Original Article

Metformin attenuates oxidative stress and liver damage after bile duct ligation in rats

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Abstract

The aim of the current study was to investigate the antioxidative effect of metformin (MTF) on bile duct ligation (BDL)-induced hepatic disorder and histological damage in rats. The rats were divided into 4 groups including sham control (SC), BDL alone (BDL surgery), MTF1 (BDL surgery and administration of 250 mg/kg of MFM) and MTF2 (BDL surgery and administration of 500 mg/kg of MTF). After BDL, the animals treated with MTF by gavage for 10 days. Hematoxylin and eosin staining, biochemical analysis and oxidative stress markers were assayed to determine histological alterations, liver functions, and oxidant/antioxidant status. Hepatotoxicity was verified by remarkable increase in plasma levels of aminotransferases and alkaline phosphatase activity and liver histology 10 days after the BDL surgery. Our finding showed that treatment with MTF markedly reduced plasma alkaline phosphatase and alleviated liver injury indices ($P \leq 0.05$). Furthermore, BDL caused a considerable increase in the protein carbonyl and malondialdehyde content ($P \leq 0.05$). However, MTF reduces oxidative stress by constraining the protein oxidation and lipid peroxidation, and increases antioxidant reserve by increasing the ferric reducing ability of plasma and reducing glutathione levels. MTF exerts antioxidative effects in the liver fibrosis and may represent a hepato-protective effect when given to rats with BDL-induced hepatic injury.

Keywords: Antioxidant; Cholestasis; Fibrosis; Metformin; Oxidative stress.

INTRODUCTION

Liver is described by a complex structure and has a critical role in the preservation, implementation, and regulation of the body homeostasis (1). Extracellular matrix proteins accumulate uncharacteristically in the perisinusoidal space in some chronic liver damages, including alcoholic, metabolic, viral, and biliary disorders (2). Cholestasis is revealed by a fault in the bile acid transportation and intensified atypical flux of bilirubin and bile acids in the hepatic tissue (1). Preservation and accretion of toxic bile salts cause oxidative stress culminating in cirrhosis and liver failure (1,3). The exact mechanism of cholestatic disease is not well clarified, however, oxidative stress is a main reason which might be involved in this process (4). An imbalance between free reactive oxygen species (ROS) and antioxidants system is known as oxidative stress, which can lead to the oxidative damage (5). ROS generated under this condition can cause distress in the lipid, protein and DNA, and depletes antioxidant enzymes (6).

Metformin (MTF), a dimethylbiguanide, is a hypoglycemic drug that is generally prescribed in the controlling of type II diabetes mellitus.

Furthermore, MTF has a direct scavenging effect against ROS, restores the antioxidant system, and therefore recovers the level of oxidative and nitrosative stress in diabetes mellitus (7,8). Earlier studies have confirmed the protecting effect of MTF in reducing oxidative stress markers such as advanced glycation end products, and restoration of ferric reducing antioxidant power (9). Furthermore, MTF decreases lipid peroxidation, protein carbonyl (PCO) content (10), and cardiac fibrosis (11). Collectively these remarks powerfully advocate MTF as a potent antioxidant agent. Bile duct ligation (BDL) is an appropriate model in animal cholestatic liver injury, which pathogenitically and etiologically is similar to the biliary fibrosis in humans (12).

Considering that the study of probable antioxidant effects of MTF has not been studied in the BDL-induced cholestasis, the present study was designed to examine the protective effect of MTF against oxidative damage, oxidative stress markers, and antioxidant status in plasma and liver tissue during BDL-induced hepatic injury.

MATERIAL AND METHODS

Chemicals

MTF was obtained from Doctor Abidi Pharmaceutical Co. (Tehran, I.R. Iran), thiobarbituric acid (TBA), 2,4,6-tris (2-pyridyl) -s-triazine (TPTZ), and 5,5′-dithiols-(2-nitrobenzoic acid) (DTNB) were purchased from Sigma Chemical Co (St Louis, MO, USA). Trichloroacetic acid (TCA), 2, 4-dinitrophenylhydrazine (DNPH), and formaldehyde were obtained from Merck (Germany) and all other chemicals and reagents used were of analytical grade.

