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Inhibition of NF-κB and the oxidative stress -dependent caspase-3 apoptotic pathway by betaine supplementation attenuates hepatic injury mediated by cisplatin in rats

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ABSTRACT

Background: Cisplatin is a major anti-cancer drug commonly used in the treatment of various cancers; nevertheless, the associated hepatotoxicity has limited its clinical application. The aim of this investigation is to test the impact of betaine supplementation on cisplatin-induced hepatotoxicity.
Methods: Animals were allocated into four groups; normal control group (control betaine group (250 mg/kg/day, *po* for twenty six days), cisplatin group (single injection of 7 mg/kg, *ip*) and betaine + cisplatin group (received betaine for twenty one days before cisplatin injection and daily after cisplatin for five days).
Results: Cisplatin-induced liver injury was confirmed by increased alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels. Cisplatin elevated lipid peroxides, and reduced the concentrations of reduced glutathione (GSH), glutathione peroxidase (GSH-Px), catalase and superoxide dismutase (SOD) in hepatic tissues. Cisplatin increased the inflammatory mediators; nitrite and tumor necrosis factor-α (TNF- α) in hepatic tissues. Increased gene expressions of the apoptotic marker, caspase-3 and nuclear factor-kappa B (NF-κB) were observed in hepatic tissues of cisplatin-treated rats. All these changes were further confirmed by histopathological findings in cisplatin group. Pre-treatment with betaine reduced serum aminotransferases (ALT and AST), and lowered hepatic concentrations of lipid peroxides, nitrite and TNF-α while increased SOD, GSH, catalase, and GSH-Px concentrations. Moreover, the histological and immunohistochemical changes were improved.
Conclusion: The suppression of NF-κβ-mediated inflammation, oxidative stress, and caspase-3 induced apoptosis are possible mechanisms to the observed hepatoprotective effect of betaine.

Introduction

Betaine known also as trimethylglycine is a quaternary ammonium compound (*N,N,N*-trimethylammonioacetate) found in animals, plants, and microorganisms. It exists in many foods including seafood, especially marine invertebrates; wheat germ or bran; spinach and sugar beets [1,2]. It is present in most tissues with the highest concentrations in liver, kidney, and testes [3]. Betaine is a source of methyl groups that are used in the methionine-homocysteine cycle involved in many biochemical

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; DAB, diaminobenzidine tetrahydrochloride; GSH, reduced glutathione; GSH-Px, glutathione peroxidase; NF-κB, nuclear factor-kappa B; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; TBS, tris buffer solution; TNF-α, tumor necrosis factor-α.

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pathways [1,3]. The dietary human intake of betaine ranges from 1.0 to 2.5 g/day [4]. Insufficient supply of methyl groups in the diet may lead to disturbance in the hepatic protein catabolism and elevation in plasma homocysteine concentrations which may cause abnormalities in hepatic fat metabolism, leading to steatosis and dyslipidemia [5]. Betaine intake is commonly used to lower homocysteine and to reduce the risk of cardiovascular disease, peripheral vascular disease and cerebrovascular disease in healthy humans [6,7]. Betaine can also improve fatty liver diseases induced by alcohols or other causes. This is accomplished by attenuating oxidative stress, steatosis, fibrosis, and lipogenesis [8,9]. Betaine improves glucose homeostasis and hepatic lipid accumulation [10,11]. Moreover, it can diminish several markers of low-grade systemic inflammation as C-reactive protein, interleukin-6, and tumor necrosis factor-α in healthy adults [12]. In addition, the hepatoprotective actions of betaine were demonstrated against α-naphthylisothiocyanate, dimethylnitrosamine and lipopolysaccharide [13–15].

Cisplatin is considered as a major effective anticancer drug against broad spectrum of leukemia and other malignancies. It is mainly used in the treatment of solid tumors including testicular, cervical, ovarian, head and neck malignancies [16]. In spite of its beneficial effects, cisplatin has substantial nephrotoxic, hepatotoxic and neurotoxic side effects [17–21]. Hepatotoxicity is not considered as a dose-limiting toxicity for cisplatin, but liver toxicity can occur when the antineoplastic drug is administered at high doses [19,21]. Numerous mechanisms including oxidative stress, inflammatory changes, DNA damage, and apoptosis have been implicated in cisplatin toxicity [18–20]. Cisplatin can damage cancer cells by releasing free radicals which, at the same time, has the potential to damage other cells including hepatic cells [20]. Many evidence indicate that cisplatin toxicity is attributed to free radical generation and that antioxidants are effective in protection against these side effects [21–23].

Prevention or even reduction of cisplatin adverse effects is the main concern in the management of cancer patients. Recently, there has been a worldwide tendency towards the use of natural products and dietary components as potential chemoprotective agents. Cisplatin-induced nephrotoxicity has been extensively studied but, hepatotoxicity is rarely characterized, and is less studied. Recently we found that betaine supplementation is protective against cisplatin-induced renal injury [17]. But, its role in cisplatin-induced hepatotoxicity is not fully explored. The objective of the current investigation is to examine the ability of betaine to protect from the hepatic injury induced by cisplatin in experimental rats. The assessment of efficacy of betaine against the cisplatin hepatotoxicity was done biochemically *via* measuring lipid peroxides, antioxidants, inflammatory and apoptotic markers and confirmed by histopathological and immuohistochemical studies.

