 4	26 ± 4
Sulfasalazine 125 µM	31 ± 13	44 ± 9	38 ± 11
Sulfasalazine 250 µM	39 ± 8	137 ± 53*	89 ± 25
Sulfasalazine 500 µM	46 ± 13	113 ± 4*	199 ± 72*
Sulfasalazine 1 mM	75 ± 10	159 ± 52*	233 ± 29*
Sulfasalazine 5 mM	601 ± 148*	306 ± 39*	355 ± 143*

Data are shown as Mean ± SEM for at least three separate experiments which were assessed after 120 mins of liver perfusion.

*Indicates significant increase as compared with control (Only buffer) group ($p < 0.05$).

of sulfasalazine (Table 1). It was found that 5 mM of sulfasalazine caused a significant elevation of AST, ALT, and LDH in the perfusate after 120 mins of liver perfusion ($p < 0.05$) (Table 1). The 5 mM concentration of sulfasala-

zine was considered as an injurious dose of the drug in this system and was applied for further investigation on sulfasalazine-induced liver injury.

The biomarkers of liver injury were assessed in different experimental groups after administering thiol-reducing agents (NAC and DTT) and antioxidants (Vitamins C and E). It was found that NAC in both concentrations of 300 µM and 500 µM significantly decreased elevation of ALT (Table 2), AST (Table 3) and LDH (Table 4) in perfusate ($p < 0.05$). Administration of DTT as another thiol-reducing agent mitigated elevation in perfusate biomarkers of liver injury at the concentration of 400 µM (Tables 2-4) ($p < 0.05$). The levels of ALT, AST and LDH were significantly lower in liver effluent as compared with sulfasalazine-treated group, when antioxidants such as Vitamin C (200 µM) and E (200 µM) were administered ($p < 0.05$) (Tables 2-4).

Lipid peroxidation and hepatic glutathione content were assessed in different groups at the end of each experiment.

Table 2. Effect of thiol-reductants and antioxidants on ALT release from liver induced by sulfasalazine

Treatment	Time (min):	Perfusate ALT level (U/L)		
		60	120	180
Control (only buffer)		13 ± 2	20 ± 4	21 ± 8
+ Sulfasalazine 5 mM		17 ± 5	677 ± 113*	1811 ± 107*
+ NAC 300 µM		52 ± 14	122 ± 23 ^a	288 ± 140 ^a
+ NAC 500 µM		6 ± 3	33 ± 7 ^a	120 ± 35 ^a
+ DTT 200 µM		4 ± 1	76 ± 36	244 ± 27
+ DTT 400 µM		4 ± 1 ^a	23 ± 4 ^a	152 ± 81 ^a
+ Vitamin E 200 µM		1.66 ± 0.66 ^a	18 ± 6 ^a	45 ± 20 ^a
+ Vitamin C 200 µM		6 ± 3 ^a	22 ± 2 ^a	59 ± 12 ^a

Data are expressed as Mean ± SEM for at least four separate experiments.

Isolated rat liver was perfused with different concentrations of drug and/or antioxidants and thiol reducing agents. NAC: N-acetylcysteine, DTT: Dithioteritol.

*Indicates significant increase in comparison with control (only liver) group ($p < 0.05$).

^aIndicates significant decrease when compared with sulfasalazine-treated liver ($p < 0.05$).

Table 3. Effect of thiol-reductants and antioxidants on AST release from liver induced by sulfasalazine

Treatment	Time (min):	Perfusate AST level (U/L)		
		60	120	180
Control (only buffer)		14 ± 2	34 ± 4	39 ± 8
+ Sulfasalazine 5 mM		38 ± 10	328 ± 36*	2707 ± 997*
+ NAC 300 µM		30 ± 11	192 ± 77 ^a	466 ± 176 ^a
+ NAC 500 µM		34 ± 4	128 ± 22 ^a	260 ± 50 ^a
+ DTT 200 µM		21 ± 6	335 ± 133	952 ± 105 ^a
+ DTT 400 µM		24 ± 3	74 ± 27 ^a	303 ± 143 ^a
+ Vitamin E 200 µM		18 ± 4 ^a	61 ± 14 ^a	197 ± 46 ^a
+ Vitamin C 200µM		12 ± 1 ^a	37 ± 2 ^a	175 ± 70 ^a

Data are shown as Mean ± SEM for at least three separate experiments.

Isolated rat liver was perfused with different concentrations of drug and/or antioxidants and thiol reducing agents. NAC: N-acetylcysteine, DTT: Dithioteritol.

