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Hepatorenal Repercussions of Alcoholic Exposure in a Rat Model: a Dose-Dependent Study of Metformin Intervention

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ABSTRACT

Background: Diabetes mellitus is an alarming life style disease in the modern world. Exploitation of the anti-diabetic drugs for the amelioration of diabetes and associated life style diseases has become an imperative concern. In this milieu, this study was designed to explore the plausible effects of metformin intervention on hepatic and renal functions in a rat model of alcoholic liver disease. **Methods:** Thirty rats were divided into five groups (n = 6): ethanol control, ethanol water and also low, moderate and high doses of metformin. Ethanol 20% v/v (1 ml/100 g) was administered by oral gavage to all five groups for 21 days. Blood and tissue samples were collected for the assessment of lipid profile, hepatic and renal functions. **Results:** After 21 days, the levels of hepatic function and lipid parameters were maintained at normalcy, especially in the high-dose metformin treated alcoholic rats as compared to the levels at day 1. Despite this, the renal biomarkers did not display any significant variation due to ethanolic exposure in any group. The histopathological score portrayed that the noxious effect of ethanol is prevented in the liver of moderate- and high-dose metformin, whereas the renal histological scores were unchanged in all the groups including ethanol control. **Conclusion:** These results suggest that the dose of ethanol required to induce hepatic dysfunction does not influence renal functions. In addition, high-dose metformin offers maximal hepatoprotection and spares kidney from *per se* toxicity, thereby advocating the beneficial intervention of the anti-diabetic drug, metformin, in alcoholic liver dysfunction. *Iran. Biomed. J. 16 (2): 101-106, 2012*

Keywords: Ethanol, Metformin, Kidney

INTRODUCTION

Diabetes and other life style diseases including alcoholic liver disease (ALD) are still among the leading causes of morbidity and mortality in the world [1, 2]. The increase in nicotinamide adenine dinucleotide concentration and acetaldehyde dehydro-genase activity may injure β-oxidation of fatty acid and the tricarboxylic acid cycle, resulting in an increase in the synthesis of triacylglycerol, followed by steatosis in hepatic tissue [3]. Alcohol manifests its harmful effects either through free radical species that react with most of the cell components, changing their structures and functions, or by contributing to other mechanisms that finally promote enhanced oxidative damage. The pathophysiology of ALD is well studied, but a successful therapeutic approach to prevent or reverse ALD is still minimal. Conventional therapy is still based largely on abstinence from alcohol and general supportive and symptomatic care [1]. However, it is very difficult for the addict to follow abstinence and usually there is relapse. Multiple treatment interventions for both the short and long term mortality and morbidity of this disease have been proposed, but strong disagreement exists [1]. Phytotherapeutic approaches are being used with limited proof for their efficacy. Few synthetic molecules are still under pipeline and are being probed for their utility in ALD [4, 5]. Therefore, search for a potential candidate in attenuating ALD is still enduring. Previous studies explore a strong relationship between hepatic insulin resistance and fatty liver as well as steatohepatitis in humans [6, 7]. In view of these evidences, an array of animal and human studies displays the insulin-sensitizing biguanide, metformin, bestow protection in non-alcoholic fatty liver disease [8]. Although the pathophysiological mechanism of non-alcoholic fatty liver disease and ALD are similar and even a previous

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study had shown the protective effect of metformin on ALD in a mouse model [6], the effect of metformin and ethanol co-exposure on the hepatic and renal functions was not investigated concurrently in a single study. With this note, the present work was designed to assess the dose-dependent effect of metformin on hepatic and renal functions simultaneously in ethanol-exposed rats.

MATERIALS AND METHODS

Drugs and chemicals. Metformin was procured from Glenmark (Mumbai, India) and ethanol was purchased from Merck (Germany). All other chemicals were of analytical grade and the organic solvents were distilled before use.