Materials and methods

Chemicals

Trimethylglycine, cis-platin, thiobarbituric acid, 5, 5-dithiobis-(2-nitrobenzoic acid), and thiobarbituric acid were obtained from Sigma Com. (MO, USA). Aminotransferases (ALTand AST) levels were measured using kits from Biomérieux Inc., (France). Superoxide dismutase and glutathione peoxidase were measured using kits from Randox Company (Antrim, UK). Antibodies used for immunohistochemical studies for nuclear factor kappa and caspase-3 were purchased from Santa Cruz Biotechnology, Inc. (TX, USA). Tumor necrosis factor-α (TNF-α) and nitrite/nitrate concentration were estimated in hepatic tissues using ELISA kits from R&D (MN, USA).

Animals

Adult male Wistar rats (220–250 g), were obtained from the Animal Care Centre, College of Pharmacy, King Saud University. All the animals were fed a standard rat chow and water ad libitum and kept at (20–22 °C) with an alternating cycle of 12-h light and dark. The animals were handled according to guideline of the NIH for experimental care and use of animals. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All animal procedures were approved by the National Institutes of Health (NIH) for experimental care and use of animals.

Experimental design

The animals were allocated into four groups (n = 8) as follow:

(1) Normal control group: the saline (2 ml/kg, *po*) was administered for 26 days.
(2) Betaine group: betaine was given at a dose of (250 mg/kg/day, *via* gavage) for 26 days [24,25].
(3) Cisplatin group: A single dose of cisplatin was given (7 mg/kg, *ip*) [26].
(4) Cisplatin + betaine group: betaine was given for 21 days before cisplatin and daily for 5 days after cisplatin.

The doses of betaine and cisplatin were chosen depending upon our preliminary studies and other publications [24–26]. At the end of the experiments, animals were sacrificed; blood and livers were collected. Blood samples were centrifuged and sera were separated and assessed for hepatic functions.

Hepatic function

Hepatic function was assessed by measuring alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (Biomérieux Inc., France).

Preparation of hepatic tissues for biochemical studies

Portions of the livers were taken for histopathological study while the remaining parts were homogenized in ice-cold saline to produce 10% (w/v) homogenates. The homogenate were centrifuged at 1000*g* for 20 min at 4 °C and the supernatants were divided into aliquots and kept at −40 °C until assayed for lipid peroxides, GSH-Px, GSH and SOD.

Assessment of thiobarbituric acid-reactive substances

Hepatic lipid peroxides were measured as thiobarbituric acid-reactive substances (TBARS) as previously described by Buege and Aust [27] and concentrations were calculated by the use of 1,1,3,3 tetraethoxypropane as a standard. The results were expressed as nmol/g tissue weight.

Determination of SOD activity

Hepatic SOD was determined according to the method of Sun et al. [28]. SOD activity was expressed as units per milligram protein.

Measurement of catalase

Hepatic catalase was measured by following the decomposition of hydrogen peroxide (H_2O_2, Aldrich, USA) spectrophotometrically at 240 nm in 50 mM potassium phosphate buffer (pH 7.05) with

19 mM H_2O_2. The specific activity of catalase was expressed as the number of μmol H_2O_2 decomposed/min/g tissue weight [29].

Determination of reduced glutathione

Hepatic GSH was determined in rat livers following the method of Ellman et al. [30], and modified by Nagi et al. [31]. The results were expressed as μmol/g tissue weight.

Assessment of GSH-Px

Hepatic GSH-Px was estimated following the method of Paglia and Valentine [32]. GPH-Px activity was defined as the number of μmol NADPH oxidized/min/g tissue weight.

Estimation of nitrite concentration

Hepatic nitrite/nitrate concentration was measured using ELISA kit. The nitrite levels were expressed as μmol/g wet tissue.

Determination of hepatic TNF-α

Hepatic TNF-α was estimated using ELISA kit according to the manufacturer's instruction.

Measurement of protein content

Total protein concentration was estimated according to the method of Bradford [33], with bovine albumin as a standard.

Histological studies

Hepatic samples were fixed in 10% buffered formaline, dehydrated, embedded in paraffin. and cut at 5 μm. Sections were stained with haematoxylin and eosin, for histological evaluation of hepatic injury. Evaluation was done on coded slides to avoid bias of the observer and using scoring system for histopathological changes ranked from 0 to 4 as follow: 0 = negative; 1 = mild; 2 = mild to moderate; 3 = moderate to severe; 4 = severe.

Immunohistochemistry for NF-kβ and caspase-3

Three-micrometer paraffin sections of livers were deparaffinized in xylene, rehydrated through graded alcohol, washed in running tap water. Antigen retrieval was done by heating the slides in 10 mM citrate buffer solution pH 6 for 10 min. Neutralization of the endogenous peroxidase was done using peroxidase block for 5 min. Sections were washed twice in Tris buffer solution (TBS) each for 5 min. The sections were incubated with protein block for 5 min. followed by double washing in TBS, 5 min. for each wash. Incubation was done with monoclonal antibodies against nuclear factor-κB subunit p 65 (NF-κB), or caspase-3 (1:200) for 2 h in a humidified chamber, followed by double washing with TBS, 5 min. for each wash. The slides were incubated in biotinylated secondary antibody for 30 min followed by double washing in TBS. Incubation with streptavidin-horseradish peroxidase for 30 min was done followed by double washing in TBS. Peroxidase localization was revealed using DAB reagent (diaminobenzidine tetrahydrochloride) as the chromogen for 5 min. The slides were washed in water and counterstained with hematoxylin for 20 min followed by tap water for 5 min, dehydrated, cleaned and mounted in depex and were examined under light microscope.