*Shows significant increase as compared with control (only buffer) group ($p < 0.05$).

^aIndicates significant decrease when compared with sulfasalazine-treated liver ($p < 0.05$).

Table 4. Effect of thiol-reductants and antioxidants on perfusate LDH level after sulfasalazine administration to isolated rat liver

Treatment	Time (min):	Perfusate LDH level (U/L)		
		60	120	180
Control (only buffer)		10 ± 3	19 ± 5	26 ± 4
+ Sulfasalazine 5 mM		138 ± 59*	387 ± 114*	1146 ± 223*
+ NAC 300 µM		14 ± 14 ^a	296 ± 23	965 ± 123
+ NAC 500 µM		17 ± 4 ^a	116 ± 38 ^a	619 ± 68 ^a
+ DTT 200 µM		30 ± 8 ^a	793 ± 266	2080 ± 185
+ DTT 400 µM		4 ± 1 ^a	258 ± 57 ^a	161 ± 50 ^a
+ Vitamin E 200 µM		17 ± 2 ^a	156 ± 80 ^a	821 ± 402 ^a
+ Vitamin C 200 µM		25 ± 4 ^a	249 ± 22 ^a	1386 ± 120

Data are shown as Mean ± SEM for at least three separate experiments.

Isolated rat liver was perfused with different concentrations of drug and/or antioxidants and thiol reducing agents. NAC: N-acetylcysteine, DTT: Dithioeritol.

*Indicates significant increase as compared with control (only buffer) group ($p < 0.05$).

^aIndicates significant decrease when compared with sulfasalazine-treated liver ($p < 0.05$).

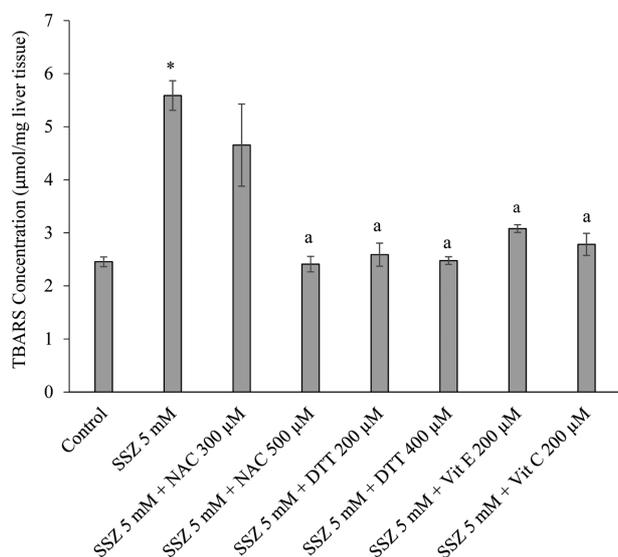


Fig. 1. Effects of antioxidants and thiol-reductants on lipid peroxidation induced by sulfasalazine in isolated rat liver. Data are represented as Mean ± SEM for at least three independent experiments. SSZ: Sulfasalazine, NAC: N-acetylcysteine, DTT: Dithioeritol. *Indicates significantly higher than control group ($p < 0.05$). ^aIndicates significantly lower when compared with sulfasalazine-treated group ($p < 0.05$).

It was found that a significant amount of thiobarbituric acid reactive substances (TBARS) were formed after sulfasalazine administration to the liver ($p < 0.05$) (Fig. 1). Moreover, a significant decrease in hepatic glutathione stores was detected in sulfasalazine-treated group ($p < 0.05$) (Fig. 2). Administration of antioxidants and thiol-reducing agents in the current investigation significantly reduced the level of lipid peroxidation in rat liver ($p < 0.05$) (Fig. 1). Furthermore, hepatic glutathione reservoirs maintained in a significantly higher level when sulfasalazine was co-administered with thiol-reducing agents and/or antioxidants ($p < 0.05$)