Animals. Thirty adult Sprague Dawley rats (Central Animal House, Bharati Vidyapeeth Deemed University Medical College, Pune, India; CPCSEA Reg. No.258/2009) of either sex, weighing 150-200 ×g were selected. The animals were housed in plastic cages under controlled conditions of 12-h light/12-h dark cycle, 50% humidity and at 25°C. They all received a standard pelleted diet (Pranav Agro Industries Ltd., Pune, Maharashtra, India) and water *ad libitum*. This study was approved by Institutional Animal Ethics Committee and performed as per CPCSEA guidelines.

Experimental regimen. The animals were randomized into five groups (n = 6) as follows: Group I, ethanol control rats received ethanol 20% v/v in water (1 ml/100 g/day, oral gavage [p.o.]); Group II, rats received ethanol 20% v/v in water (1 ml/100 g/day, p.o.) + water; Group III, low-dose metformin (125 mg/kg/day, p.o.); Group IV, moderate-dose metformin (250 mg/kg/day, p.o.) and Group V, high-dose metformin (500 mg/kg/day, p.o.). Ethanol 20% v/v in water (1 ml/100 g; p.o.) was administered to all five groups. All the treatments were given once per day for 21 days. On day 1 before drug administration, the blood was collected by retro-orbital puncture to assess various biochemical functions. The body weight of animals was recorded on day 1 and day 21. After the final doses of drugs (i.e. on day 21), the animals were anaesthetized and the blood was collected by retro-orbital puncture for the estimation of biochemical parameters and later the animals were sacrificed by cervical dislocation. The livers were removed and the weight and volume were also recorded. Then, the kidneys were also dissected and both organs were used for the histopathological assessments.

Biochemical assays. The levels of aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) in serum were estimated by using commercial kits (ERBA diagnostic Mannheim GmbH, Germany). Total bilirubin, total protein and albumin were measured spectrophotometrically by commercially available kits and prothrombin time by using auto analyzer. Serum lipid profiles such as triglycerides were measured enzymatically by the glycerophosphate oxidase assay using a commercial kit (ERBA diagnostic Mannheim GmbH, Germany). Total cholesterol and high density lipoprotein levels were measured enzymatically by cholesterol oxidase assay using commercially available kits (ERBA diagnostic Mannheim GmbH, Germany). Blood glucose, urea and serum creatinine levels were measured by using the kits obtained from ERBA diagnostic Mannheim GmbH (Germany).

Histopathological analysis. Small portions of the liver and kidney were dissected and fixed in 10% neutral buffered formalin solution for 24 h. The fixed tissues were embedded in paraffin, sectioned to 3-5 µm thickness, deparaffinized, and rehydrated using standard techniques. The extent of alcohol-induced necrosis and steatosis was evaluated by assessing morphological changes in liver sections stained with hematoxylin and eosin using standard techniques. Liver sections were graded numerically to assess the degree of histological changes in acute hepatic injury. The scoring of liver damage was done [9, 10] as follows: portal fibrosis (0-6), lobular infiltration and necrosis (0-3), mallory bodies (0-3), hepatocyte ballooning (0-3) and fatty changes (0-3). The parameters were graded from score 0 to 6, with 0 indicating no abnormality, 1 to 2 mild injury, 3 to 4 moderate injury and 5 to 6 with severe liver injury. Scoring of kidney damage was done [11] as follows: tubular epithelial cell degeneration (0-3), tubular epithelial cell necrosis (0-3), atrophic glomerulus and tubulus (0-3), eosinophilic secretion in the tubulus lumen (0-3), interstitial mononuclear cell titration (0-3), increased fibrous tissue (0-3), hyperemic vessels in the interstitium (0-3).

Statistical analysis. All the data were expressed as mean ± SEM. The results were evaluated using a paired *t* test and analysis of variance (ANOVA) using the SPSS software.