Statistical analysis

Statistical analysis was performed using Graph prism statistical software. Analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparisons test was done and data were considered significant at $p < 0.05$. Results were expressed as mean ± SE.

Results

Initial and final body weights

No significant differences in the initial body weights were noted between all four groups (Table 1). Cisplatin-treated rats showed a significant decline in the final body weights when compared to the control group. Prior treatment with betaine prevented the decrease in the body weight of cisplatin-treated animals and the rats showed a significant increase in the final body compared to cisplatin alone rats.

Effect of betaine on hepatic functions in cisplatin-treated rats

A single cisplatin injection (7 mg/kg, *ip*) resulted in a significant increase in serum ALT (Fig. 1A) and AST levels (Fig. 1B). No significant difference in levels of aminotransferases was noted between normal control and betaine control groups ($p < 0.05$). Betaine improved hepatic functions in cisplatin-treated rats as manifested by the significant ($p < 0.05$) reduction in serum ALT and AST levels (Fig. 1A and B).

Effects of betaine on hepatic TBARS concentration

Lipid peroxides measured as TBARS were similar in control and betaine groups (Fig. 2A). TBARS concentrations were increased in cisplatin-treated rats (612 ± 27 nmol/g tissue) as compared to normal control rats (182 ± 12 nmol/g tissue). Prior-treatment with betaine inhibited cisplatin-induced lipid peroxidation and resulted in a significant decrease in hepatic TBARS concentration (285 ± 14 nmol/g tissue) respectively as compared to cisplatin group alone (Fig. 2A).

Effects of betaine on hepatic SOD activity

Administration of cisplatin alone caused a significant inhibition in hepatic SOD activity (21.8 ± 1.9 U /mg tissue, $p < 0.05$) as compared to normal control rats (48.7 ± 2.7 U/mg tissue) (Fig. 2B). Betaine alone caused no change in SOD activity compared to control group. Treatment with betaine provided protection against the decline in SOD activity induced by cisplatin in hepatic tissues (40.5 ± 4.1 U/mg wet tissue, $p < 0.05$) (Fig. 2B).

Effect of betaine on hepatic GSH concentration

The effect of treatment of adult rats with betaine on cisplatin-mediated changes in hepatic glutathione concentration is shown in (Fig. 2C). There was significant GSH depletion ($p < 0.05$) in cisplatin-treated rats (1.5 ± 0.3 μmol /g tissue, $p < 0.05$) as compared to normal control group (4.8 ± 0.6 μmol /g tissue)

Table 1
Initial and final body weight of all experimental groups.

Groups	Initial Body Weight (gm)	Final Body Weight (gm)
Control	244 ± 8	275 ± 4
Betaine	251 ± 7	277 ± 3
Cisplatin	248 ± 6	211 ± 4*
Cisplatin + Betaine	250 ± 3	270 ± 5#

Values are expressed as means ± SEM, n = 8. Statistical analysis was performed using one way analysis of Variance (ANOVA) followed by Tukey-Kramer multiple comparison test.
* $p < 0.05$ compared with normal control group.
# $p < 0.05$ compared with cisplatin-treated group.

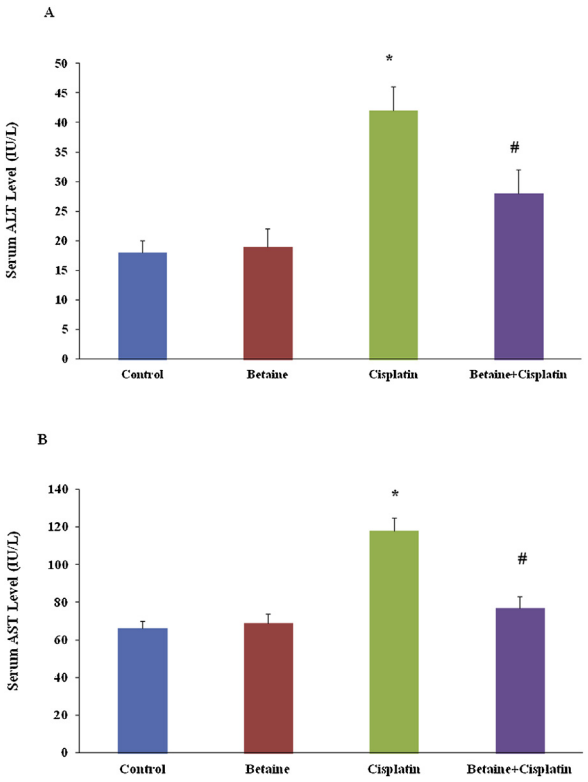

Fig. 1. Effect of betaine supplementation (250 mg/kg/day, orally) for 21 days on liver functions in cisplatin-treated rats. (A) Serum ALT level; (B) serum AST level. Values are expressed as mean ± SEM, n = 8; * $p < 0.05$ compared with control group, # $p < 0.05$ compared with cisplatin-treated group.