Experimental design

Male Wistar rats weighing 180-250 g were obtained from Razi Institute (Tehran, I.R. Iran) and acclimatized for 1 week before the experiments. The animals were kept in the animal house of Yasuj University of Medical Sciences with 12:12-h light/dark cycles. The rats had free access to a normal diet and water, *ad libitum*. The Ethics Committee of Yasuj University of Medical Sciences approved the study. A total of 28 rats were randomly divided into 4 groups of 7 animals each as follows: group 1, sham-control (SC) rats experienced laparotomy without BDL; group 2 (BDL), rats with BDL alone; group 3 (BDL and 250 mg/kg of MTF), and group 4 (BDL and 500 mg/kg of MTF). BDL was performed under common anesthesia induced by a combination of ketamine HCl (50 mg/kg) and xylazine HCl (10 mg/kg) (13). The day after surgery, the animals received normal saline orally or MTF (250 or 500 mg/kg/day) for 10 consecutive days. Finally, the animals were killed by puncturing the heart under deep anesthesia and the blood was taken. Liver tissue from each rat were removed and divided into two parts; one part was used for preparing tissue homogenate and the other part was fixed in buffered 10% formalin for liver histology.

Biochemical examination

Plasma total bilirubin (TBIL), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and alanine aminotransferase (ALT) were measured by standard diagnostic kits (Bionik Diagnostic Co., I.R. Iran) and an automatic biochemical analyzer (Roche COBAS, Mira Plus, USA).

Determination of oxidative stress biomarkers

Nitrite and nitrate levels were measured as an index of nitric oxide (NO) formation according to the Griess reaction (14). The level of NO metabolites was expressed as μmol/L for plasma and μmol/mg protein for tissue using sodium nitrite as standard (0-100 μmol/L). Concentration of reduced glutathione (GSH) in tissue homogenate and plasma was determined using DTNB, which develops a yellow color complex with GSH (15). The content of GSH was calculated using a molar absorption coefficient of 1.36×10^3 M^{-1}cm^{-1} and expressed as μmol/L for plasma and nmol/mg protein for tissue.

Plasma and tissue malondialdehyde (MDA) were determined based on the reaction with TBA (15). The MDA content was determined using a molar absorption coefficient of 1.56×10^5 M^{-1}cm^{-1} and expressed as μmol/mg

protein for tissue and µmol/L for plasma. The ferric reducing antioxidant power (FRAP) was determined on the ferric reducing ability of plasma; which is estimated from the reduction of a Fe^{3+-}TPTZ complex to the Fe^{2+} form at low pH (16). The FRAP content was expressed as µmol/L for plasma using FeSO4.7H2O solution as standard (0-1500 µmol/L).

PCO content was assayed using a spectrophotometric method based on the color produced by the reaction of DNPH and the carbonyl groups reaction (17). PCO level was calculated using a molar absorption coefficient of 2.2×10^4 $M^{-1}cm^{-1}$ and demonstrated as µmoL/mL protein for plasma and µmol/g for tissue. Total thiols (TSH) content was determined based on the reaction with DTNB (15). Total TSH was calculated using the molar absorption coefficient of 13,600 M^{-1} cm^{-1} and expressed as µmol/mg protein for tissue.

Histological evaluation

Liver samples were fixed in a 10% formalin solution. After dehydration in graded alcohol series, the liver tissues were cleared in xylene. Then, the samples embedded in paraffin and 5-µm sections were attained using a microtome. Routine staining with hematoxylin & eosin, was prepared for each liver section (13).

Statistical analysis

The results are expressed as mean ± SEM. Statistical analysis was done using one-way analysis of variance (ANOVA) followed by a Tukey's post hoc test. Significance was considered at $P \leq 0.05$.