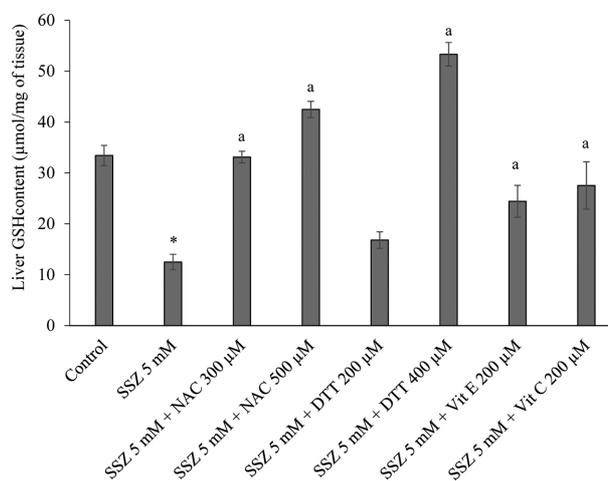


Fig. 2. Protective effects of antioxidants and thiol-reductants on hepatic glutathione depletion induced by sulfasalazine in isolated perfused rat liver. Data are given as Mean ± SEM for at least three independent experiments. SSZ: Sulfasalazine, NAC: N-acetylcysteine, DTT: Dithioeritol. *Indicates significantly lower than control group ($p < 0.05$). ^aIndicates significantly higher as compared to sulfasalazine-treated livers ($p < 0.05$).

(Fig. 2).

At the end of each experiment, livers were evaluated for histopathological lesions. It was found that sulfasalazine (5 mM) caused sinusoidal dilation, hepatocellular vacuolization, and tissue necrosis in comparison with control group (Fig. 3). Administration of NAC (500 µM), DTT (400 µM), vitamin C (200 µM) and vitamin E (200 µM), significantly alleviated liver histopathological changes induced by sulfasalazine (Fig. 3).

DISCUSSION

The objective of the present study was to investigate the

Table 5. Graded histopathological changes of sulfasalazine-treated perfused rat liver

Treatment	Hydropic changes	Vacuolization	Sinusoidal dilation	Necrosis
Control	–	–	++	–
Sulfasalazine 5 mM	–	+++	+++	+++
Sulfasalazine 5 mM + NAC 500 μ M	–	–	++	–
Sulfasalazine 5 mM + DTT 400 μ M	–	+	++	–
Sulfasalazine 5 mM + Vitamin E 400 μ M	–	++	++	–
Sulfasalazine 5 mM + Vitamin C 400 μ M	–	++	++	–