(Fig. 2C). Betaine was able to abrogate the decline in GSH content in cisplatin-treated rats.

Effect of beatine on hepatic GSH-Px activity

Hepatic GSH-Px activity was reduced in hepatic tissues in cisplatin-treated rats (14 ± 2.04 μmol/min/g tissue) as compared to normal control rats (25.34 ± 2.2 μmol/min/g tissue) (Fig. 2D). Betaine alone caused no change in GSH-Px activity (24.2 ± 2.5 μmol/min/g tissue) compared to control groups. Treatment with betaine ameliorated cisplatin-induced decreased GSH-Px activity in liver tissues (20.2 ± 1.86 μmol/min/g tissue, $p < 0.05$) respectively as compared to cisplatin group (Fig. 2D).

Effect of betaine on hepatic catalase concentrations

As depicted in Fig. 2E, there was a decline in hepatic catalase activity in cisplatin group that was statistically significant (178 ± 9 μmol/min/g tissue) compared to normal control hepatic values respectively (258 ± 8 μmol/min/g tissue). Prior administration of betaine was capable to reverse the decrement in catalase activity in cisplatin-treated rats.

Effect of betaine on hepatic TNF-α and nitrite in cisplatin-treated rats

The anti-inflammatory action of betaine was tested by evaluating TNF-α and nitrite in hepatic tissues of all treated animals. As shown in Table 1 hepatic TNF-α and nitrite concentrations were elevated in cisplatin treated rats. There was no difference between normal control and betaine control groups. Betaine administration to cisplatin treated rats produced significant decrease ($p < 0.05$) in hepatic concentrations of TNF-α and nitrite compared to cisplatin-treated rats alone. This confirms the anti-inflammatory ability of betaine (Table 2).

Effect of betaine on hepatic histological changes

Semi quantitative analyses of histology of liver of all experimental groups were demonstrated in (Table 3 and Fig. 3). Control hepatic tissue showed normal large polygonal cells with prominent round nuclei and eosinophilic cytoplasm, and few spaced hepatic sinusoids arranged in-between the hepatic cords with fine arrangement of Kupffer cells (Fig. 3A, Table 3). No histological changes were noted in rats treated with betaine alone (Fig. 3B, Table 3). Cisplatin administration showed histopathological alterations including cellular infiltration, marked degeneration of hepatic cords, increased incidence of vacuolar degeneration and apoptotic cell death (Fig. 3C, Table 3). Minimal histopathological changes were noted in animals treated with betaine and cisplatin (Fig. 3D, Table 3).

Effect of betaine on hepatic NF-κB immunostaining

Nuclear factor kappa–β was undetectable in normal control, and control betaine (Fig. 4 A&B). Rats which received cisplatin showed a significant increase in the immunoreactivity of NF-κB in the cytoplasm of hepatocytes compared with the normal control group (Fig. 4C). On the other hand, animals treated with betaine prior to cisplatin demonstrated markedly reduced staining for NF-κB in their hepatic tissues compared to the cisplatin treated rats (Fig. 4D).

Effect of betaine on caspase-3 immunostaining

Caspase-3 was undetectable in normal control (Fig. 5A). No staining was noted in control betaine group (Fig. 5B). On the other hand, livers obtained from rats treated with cisplatin alone demonstrated marked increase of caspase-3 in liver cells (Fig. 5C). Livers obtained from rats treated with betaine demonstrated marked reduction in staining for caspase-3 (Fig. 5D).

Discussion

Cisplatin is a major anti-neoplastic drug commonly used as front-line therapy for cancers such as small cell lung cancer, gut cancer, bladder cancer, stomach cancer, ovarian cancer and germ cell tumors [34]. Although cisplatin nephrotoxicity is well documented, there is a shortage of literature on hepatotoxicity and how to protect against it. The aim of this study was to examine the impact of betaine supplementation on cisplatin-induced hepatotoxicity. In the current investigation, a single dose of cisplatin (7 mg/kg) provoked loss of body weight, and hepatic dysfunction that was evident by elevation in serum levels of AST, and ALT. Prior-treatment with betaine antagonized the loss of body weight and elevation of hepatic enzymes. This amelioration of the body weight reduction induced by cispaltin is an indication of the protective effect of betaine on the hepatotoxicity. There was an increase in oxidative stress in cispaltin-treated rats as measured by the increase in hepatic lipid peroxides (TBARS) and the decline in the hepatic concentrations of antioxidants SOD, reduced glutathione, GSH-Px, and catalase. Cisplatin induced elevation of inflammatory mediators (NO, and TNF-α), and over-expression of caspase-3 and NF-κB signaling. Prior-administration with betaine was able to reduce lipid peroxides, corrected the decline in the antioxidant enzymes and mitigates hepatic dysfunction. Inflammatory mediators (NO and TNF-α) were reduced in hepatic tissues. Over-expression of caspase-3 and NF-κB signaling pathways in cispaltin treated rats were also reduced by prior betaine treatment.