RESULTS

Histopathological changes

No unusual histopathological alterations were noted in the livers of SC animals, in contrast to the BDL group that revealed different degrees of liver injury. A mild reduction in liver changes was detected in rats receiving MTF when compared to the BDL group (Fig. 1).

Biochemical parameters

As displayed in Table 1, 10 days after the experiment, the levels of TBIL, ALT, AST, and ALP of rats in the BDL group was significantly higher than that of the SC group ($P < 0.05$). In addition, MTF at a dose of 500 mg/kg in BDL rats caused reduction in ALP activity compared to BDL alone group ($P < 0.05$).
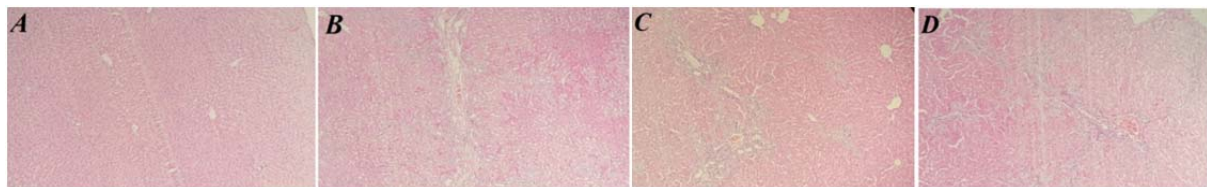

Fig. 1. Histopathological findings of liver tissue stained with hematoxylin and eosin (×10). (A) Sham control rat, (B) bile duct ligated rat, (C) bile duct ligated rat treated with 250 mg/kg metformin, and (D) bile duct ligated rat treated with 500 mg/kg metformin.

Table 1. Effect of MTF on plasma biochemical parameters in BDL-induced cholestasis in rats. Each value represents the mean ± SEM. *P value ≤ 0.05 different from SC; #P value ≤ 0.05 Significantly different from BDL alone group.

Groups	SC	BDL alone	BDL + MTF (250 mg/kg)	BDL + MTF (500 mg/kg)
ALT (U/L)	45.40 ± 1.24	107.80 ± 12.27*	162.66 ± 16.95*#	144.00 ± 16.63*
AST (U/L)	118.80 ± 8.57	377.80 ± 64.14*	415.80 ± 28.73*	528.40 ± 85.47*
ALP (U/L)	844.00 ± 45.00	1719.60 ± 99.84*	1700.28 ± 62.58*	1232.27 ± 92.99#
TBIL (mg/dl)	0.19 ± 0.01	9.03 ± 0.54*	9.17 ± 0.16*	8.57 ± 0.48*

ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TBIL, total bilirubin; SC, sham control; BDL, bile duct-ligated rats; MFT, metformin.

Liver tissue oxidative stress markers

Our findings indicated markedly reduced level of TSH in the BDL rats compared to SC group (Table 2). PCO, a specific marker of protein oxidation (18), was decreased by 48% in the BDL plus 500 mg/kg of MTF group in relation to the BDL alone group. Furthermore, MDA contents were 71% lower in the BDL plus 500/kg mg of MTF group compared to the BDL group (P < 0.05). Moreover, the liver NO metabolites level significantly decreased by 120% and in the BDL plus 250 mg/kg of MTF and 90% in BDL plus 500 mg/kg of MTF group compared to the BDL alone rats (Table 2).

