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Exploratory Studies of Alcohol Intoxication on Renal Antioxidant Enzymes in Diabetic Rats: A Study with Reference to Lipid Metabolic Profiles and Gene Markers

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Abstract

In the present study, we investigated the impact of alcohol on antioxidant enzymes, lipid metabolic profiles and gene markers in diabetic rats. Oxidative stress has been suggested to be a contributing factor to complications of diabetes and alcoholic disorders. Oral administration of alcohol (2gm/kg BW) to diabetic rats for 30 days resulted in a significant increase in blood glucose and a decrease in insulin levels. In alcoholic rats, diabetic rats, glutathione metabolism enzymes like glutathione peroxidase (GPx), glutathione reductase (GR), reduced glutathione (GSH), levels are depleted, and Glutathione-s-Transferase (GST) and Malondialdehyde (MDA) levels are elevated. Whereas with alcohol supplementation in diabetic rats, all the antioxidant enzymes are depleted, but GST, MDA levels are elevated. Lipid metabolic profiles like Low-Density Lipoprotein cholesterol (LDL), Triglyceride (TG), total cholesterol (TC) and Very Low-Density Lipoprotein cholesterol (VLDL) levels are upregulated, and High-Density Lipoprotein cholesterol (HDL) levels are downregulated in alcoholic, diabetic and alcohol treated diabetic rats. Gene markers like TNF-α, IL-6 and TGF-β are also upregulated in alcohol, diabetic and alcohol treated diabetic rats. The serum markers Alkaline Phosphatase (ALP), Aspartate Aminotransferase (AST) and aspartate alanine Aminotransferase (ALT) are elevated in all the groups. Our findings reveal that in alcohol intoxicated rats, the renal antioxidant enzymes are depleted, lipid profiles and gene markers are altered in diabetic rats. Furthermore, our histopathological studies also prove that alcohol administration in diabetic rats caused more renal tissue damage.

Major Findings: 1. Alcohol supplementation leads to alterations in antioxidant enzymes, lipid metabolic profiles and gene markers in diabetic rats. 2. We reported that alcohol toxicity leads renal tissue damage in diabetic rats.

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1. Introduction

Diabetes is a metabolic disorder that affects many organs in the human body, and it is also one of the major causes of death in the world[1]. As per the World Health Organisation (WHO) report, there are 830 million diabetic people in the world[2]. Diabetes is stress stress-related disorder, where free radicals are produced, which leads to tissue damage. In a diabetic condition, the formation of free radicals causes oxidative stress, which leads to alterations in antioxidant enzymes[3].

Alcohol intoxication is related to mental, physical and social harms. Many studies prove that alcohol intake affects many organs in the human body. Alcoholism was linked to more than 60 disease conditions[4]. Alcoholism is a main health problem which have pathological effects on the kidney, heart, liver and nervous system. Alcohol metabolites prompt the increase in free radical production in the human body, and they deplete antioxidant levels and lead to alcohol related diseases[5].

There are very few reports on the effect of alcohol intoxication in diabetic rats. Also, there are no reports on the impact of alcohol on oxidative stress markers, lipid profiles and gene markers during alcohol toxicity in diabetic rats. The objective of the present study was to monitor the toxic effects of alcohol in diabetic rats.

2. Materials and Methods

2.1 Animals

Young male Wistar strain albino rats weighing 180±20, three months old age are purchased from the Indian Institute of Sciences (IISc), Bangalore. All the rats are housed in cages and maintained under controlled room temperature with a photoperiod of 12 hours light and 12 hours dark. The rats are provided with diet and water *adlibitum*.

2.2 Chemicals

The chemicals used in this study are obtained from sigma aldrich, qualigens, himedia and fisher scientific companies.

2.3 Diabetes Induction in Rats

The rats are given a single intraperitoneal injection (ip) of STZ (50 mg/kg) for the induction of diabetes. All the STZ-induced rats are given a 20% glucose solution for 24 hours to prevent hypoglycemic deaths. Accucheck glucometer (Roche- Germany) was used to monitor the blood glucose levels. After 3 days of STZ injection, the rats with blood glucose levels more than 250 mg/dl are selected for the study. All the experiments in this study are approved and accepted by the Institutional Animal Ethics Committee (No: 09(i)/a/CPCSEA/IAEC/SVU/ZOOL/Prof.KSR/Dt. 08-07-2012).

2.4 Grouping of Animals

Rats are separated into five groups, each group containing six rats.

I – Normal Control (NC): rats receive saline for 30 days.

II – Alcohol treatment (At): rats receive the alcohol (2% bodyweight) for 30 days.

III – Diabetic Control (DC): Streptozotocin (STZ-50mg/kg) was used for the induction of diabetes.

IV – Diabetic + Alcohol administration (D+At): diabetic rats receive the alcohol for a period of 30 days.