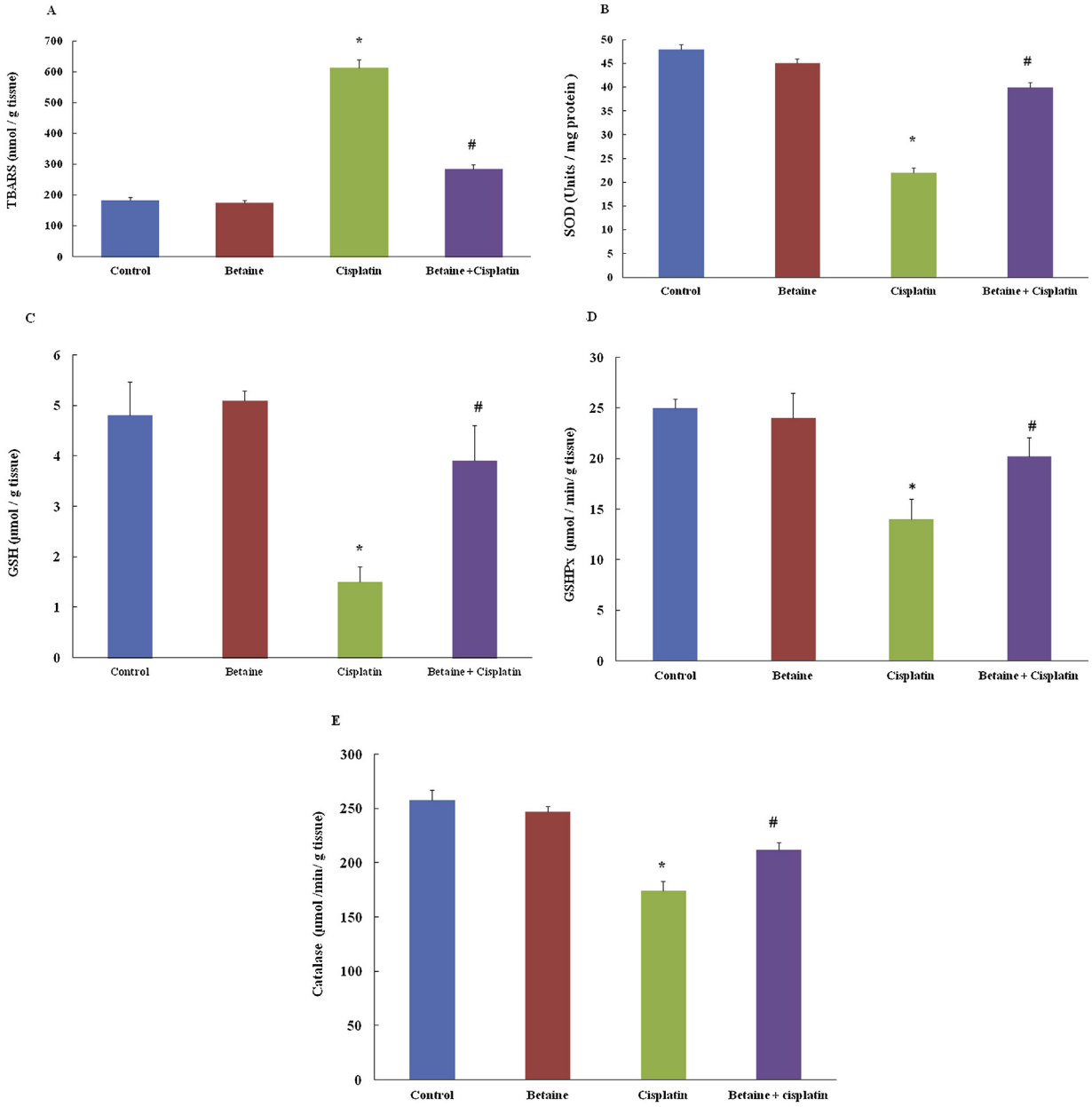

Fig. 2. Effects of betaine supplementation (250 mg/kg, orally) for 21 days on concentrations of (A) Thiobarbituric acid reactive substances (TBARS); (B) superoxide dismutase (SOD); (C) Reduced glutathione (GSH); (D) Glutathione peroxidase (GSH-Px); and (E) Catalase in hepatic tissues in cisplatin -treated adult male rats. Values are expressed as means ± SEM, n = 8; * $p <$ 0.05 *vs.* control group, # $p <$ 0.05 compared *vs.* cisplatin group.

Table 2
Effects of betaine supplementation (250 mg/kg, orally) for 21 days on tumor necrosis factor-α (TNF-α) and nitrite concentrations in hepatic tissues of cisplatin-treated adult male rats.

Groups	TNF-α concentration (ng/g tissue)	Nitrite concentration (µmol/g tissue)
Control	0.20 ± 0.06	10 ± 2
Betaine	0.18 ± 0.04	12 ± 3
Cisplatin	52 ± 8*	2.5 ± 0.2*
Cisplatin + Betaine	1.25 ± 0.08#	35 ± 2#

Values are expressed as means ± SEM, n = 8. Statistical analysis was performed using one way analysis of Variance (ANOVA) followed by Tukey-Kramer multiple comparison test.
* $p <$ 0.05 compared with normal control group.
# $p <$ 0.05 compared with cisplatin-treated group.