Plasma oxidative stress markers

Fig. 2 shows significant increase in plasma NO metabolites, MDA, FRAP, GSH, and PCO in BDL alone rats compared to SC group (P < 0.05). MTF at 500 mg/kg caused a significant reduction in MDA content compared to BDL alone group (P < 0.05). In addition, treatment of MTF (at doses of 250 and 500 mg/kg) in BDL group significantly increased the GSH content and decreased the PCO production (P < 0.05) compared to the BDL rats. Our analysis revealed that MTF had no significant effect on NO metabolite levels in plasma compared to BDL alone group (Fig. 2).
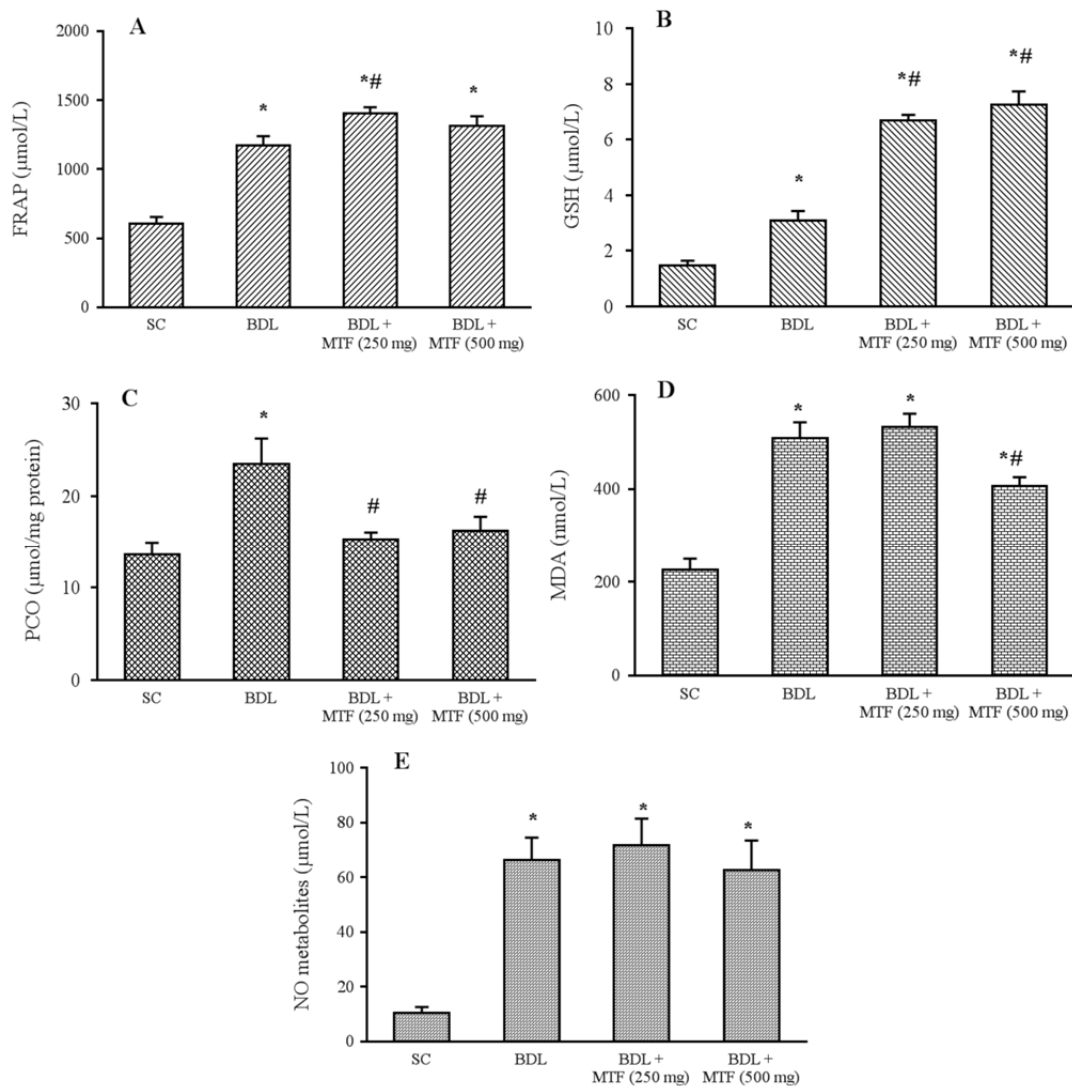

Fig. 2. Effect of MTF on (A) FRAP, (B) GSH, (C) PCO, (D) MDA, and (E) NO metabolites content in the plasma of BDL-induced cholestasis rats. Each value represents the mean ± SEM.. *Significantly different from SC, P value ≤ 0.05. #Significantly different from BDL, P value ≤ 0.05. FRAP, ferric reducing antioxidant power; GSH, glutathione; PCO, protein carbonyl; MDA, malondialdehyde; NO, nitric oxide; SC, sham control; BDL alone; bile-duct-ligated rats; MTF, metformin.