RESULTS

Effects of metformin and ethanol on body weight, total protein, blood urea, serum creatinine and blood sugar levels. Effects of metformin and ethanol on body weight, total protein, blood urea, serum creatinine and blood sugar have been depicted in Table 1. Ethanol-exposed rats in group I showed an increase in body

Table 1. Effects of metformin and ethanol on body weight, total protein, total albumin, blood urea, serum creatinine and blood glucose.

Parameter		Body weight (g)	Total protein (g%)	Total albumin (g%)	Blood urea (mg%)	Serum creatinine (mg%)	Blood glucose (mg%)
Ethanol	Day 1	164.17 ± 19.07	8.58 ± 0.31	4.47 ± 0.68	36.83 ± 4.62	0.65 ± 0.005	72.33 ± 13.68
	Day 21	175.83 ± 17.77**	6.60 ± 0.41***	2.73 ± 0.35***	36.83 ± 4.79	0.70 ± 0.005	72.83 ± 13.35
ethanol + water	Day 1	194.83 ± 11.20	8.53 ± 0.33	4.77 ± 0.24	40.17 ± 6.68	0.63 ± 0.008	89.83 ± 8.42
	Day 21	199.00 ± 15.90	7.13 ± 0.54***	3.57 ± 0.40***	40.17 ± 7.73	0.68 ± 0.008	91.00 ± 11.38
ethanol + met (125)	Day 1	172.17 ± 17.37	8.65 ± 0.47	4.22 ± 0.55	34.00 ± 3.29	0.63 ± 0.14	77.50 ± 16.69
	Day 21	174.33 ± 8.69	8.22 ± 0.61*	3.92 ± 0.69*	34.50 ± 3.99	0.65 ± 0.12	76.17 ± 20.02
ethanol + met (250)	Day 1	174.17 ± 18.00	8.75 ± 0.49	4.20 ± 0.87	32.17 ± 3.06	0.67 ± 0.005	96.67 ± 8.52
	Day 21	179.83 ± 15.79	8.45 ± 0.50*	4.07 ± 0.93*	32.50 ± 2.07	0.67 ± 0.005	89.17 ± 8.98*
ethanol + met (500)	Day 1	198.33 ± 30.07	8.05 ± 0.26	4.25 ± 0.71	66.00 ± 29.13	0.62 ± 0.01	81.67 ± 6.5
	Day 21	201.83 ± 32.05	7.92 ± 0.34	4.23 ± 0.74	66.83 ± 29.39	0.63 ± 0.005	67.17 ± 16.73**

Values are represented as mean ± SEM; 6 animals in each group; Comparisons were made between day 1 vs. day 21 within each group. **P*<0.05, ***P*<0.01, ****P*<0.001. met, metformin

weight significantly (*P*<0.01) at day 21 when compared to rats administered with ethanol at day 1. However, ethanol given with water (group II), and low to high dose of metformin (group III, IV and V) did not show any significant alteration in body weight between day 1 and day 21. Levels of total protein and albumin were significantly (*P*<0.001) reduced in groups I and II rats at day 21 when compared to the rats of the same group at day 1. Such reduction (*P*<0.05) was also seen in the group III and group IV (low and moderate dosed metformin). While in high-dose metformin (group V), no significant decrease in protein and albumin levels were seen. The blood urea and serum creatinine were not significantly altered in any of the groups (group I-V). The results indicate that the dose of ethanol that causes liver damage did not cause any deleterious effect on kidney when given for 21 days. Blood glucose levels were not significantly altered by ethanol (group I) or ethanol given with water (Group II) or with low-dose metformin with ethanol (group III) treatment. Conversely, there was a significant decrease in blood glucose level in moderate (group IV) and high (group V) doses of metformin, which indicates the anti-diabetic potential of metformin.