Betaine supplementation to cisplatin-treated rats prevented not only hepatic lipid peroxide formation (TBARS) but also the decline in enzymatic and non–enzymatic antioxidants (GSH, SOD, catalase and GSH-Px) concentrations in hepatic tissues. These results imply that the conservation of antioxidant defense capacity may explain the hepatoprotective activity of betaine. Betaine may reduce cisplatin-induced lipid peroxidation by its antioxidant action [35,36]. Earlier reports [37,38], indicated that betaine had a

Table 3
Semi quantitative analyses of histology of livers of experimental rats.

Groups	Sinusoidal expansion	Kupffer cell activation (Hypertrophy)	Hepatocyte vacuolization & degeneration
Control	0	0	0
Betaine	0	0	0
Cisplatin	$1.8 \pm 0.1^{*}$	$2.5 \pm 0.2^{*}$	$3.1 \pm 0.1^{*}$
Cisplatin + Betaine	$0.7 \pm 0.02^{\#}$	$0.9 \pm 0.06^{\#}$	$1.1 \pm 0.02^{*}$

Rats were treated with cisplatin and betaine and histological grading was performed as described in materials and methods section. Values are expressed as means ± SEM, n = 8.
* $p < 0.05$ compared with normal control group.
# $p < 0.05$ compared with cisplatin-treated group.

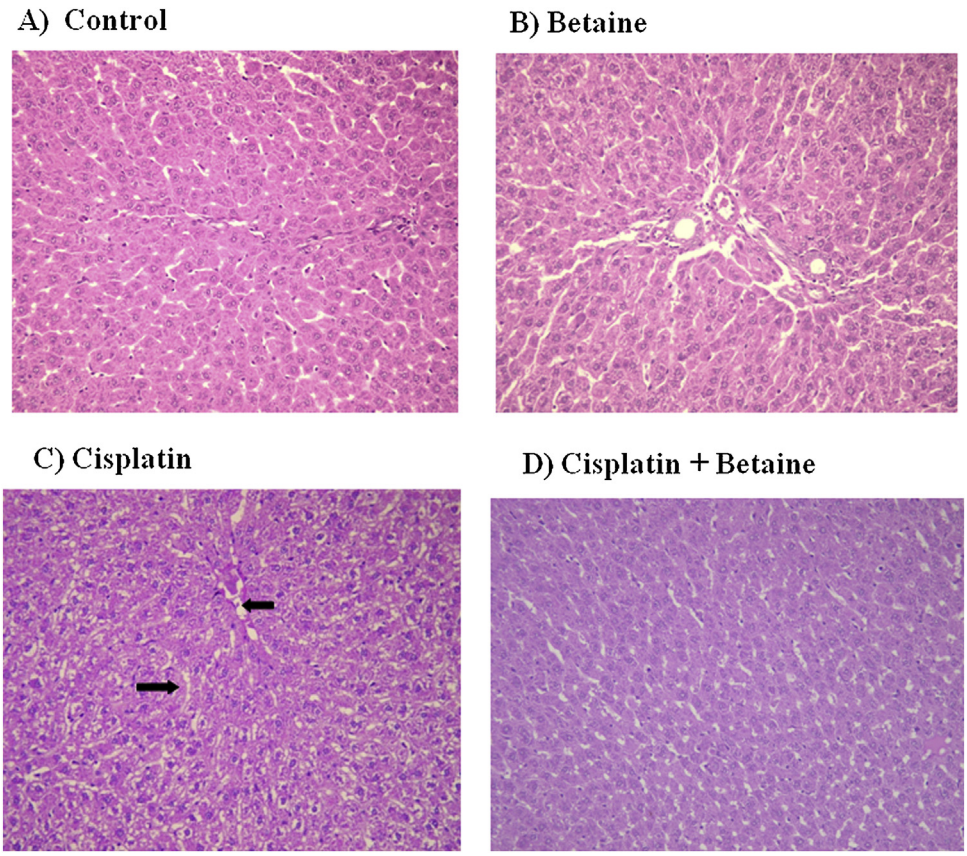

Fig. 3. Photomicrographs of rat liver (H&E, 200×) from: (A) Control group showing normal architecture (B) Betaine alone treated group where there is no significant pathology noted ;(C) Cisplatin group showing hepatocyte vacuolization and degeneration. (D) Betaine + cisplatin group displaying marked improvement in the histological picture.

potent reducing effect on the production free radicals in rats exposed to cytotoxicity. In previous studies, betaine administration exhibited protective effects against oxidative injury in liver [13], and other organs as heart [39], and kidney [17]. Betaine accentuates oxidative hepatic injury induced by ischemia/reperfusion [40], and by hepatotoxicants such as ethanol [41], chloroform [13], lipopolysaccharide [35,15], dimethylnitrosoamine [14], and α-naphthylisothiocyanate [13]. Betaine was also protective in alcoholic and non-alcoholic fatty liver disease [8,9,42,43]

Betaine can limit reactive oxygen species via the regulation of Kupffer cells [44] which play a central role in the production of reactive oxygen radicals and cytokines in both acute and chronic liver injury [45]. Another possibility is that betaine may acts *via* its role in sulfur-containing amino acids metabolism in the trans-sulfuration pathway in the liver [13,14,43]. Betaine plays an important role in methionine synthesis that acts as a principal provider of cellular cysteine *via* trans-sulfuration pathway required for reduced glutathione synthesis [36]. GSH is the major antioxidative tripeptide in the cell that plays a pivotal role in the protection from reactive metabolites [1]. In addition, it is essential in the detoxification of toxicants including chemotherapeutic drugs, and the regulation of cellular homeostasis [46]. Detoxification of lipid peroxides occurs by the glutathione redox cycle that includes reduced GSH together with GSH-Px leading to the maintenance of mitochondria and cell membranes integrity. This might explain the observed reduction in hepatic GSH and GSH-Px activity induced by cisplatin and give a clue to thiol-enhancing action of betaine that might attenuate the oxidative stress and maintained the antioxidant defense system in cisplatin-treated animals.