Table 2. Effect of MTF on the tissue oxidative stress markers in BDL-induced cholestasis in rats. Each value represents the mean ± SEM. *P value ≤ 0.05 different from SC; #P value ≤ 0.05 significantly different from BDL alone group.

Groups	SC	BDL alone	BDL + MTF (250 mg/kg)	BDL + MTF (500 mg/kg)
GSH (nmol/mg protein)	186.74 ± 15.08	200.16 ± 22.32	261.09 ± 33.62	253.23 ± 36.99
TSH (µmol/mg protein)	2.69 ± 0.07	1.87 ± 0.22*	2.21 ± 0.30	2.00 ± 0.21
PCO (µmol/g tissue)	27.72 ± 2.82	31.06 ± 2.23	29.59 ± 2.86	20.96 ± 1.23#
MDA (nmol/mg protein)	25.86 ± 3.39	44.32 ± 13.11	25.28 ± 3.30	18.36 ± 1.87*#
NO metabolites (µmol/mg protein)	1.39 ± 0.14	1.43 ± 0.20	0.65 ± 0.09*#	0.75 ± 0.09#

GSH, glutathione; TSH, total thiol; PCO, protein carbonyl; MDA, malondialdehyde; NO, nitric oxide; MTF, metformin.

DISCUSSION

BDL causes modifications that nearly resemble to those observed in cirrhosis in humans, and therefore this model is commonly used to induce cholestasis and biliary cirrhosis in animals (13). Cholestatic liver damage is developed four days after BDL and extends significantly between the first and second week after the operation (19). ALT, AST, and ALP enzyme activity are appropriate tests for estimating the extent of hepatic injury (20). Our results showed that rats revealed an evident cholestatic liver damage ten days after BDL surgery, in accordance with the high levels of ALT, AST, ALP, and total bilirubin concentrations in the plasma. Although MTF at a high dose (500 mg/kg/day) reduced the level of ALP, it had no reducing effect on the contents of ALT, AST, and total bilirubin.

It has been reported that BDL increases ROS formation and reduces antioxidant reservoir, which promotes oxidative stress (12). Zhao *et al.* exhibited that in the BDL animals, the content of MDA were augmented (21). Our results showed that MDA as a final product of lipid peroxidation slightly was increased in hepatic tissue but significantly increased in the plasma of BDL group as compared to SC group. A previous study has reported hepato-protective effects of antioxidants in BDL-induced liver damage (22). In the current study, administration of BDL rats with MTF significantly reduced the MDA contents in both tissue homogenate and plasma samples compared to BDL alone rats. In agreement with these results, Salman *et al.* showed that treatment of diabetic rats with MTF significantly decreased hepatic MDA concentrations compared to diabetic animals

(23). MTF may attenuate MDA levels by inhibiting the mitochondrial complex-I of respiratory chain, and lowering the production of ROS (24).

In this study BDL rats showed a significant increase in plasma GSH and a slight increase in hepatic GSH compared with SC group. These findings are consistent with Orellana *et al.*, which found an apparent increase in GSH in BDL rats compared to SC group (4) that may be related to reducing removal of GSH in bile acids or to lowering activity of microsomal uridine 5'-diphospho-glucuronosyltransferase enzyme (25). Our finding showed that ten days after administration of MTF at doses of 250 and 500 mg/kg to BDL group, plasma GSH content was considerably higher as compared to BDL alone group ($P < 0.05$), as well as hepatic GSH content tended to increase in BDL rats receiving MTF. Alhaider *et al.* reported that administration of MTF (at a dose of 500 mg/kg) in diabetic rats with nephropathy, considerably recovered the depletion of GSH in the kidney homogenates of normoglycemic control rats (7). The enhancement of GSH is evidently through producing NADP in pentose phosphate shunt by MTF which is used in GSH restoration (10).