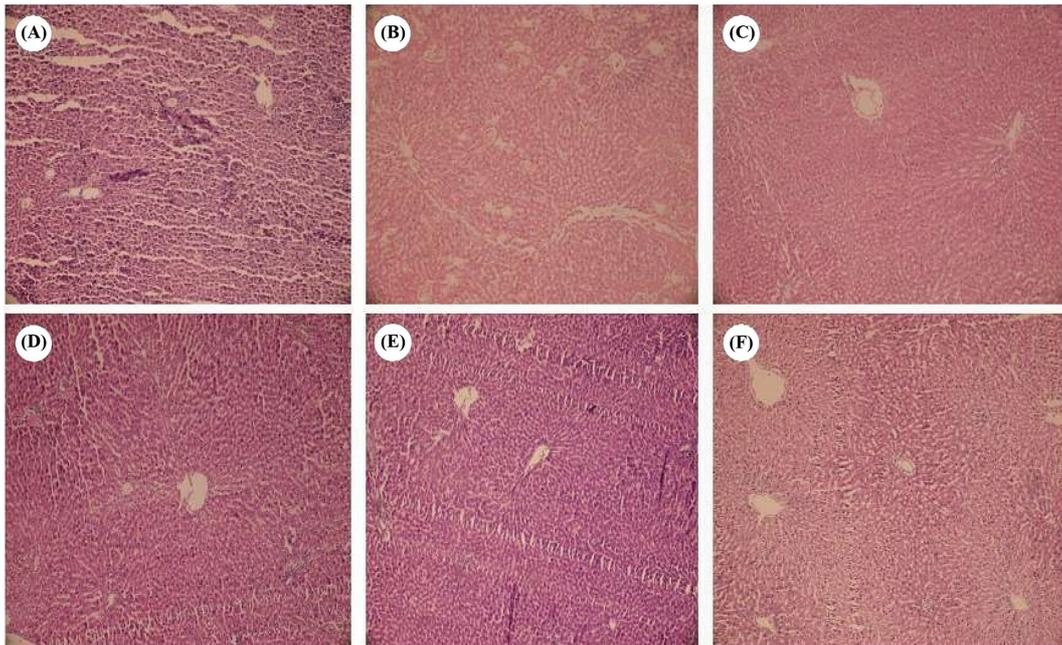


Fig. 3. Histopathological changes of perfused rat liver treated with sulfasalazine (H&E staining). **(A)** control group perfused with Krebs-Henseleit buffer for 180 mins. No significant pathological changes except for a mild sinusoidal dilation was detected in this group. **(B)** Sulfasalazine (5 mM) was perfused to isolated rat liver for 180 mins. Sinusoidal space dilation, hepatocellular vacuolization (Blue arrow) and cell necrosis (Yellow arrow) were observed in this group. **(C)** N-acetylcysteine (500 μ M) was co-administered with sulfasalazine for 180 mins. Only a mild sinusoidal space dilation was observed in this group. **(D)** Isolated rat liver was perfused by sulfasalazine (5 mM) and dithioeritol (400 μ M) for 180 mins. A mild degree of hepatocellular vacuolization (Blue arrow) and sinusoidal dilation were pathological findings in this group. **(E)** Vitamin C (200 μ M) was administered with sulfasalazine (5 mM) during 180 mins of liver perfusion. **(F)** effect of vitamin E (200 μ M) on sulfasalazine (5 mM)-induced histopathological lesions in perfused rat liver. Mild hepatocellular vacuolization and sinusoidal space dilation was found in groups **(E)** and **(F)**.

role of thiol reducing agents and antioxidants against sulfasalazine-induced hepatic injury in an *ex vivo* model of isolated perfused rat liver. Sulfasalazine (5 mM) caused hepatotoxicity as judged by a significant increase in perfusate biomarkers of liver injury, rise in tissue lipid peroxidation and depletion of liver glutathione reservoirs. Moreover, drug administration caused histopathological changes in the liver. The abovementioned markers of liver injury caused by sulfasalazine were alleviated by administration of thiol-reducing agents and antioxidants.

Previous investigations proposed oxidative stress as a mechanism for sulfasalazine-induced liver injury (12,14). In current study, we found that sulfasalazine caused a significant amount of lipid peroxidation as an index of oxidative

biomembranes injury (Fig. 1). Moreover hepatic glutathione stores were decreased (Fig. 2), which might be a consequence of oxidative stress in the liver. The antioxidants and thiol-reducing agents effectively mitigated sulfasalazine-induced liver injury in our study. This might implicate the important role of thiol groups and oxidative stress in the organ injury caused by sulfasalazine. Thiol reductants and antioxidants might provide protection against sulfasalazine by counteracting oxidative stress in hepatocytes. Interestingly, it has been reported that NAC administration significantly alleviated severe sulfasalazine-induced reactions in human (22). This report might endorse the potential therapeutic capability of thiol-reductants against adverse events accompanied with sulfasalazine administration. It has been

found that sulfasalazine blocks cysteine transport to glioma cells and consequently prevents glutathione synthesis (23), a mechanism which finally sensitizes tumor cells to radio/chemotherapy (23). Although such mechanism has not been proved in hepatocytes, it might be involved in sulfasalazine-induced liver injury and also in the potential protective effect of thiol reductants against sulfasalazine hepatotoxicity.

In large intestine, bacterial azoreductase enzymes cleave sulfasalazine to sulfapyridine and mesalazine. 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 (24). In current investigation we aimed to reproduce sulfasalazine-induced hepatic injury in *ex vivo* model. Hence, we administered a higher concentration of sulfasalazine. This is actually different from *in vivo* conditions where only 10~30% of intact sulfasalazine reaches the liver (24).

It is not clear that 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. Hence, further future investigations on the metabolic pathways of sulfasalazine and its intestinal metabolites might shed light on the mechanisms of liver injury induced by this drug.

Conditions in animal models and human subjects are highly different from *in vitro/ex vivo* experiments because of pharmacokinetic/pharmacodynamic parameters. However, results such as protective effects of thiol reducing agents and antioxidants against sulfasalazine-induced toxicity could help to clear the mechanisms of liver injury and may provide therapeutic strategies against drug hepatotoxicity. Furthermore, isolated organs are useful models for examining xenobiotics-induced injury without the complication of many interacting factors which are difficult to control in other experimental models. Hence, *ex vivo* models are effective tools for screening hepatoprotective compounds and studying the mechanisms of drug-induced liver injury. The protective agents in this model are potential subjects of