Effects of metformin and ethanol on serum lipid profiles. Effect of metformin and ethanol on serum cholesterol, triglycerides and HDL has been depicted in Figure 1. There was no significant alteration in the level of total cholesterol in any of the groups (groups I-V). Triglyceride levels were significantly increased in all the groups. However, in ethanol-treated rats (group I) it was highly significant (*P*<0.001). Conversely, in high-dose metformin (500 mg/kg)-treated rats the significance (*P*<0.05) was low, which indicates its lipid-lowering effect. Level of the protective lipoprotein, HDL, was significantly diminished in ethanol, ethanol with water and ethanol with metformin low-dose groups. But in moderate and high dose of metformin-treated groups, no significant alteration in HDL levels were observed.

Effects of metformin and ethanol on hepatic biochemical markers. Effects of metformin and ethanol on hepatic biochemical markers have been displayed in Table 2. Ethanol control and ethanol with water rats displayed a significant increase in the levels of AST, ALT, ALP, total bilirubin and prothrombin time, which reveals the hepatocellular membrane damage. In contrary, metformin-treated rats (moderate and high dose) did not show any significant alteration

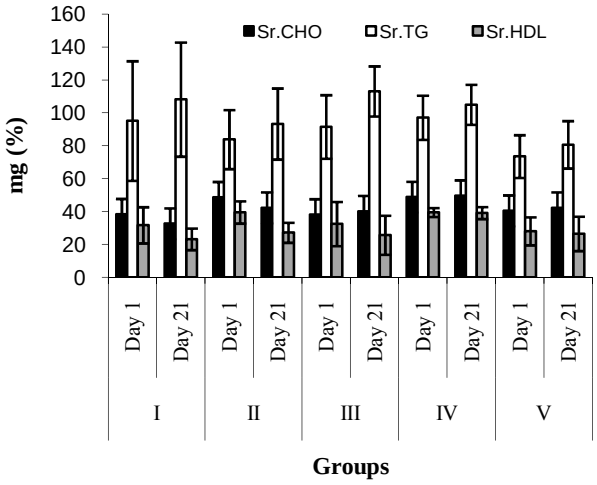

Fig. 1. Effect of metformin and ethanol on serum cholesterol, triglycerides and HDL. Values are represented as mean ± SEM; 6 animals in each group; Comparisons were made between day 1 vs. day 21 within each group. **P*<0.05, ***P*<0.01, ****P*<0.001.

Table 2. Effect of metformin and ethanol on hepatic biomarkers.

Parameter		AST (IU/L)	ALT (IU/L)	ALP (IU/L)	Total bilirubin (mg%)	Prothrombin time (s)
ethanol	Day 1	272.8 ± 47.58	45.50 ± 10.31	288.7 ± 113.68	0.300 ± 0.160	11.33 ± 0.52
	Day 21	491.7 ± 165.32**	62.70 ± 15.62**	316.5 ± 117.59*	1.210 ± 0.41***	19.83 ± 4.26**
ethanol + water	Day 1	365.5 ± 68.53	50.30 ± 9.61	219.3 ± 47.08	0.235 ± 0.008	11.50 ± 0.55
	Day 21	551.3 ± 140.95**	72.50 ± 11.93**	255.2 ± 46.01**	1.010 ± 0.26***	13.83 ± 1.94*
ethanol + met (125)	Day 1	300.0 ± 41.42	46.00 ± 8.81	213.3 ± 86.88	0.270 ± 0.01	11.50 ± 0.55
	Day 21	322.8 ± 40.32**	51.20 ± 7.14*	211.3 ± 72.42	0.400 ± 0.17	13.67 ± 1.63**
ethanol + met (250)	Day 1	289.0 ± 52.92	47.30 ± 8.41	224.2 ± 50.44	0.220 ± 0.007	11.67 ± 0.52
	Day 21	297.2 ± 48.89	50.30 ± 7.69	227.3 ± 49.15	0.280 ± 0.11	12.33 ± 0.52
ethanol + met (500)	Day 1	297.5 ± 95.56	53.20 ± 4.75	197.5 ± 66.74	0.280 ± 0.13	11.50 ± 0.55
	Day21	300.2 ± 92.83	53.50 ± 4.59	199.8 ± 63.88	0.300 ± 0.12	11.83 ± 0.75