The main findings of the current study is increased plasma PCO in the BDL rats compared to the SC group and decreased PCO levels compared to the BDL group when rats treated with MTF at 250 and 500 mg/kg. Moreover, MTF at 500 mg/kg considerably decreased the abnormal elevating hepatic levels of PCO in the BDL-induced rats. Among the numerous oxidative alterations of the amino acids in proteins, PCO production

may be an early marker of ROS-mediated protein oxidation (26). According to Sundari *et al.* (27) and Terzioglu *et al.* (28) reports, oxidative injury of proteins happens in CCl₄ and BDL-induced liver injury, and it may contribute in the pathogenesis of liver damage. Although oxidative injury of DNA, protein, or lipid may be extremely detrimental, proteins are more susceptible because they often act as catalysts inside the cells. Advanced glycation end products (AGEs) can accumulate as protein modifications (29) Beisswenger *et al.* observed that MTF decreases methylglyoxal, an important AGE with α-dicarbonyl structure, in type 2 diabetes (30). Also, Ruggiero-Lopez *et al.* showed that MTF as a guanidine-like compound decreases AGE creation by reacting with α-dicarbonyl groups (31). We conclude that MTF can react with carbonyl groups and reduce these compounds.

TSH, an organosulfur containing endogenous antioxidant compounds, is a sensitive indicator of oxidative stress that plays a vital role in defense against ROS (28). Our findings revealed that the hepatic TSH groups were reduced significantly in BDL rats compared to SC, while MTF had no significant impact on it. In accordance with our findings, Kabirifar *et al.* have demonstrated a decrease in TSH group and an increase in PCO level in BDL rats compared to the SC (32).

Total anti-oxidant capacity measures the antioxidants such as bilirubin, uric acid, ascorbic acid, polyphenols, and β-carotene (26). Hence, we estimated the antioxidant/reducing potential of plasma using FRAP assay. In the current study, BDL Plus MTF rats indicated a significant increase in plasma FRAP content compared to the SC and BDL alone groups. In agreement with our results, Esteghamati *et al.* reported that FRAP contents increased (about 27%) following MTF administration in type 2 diabetic patients (9). The elevated levels of plasma FRAP in BDL rats may be due to an increase in the content of bilirubin and other endogenous antioxidants (33).

NO is a highly reactive molecule produced by a variety of cells such as endothelial, macrophage, hepatocyte, and Kupffer cells in liver and contributes as the principal mediator of hepatic cell damage (34). Therefore, we estimated nitrate and nitrite levels as an indicator of NO formation. In accordance with our previous study (35), the current investigation showed significant elevation in NO metabolites level in the plasma of BDL rats. Furthermore, our results showed that MTF at both 250 and 500 mg/kg caused a marked decrease in the hepatic NO metabolites level while it had no effect on NO metabolites in the plasma compared to BDL alone group. Salman *et al.* reported that treatment with MTF significantly lowered nitrite/nitrate levels compared to the untreated diabetic rats (23). MTF may reduce oxidative stress by arresting reactive nitrogen species such as NO at an intracellular level (9).

CONCLUSION

In summary, the present results suggest that 10-day administration of MFT had no effect on conventional biochemical indicators such as AST, ALT, and TBIL of liver injury of BDL rats. In addition, the current study provides more data that MTF decreases oxidative stress by preventing the protein oxidation and lipid peroxidation, as well as escalating antioxidant restoration by increasing plasma GSH and FRAP levels. MTF treatment may prevent hepatic damage via the suppression of specific enzymes that are responsible for protein oxidation and lipid peroxidation after BDL.

ACKNOWLEDGMENTS

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