V - Diabetic + Glibenclamide supplementation (D+Glb): Diabetic rats receive glibenclamide for 30 days.

Treatment was given for 30 days, and after 24 hours of the last treatment, the rats were decapitated by cervical dislocation. Kidney tissues are collected at 4°C and used for the estimation of antioxidant enzymes, and part of the tissues is used for histopathological investigation.

2.5 Biochemical Estimations

The antioxidant enzymes like GPx, GR GSH, GST and MDA levels are monitored by the methods of Flohe and Gunzler[6], Carlberg and Mannervik[7], Akerboom and Sies[8], Habig *et al.*[9], and Ohkawa *et al.*[10], respectively.

The blood glucose levels are monitored by a glucometer. Insulin, AST, ALT and ALP activities are measured by standard kits.

2.6 Estimation of Lipid Metabolic Profiles

LDL, VLDL, HDL, triglyceride and total cholesterol in the serum samples are assayed by standard kits. Results are expressed as µg/ml for HDL, LDL, VLDL, triglyceride and total cholesterol in mg/dl.

2.7 Gene Markers

Analysis of Total RNA and cDNA Synthesis

Total RNA was prepared by using standard kits (TAKARA)

Quantitative PCR (qPCR)

Quantitative analysis of mRNA expression of cytokine and stress markers are monitored by qPCR in the ABI Prism 7300 system (Applied Biosystems)

Primers of TNF-α, TGF-β and IL-6 are bought from Sigma (Bangalore, India). Total reaction mixture is 20µl and contains 5µl of cDNA sample, 1µl of nuclease-free water, 2µl of forward primer, 2µl of reverse primer and 10µl of SYBR Green Universal Master mix (Applied Biosystems).

The quantitative PCR information was assayed by using the relative gene expression method as reported by Livak and schmittgan[11]. All experiments are conducted twice.

2.8 Histopathological Studies

The kidney tissues are collected immediately after sacrificing the rats. 3 µm-thickness sections of the kidney are prepared and they are stained with haematoxylin and eosin (H and E-10x) for histopathological investigation.

2.9 Statistical Analysis

In this study, the results are represented as Mean and SD. In our study, one-way ANOVA analysis with Dunnett's multiple comparison test, MS Office, and Excel software are also used for analysis of the data. SPSS (Version 13.5; SPSS Inc., Chicago, IL, USA) was also used for the analysis of data. Differences were considered significant at $p<0.001$.

The Primers used in the amplification of TGF-β, IL-6 and TNF-α and mRNA

Gene mRNA	Primer sequence (5'→3')
TGF-β	FP:AAGAAGTCACCCGCGTGCTA RP:TGTGTGATGTCTTTGGTTTTGTCA
IL-6	FP:TGAAACCCTAGTTCATATCT TCAAACAA RP:GCCACTCCTTCTGTGACTCTAACTT
TNF-α	FP:AAGTTTTCCGCGGGTTGAAT RP:TGTACCGGCTGCCTTTATAGC
GAPDH	FP:ATGGCCTCCAAGGAGTAAGAAAC RP:ACTGAGGGCCTCTCTCTTGCT

3. Results

3.1 Effect of Alcohol on Blood Glucose, Body Weight and Insulin Levels in Diabetic Animals

In alcoholic rats, diabetic rats, we reported increased levels of blood glucose. But diabetic rats which received alcohol showed elevated levels of blood glucose than diabetic control rats (Table 1). The body weights are reduced in the alcohol, diabetic groups. However, the body weights are further decreased in alcohol treated diabetic rats (Table 1).

The insulin levels are decreased in alcoholic rats, diabetic rats. However, diabetic rats which received alcohol showed depleted levels than diabetic control rats (Table 1).

3.2 Impact of Alcohol on Antioxidant Enzymes in Diabetic Rats

A significant depletion in GPx, GR, GSH levels and elevated activity of GST and MDA, in the alcoholic, diabetic rats are observed. But GPx, GR, and GSH are further downregulated, whereas GST activity and MDA levels are upregulated in alcohol administered diabetic animals (Figure 1).

3.3 Effect of Alcohol on mRNA Expression of Proinflammatory Cytokines Genes

IL-6, TNF-α, and TGF-β levels are up-regulated in alcoholic and diabetic rats. Whereas in alcohol administered diabetic rats, expression of TNF-α, IL-6 and TGF-β is more elevated than in alcohol and diabetic rats. This is very strong evidence that alcohol alters tissue damage in diabetic conditions (Figure 2).