Values are represented as mean ± SEM; 6 animals in each group; Comparisons were made between day 1 vs. day 21 within each group. **P*<0.05, ***P*<0.01, ****P*<0.001. met, metformin, AST, aspartate transaminase; ALT, alanine transaminase; ALP, alkaline phosphatase

in the levels of hepatic biochemical markers between day 1and day 21, which underscored the hepatic membrane protection offered by metformin. In this study, the significant variations in AST, ALT and prothrombin time, pointed out that the low-dose metformin failed to protect the hepatic tissue.

Effects of metformin and ethanol on liver and kidney histopathology, liver weight, liver volume and liver ratio. The biochemical observations were further confirmed by histological studies and the scores have been depicted in Table 3. In the present study, the scores of liver damage were significantly (*P*<0.01 and *P*<0.001, respectively) low in moderate- and high-dose metformin groups when compared with the ethanol-exposed group (group I). The liver weight was significantly reduced (*P*<0.05) in high-dose metformin rats when compared to the ethanol rats. Also, the liver volume was significantly (*P*<0.001) low in metformin moderate and high doses when compared with ethanol administered group. But metformin low dose had minimal effects on liver weight, thereby indicating its scarce hepatoprotection. In contrast, the kidney histopathological scores were not significantly altered by ethanol and metformin.

DISCUSSION

Kotronen *et al*. [12] reported that ALD may be a risk for diabetes and other metabolic disorders. Though a comprehensive mechanism remains to be unraveled, many studies [1, 3] had shown that ethanol oxidation generates toxic metabolites, free radicals and induces a state of oxidative stress which contributes to the pathogenesis of ALD. Importantly, oxidation of ethanol through the cytochrome P450 2E1 (CYP 2E1) generates superoxide anion radical and hydrogen peroxide [13-15]. These free radicals are capable of damaging many cellular components such as DNA, proteins and lipids [16].

In the present study, 20% ethanol (1 ml/100 g) daily for 21 days was used to induce ALD. The hepatic damage induced by ethanol was clearly visible from the leakage of hepato-specific marker enzymes (AST, ALT and ALP) from the hepatocytes into the blood stream, which was observed by the escalated levels of liver enzymes [17]. Based on this view point, it could be proposed that treatment with metformin at low dose displayed a minimal hepatocellular membrane protection when compared to that of the ethanol-treated

Table 3. Effects of metformin and ethanol on liver weight and liver volume and histopathology scores of liver and kidney damage.

Parameter	Ethanol	Ethanol + water	Ethanol + met (125)	Ethanol + met (250)	Ethanol + met (500)
Liver weight (g)	7.33 ± 1.03	8.33 ± 0.52	6.50 ± 0.55	5.83 ± 0.75	5.67 ± 0.52*
Liver volume (ml)	6.83 ± 0.75	7.33 ± 0.52	6.67 ± 0.82	5.67 ± 0.52*	4.83 ± 0.75**
Liver ratio (weight/volume)	1.07 ± 0.08	1.14 ± 0.13	0.99 ± 0.19	1.03 ± 0.13	1.20 ± 0.21
Histopathology scores of liver damage	9.17 ± 1.72	9.33 ± 1.37	6.17 ± 1.83	4.50 ± 1.76**	2.83 ± 1.17***
Histopathology scores of kidney damage	1.17 ± 1.33	1.33 ± 1.03	2.67 ± 1.86	2.17 ± 1.17	2.83 ± 2.71