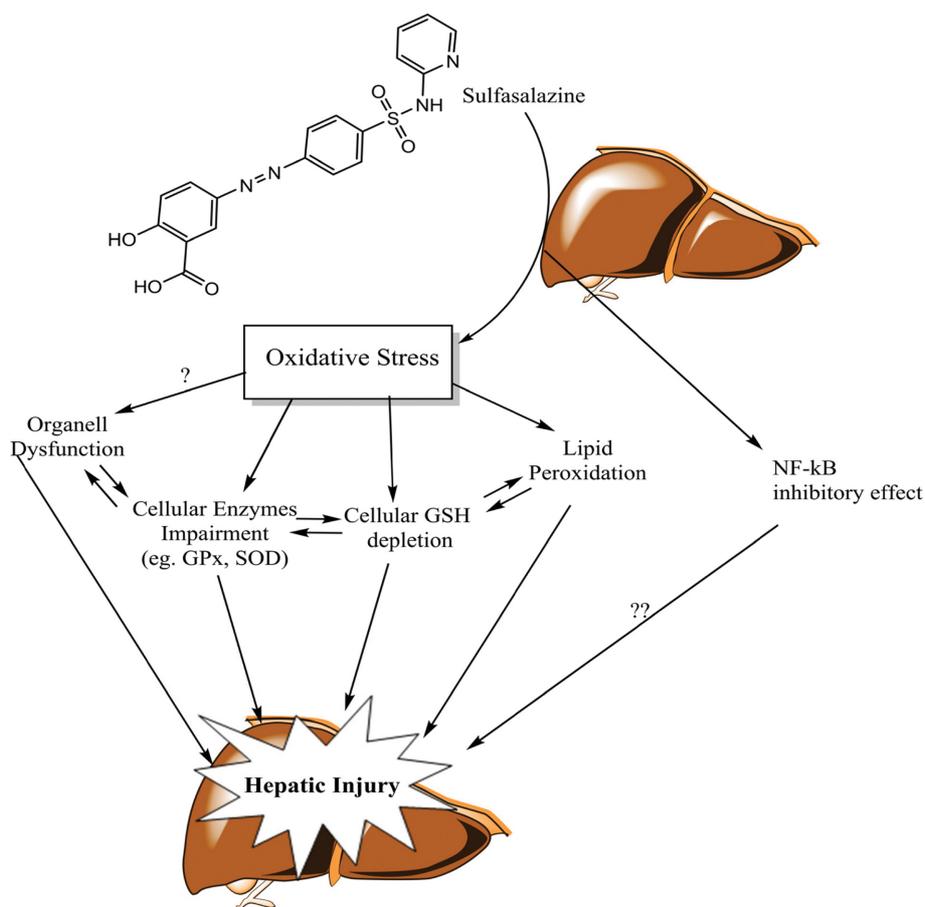


Fig. 4. The proposed mechanism(s) of liver injury induced by sulfasalazine and the hepatoprotection provided by thiol-reducing agents and antioxidants. Oxidative stress and its consequences such as biomembranes injury (lipid peroxidation) and glutathione reservoirs depletion are proposed to be involved in sulfasalazine-induced hepatotoxicity. Other complications such as cellular defense mechanisms impairment and organelle dysfunction might also be involved in sulfasalazine-induced liver injury. Antioxidants and thiol-reducing agents might provide hepatoprotection by counteracting sulfasalazine-induced oxidative stress in liver.

further investigation in other models such as *in vivo* experiments.

The polymorphism in the N-acetyl transferase (NAT) enzyme in different populations is postulated to be responsible in sulfasalazine adverse effects (25). However, the role of un-acetylated metabolites of sulfasalazine in the oxidative stress and the liver injury induced by this drug is not tested so far.

Some studies indicate the role of sulfasalazine in inhibiting nuclear factor kappa-B (NF- κ B) in different cells. It has been shown that sulfasalazine but not its metabolites, is a potent and specific inhibitor of NF- κ B (26). This event might be related to the cytotoxicity of this chemical against cells such as lymphocytes (27), which might be related to the pharmacological effects of the drug (28). NF- κ B is involved in the activation of the anti-apoptotic genes transcription and cell proliferation (29). It is not clear whether inhibition of NF- κ B contribute in the liver injury induced by sulfasalazine (Fig. 4).

At least a part of sulfasalazine-induced toxic reactions including hepatic injury, seems to be immune-mediated (30). Due to the absence of immune system and its interactions with liver in current model, this investigation cannot predict the immune-mediated mechanisms of sulfasalazine-induced liver injury.

Although there is no clear consensus that they are effective, steroids have been used against sulfasalazine-induced liver injury in human cases (31). The data obtained from the current investigation suggest that antioxidants and thiol-reducing agents could be a potential therapeutic choice against sulfasalazine-induced liver injury.

Altogether, the data obtained from current investigation indicate the role of oxidative stress in sulfasalazine-induced liver injury and the potential therapeutic properties of thiol reducing agents and antioxidants against this complication. The hepatoprotective properties of these agents deserve further investigation in other models of drug-induced liver injury. The proposed mechanisms of liver injury induced by sulfasalazine and the hepatoprotective role of antioxidants and thiol-reducing agents are summarized in Fig. 4.

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