NF-κB, is an oxidative stress-sensitive transcriptional factor. In the current study, cisplatin increased NF-κB expression in hepatic tissues, an action that was abrogated by prior betaine supplementation. The ability of betaine to suppress NF-κB expression may be linked to its capability to modify oxidative stress/NF-κB signaling

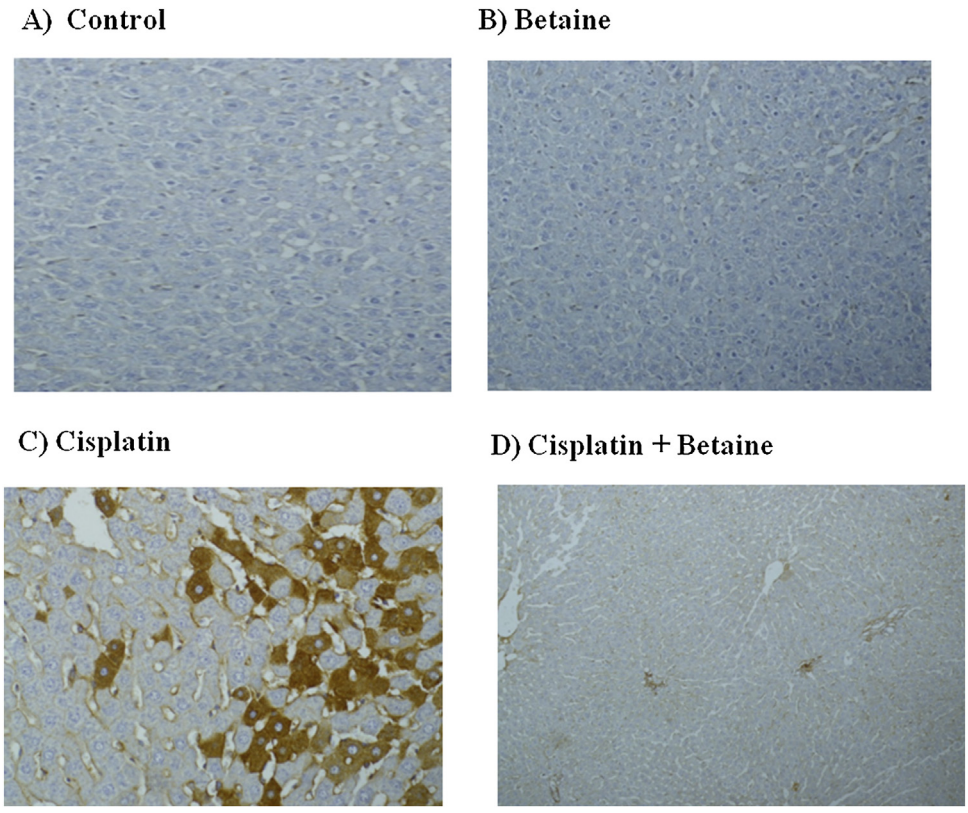

Fig. 4. Immunohistochemical staining of nuclear factor-B (NF-B) in rat liver (100×) from:(A) Normal control group and (B) Betaine alone treated group showing no expression of NF-κβ; (C) Cisplatin group without betaine treatment showing a significant increase in NF-κβ immunoreactivity; (D) Betaine + cisplatin group demonstrating a significant reduction in NF-κβ immunostaining. Brown color indicates NF-κβ positivity.