Values are represented as mean ± SEM; 6 animals in each group; on day 21, comparisons were made between ethanol vs. other groups; **P*<0.05, ***P*<0.01, ****P*<0.001. met, metformin

groups; however, metformin treatment at moderate and high doses elicited a better hepato membrane protection by decreased serum marker enzymes' levels when compared to the ethanol controls. The preservation of hepatic marker enzymes levels by metformin is attributed to its antioxidant and free radical scavenging potential which is in harmony with the previous report [18]. Serum bilirubin is one of the most sensitive tests employed in the diagnosis of hepatic diseases. It offers useful information on how well the liver is functioning [19]. In the present study, ethanol alone as well as ethanol and water-fed rats had shown a significant increase in the level of total bilirubin at day 21 when compared to the level of total bilirubin at day 1 of the same group. Treatment with metformin at three doses (125, 250 and 500 mg/kg) had not significantly altered total bilirubin levels when compared within the groups and exerted a significant protection when compared with alcohol-fed groups indicating the effectiveness of the metformin in the maintenance of normal functional status of the liver. In the current study, elevated prothrombin time in ethanol, ethanol with water and metformin low-dose groups had reflected the decreased ability of liver to synthesize coagulation factors [20]. High-dose and moderate-dose metformin, maintained the normal prothrombin time between day 1 and day 21 which imply the maintenance of hepatic function and homeostasis by metformin.

In the current study, significant weight gain was seen in ethanol-treated group which may be due to the appetite stimulant action of ethanol, which had kindled the rats to consume more food. Metformin-treated groups did not show any significant weight change which may be due to the metformin induced decrease in calorie intake and weight loss against ethanol-induced weight gain [21]. We observed a significant decrease in total protein and albumin in ethanol and ethanol plus water administered rats as reported earlier [22] which demonstrate the functional disability of hepatocytes due to ethanolic insult. Though the low and moderate doses of metformin failed to uphold the TP and albumin levels, high-dose metformin maintained these levels thereby confirming the beneficial role of high-dose metformin on liver [18].

Ethanol acts as a surfactant and suppresses the action of enzyme lecithin cholesterol acyl transferase (LCAT) to block the uptake of lipoprotein from circulation by extra hepatic tissue, resulting in an increase in lipid levels. Though there was no significant alteration in the serum cholesterol level of any group, the HDL level was appreciably decreased in the ethanol control and ethanol plus water groups. This may be due to the oxidant inference with the HDL synthesis. Conversely, moderate and high-dose metformin treatment sustained the HDL levels against ethanolic toxicity which may be due to the antioxidant and anti-lipid peroxidative effect of metformin as reported in earlier studies [18, 23]. Metformin treatment at low dose had no favorable effect on HDL level which may be due to the inadequacy of the metformin dose to combat against oxidative stress. Triglyceride levels were increased significantly in all the groups including high-dose metformin group. But, it was observed that these abnormalities were somewhat low in metformin high-dose group indicating an effort to lower triglycerides. However, from the references [24, 25] it has become clear that metformin has controversial effects on triglycerides.

In the present study, ethanol and metformin-fed rats did not generate any significant change in blood urea, serum creatinine and kidney histopathological scores which highlight that both metformin and ethanol did not affect kidney stability. In addition, the blood glucose levels were significantly decreased in moderate and high-dose metformin groups, which reflect the effective anti-hyperglycemic activity of metformin. The observed data clearly advocates that the liver weight of high-dose metformin rats were significantly reduced ($P<0.05$) as compared to that of ethanol controls and here, the underpinning reason may be the adenosine monophosphate activated protein kinase-mediated suppression of lipogenic factors by metformin against ethanol-induced fatty liver and increased liver weight [26]. Histopathological assessment of liver tissues confirmed the membrane stabilizing and cytoprotective potential of metformin at a dose of 500 mg/kg against the toxic manifestations induced by ethanol.

Throughout the study, there exists a dose-dependent variation in hepatoprotection and also, this article strongly underscores that high-dose metformin may be a potential therapeutic candidate in attenuating the ALD with no *per se* toxicity on renal tissues. Furthermore, this study may serve as an imperative tool to provoke an interest in screening metformin against various metabolic disorders.

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