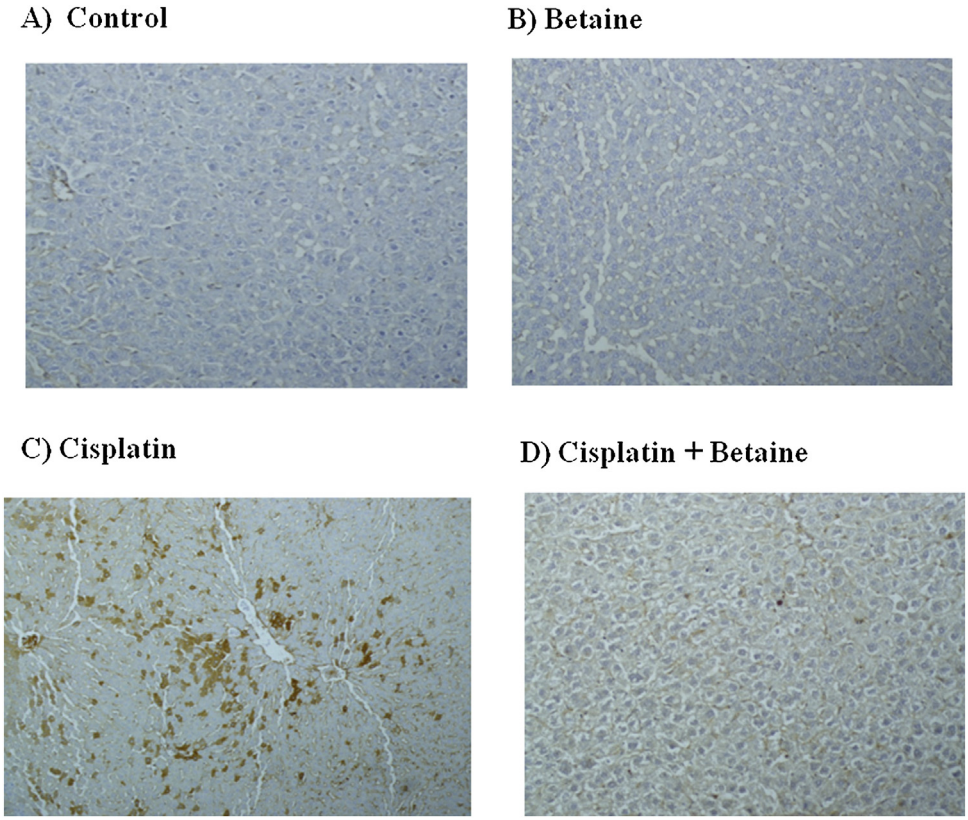

Fig. 5. Immunohistochemical staining of caspase-3 in rat liver (100×) from:(A) Control group and (B) Betaine alone treated group showing no expression of caspase 3; (C) Cisplatin group without betaine treatment showing a significant increase in caspase-3 immunoreactivity; (D) Betaine + cisplatin group demonstrating a significant reduction in caspase-3 immunostaining. Brown color indicates caspase-3 positivity.

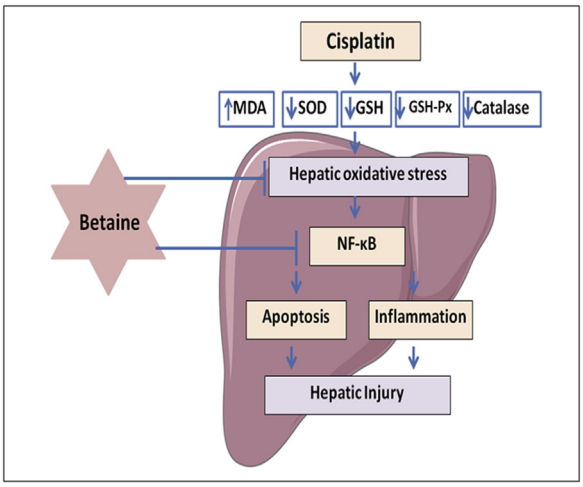

Fig. 6. Proposed mechanism of action of betaine in mitigating cisplatin induced liver injury.

pathway through correction of redox imbalance. Consistently, betaine suppressed NF-κB *in vitro* and *in vivo* in other studies [36,47].

Increased NF-κB can upregulate pro-inflammatory cytokines expression like TNF-α, inducible NO synthase, and adhesion molecules [36,48]. In the present study, cisplatin induced elevation of NO and TNF-α in hepatic tissues suggesting implication of inflammation in mediating cisplatin-induced hepatic injury. TNF-α is released from the activated Kupffer cells and can aggravate not only inflammation, but also the oxidative stress [29]. NO can combine with superoxide anion to form peroxynitrite radical that causes protein nitration and hepatic injury [43]. In the present study, the hepatoprotective action of betaine can be attributed to both the correction of oxidative stress and inhibition of NF-κB pathway that in turn enhances many inflammatory mediators' transcriptions. Betaine might inhibit inducible NO synthase indirectly *via* suppressing NF-κB [15,36]. The anti-inflammatory action of betaine on NF-κB activity and TNF-α expression has been demonstrated *in vitro* in aged rat kidney cells [15,36] and *in vivo* during cisplatin nephrotoxicity [17]. Moreover, betaine attenuated the expression of adhesion molecules in aged rat aorta [47]. Betaine diminished the markers of systemic inflammation in healthy adults as C-reactive protein, interleukin-6, and tumor necrosis factor-α [12].

The contribution of reactive oxygen species, pro-inflammatory cytokines and activation of caspase family of proteases particularly caspase-3 in cisplatin has been studied [49]. In our study, cisplatin-induced upregualtion of caspase-3 immunostatining was suppressed by prior betaine treatment. The inhibitory action of betaine on caspase-3, 8, and 9 activities was demonstrated in Madin Darley canine kidney cells after osmotic stress [50,51], in hepatocytes following bile acid-induced apoptosis [52], in cisplatin-induced nephrotoxicity in rats [17].

In conclusion, betaine supplementation can attenuate cisplatin-induced liver injury. Betaine was able to re-establish redox balance by preserving thiol homeostasis, thereby repressing inflammatory mediators, and over-expression of caspase-3 and NF-κB during cisplatin toxicity (Fig. 6). Our findings open a new hopeful approach for prevention of hepatotoxicity in cisplatin-based chemotherapy using betaine that has many advantages as it is stable, non-toxic, and highly soluble in water. However, the results of the present study are preliminary, further studies using animals and patients are wanted.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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