3.4 Effect of Alcohol on Lipid Metabolic Profiles in Diabetic Rats

LDL, HDL, total cholesterol and triglyceride levels are significantly higher in the alcohol and diabetic group compared to the normal group ($p<0.05$). Whereas with alcohol administration in diabetic rats, we observed elevated levels of LDL, total cholesterol and triglyceride. ($p<0.05$). The HDL levels are significantly decreased in alcoholic diabetes control groups compared to the normal control group ($p<0.05$). However, with alcohol supplementation in diabetic rats, HDL levels are significantly depleted than the alcohol and diabetic groups ($p<0.05$) (Table 2).

3.5 Impact of Alcohol on Liver Marker Enzymes in Diabetic Rats

The serum ALP, ALT, and AST activities are represented in Table 1. The ALT, ALP and AST are upregulated significantly in alcohol, diabetic control groups compared to the normal group ($p<0.05$). Whereas ALT, AST and ALP levels were significantly elevated in the alcohol treated diabetic group than the alcohol and diabetic groups ($p<0.05$) (Table 3).

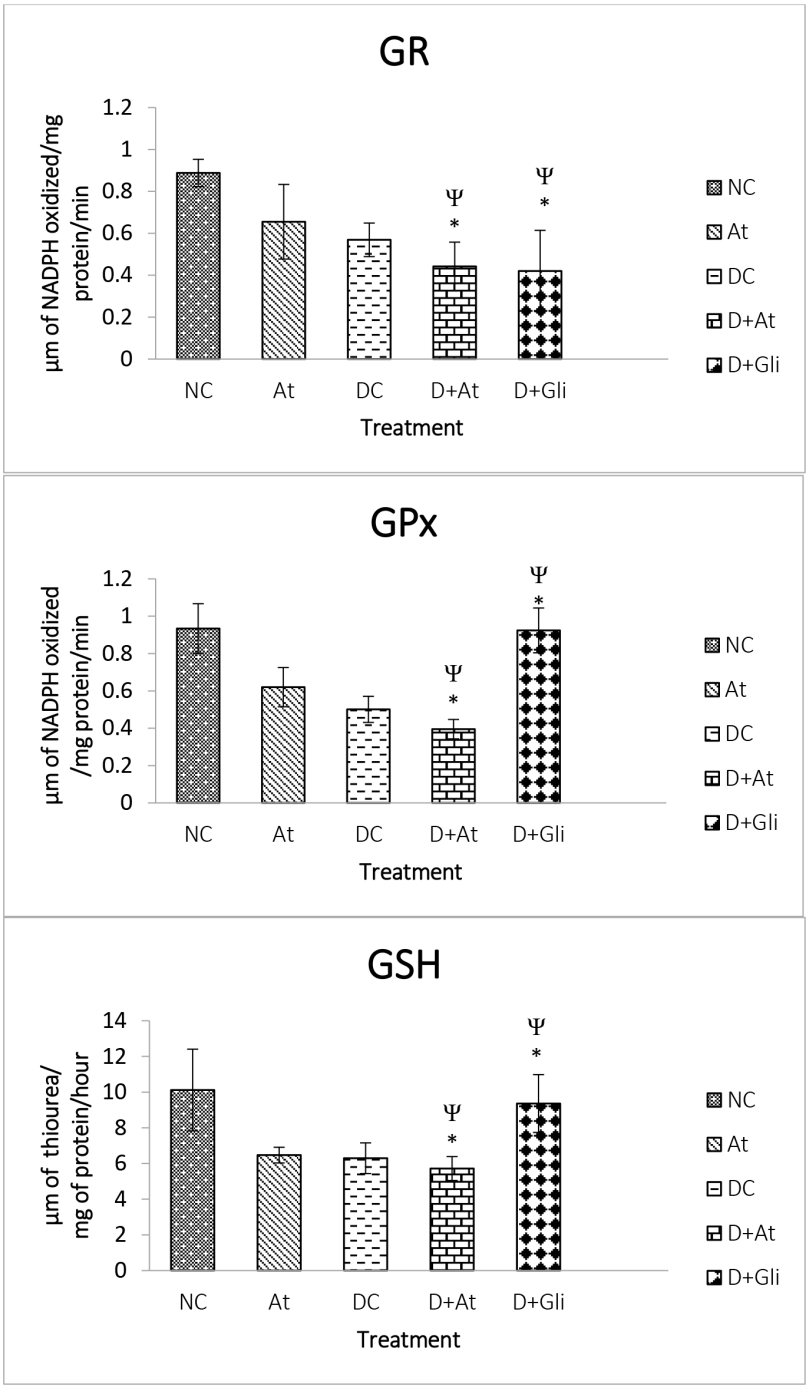


Figure 1. Effects of alcohol on GR, GPx, GST activities & GSH, MDA levels in the kidney tissues of normal and diabetic rats. Data are expressed as means ± SD (n = 6). * The values are significant compared to the following: control (*$p < 0.001$), diabetic (*$p < 0.01$) (Dunnett's multiple comparison test).

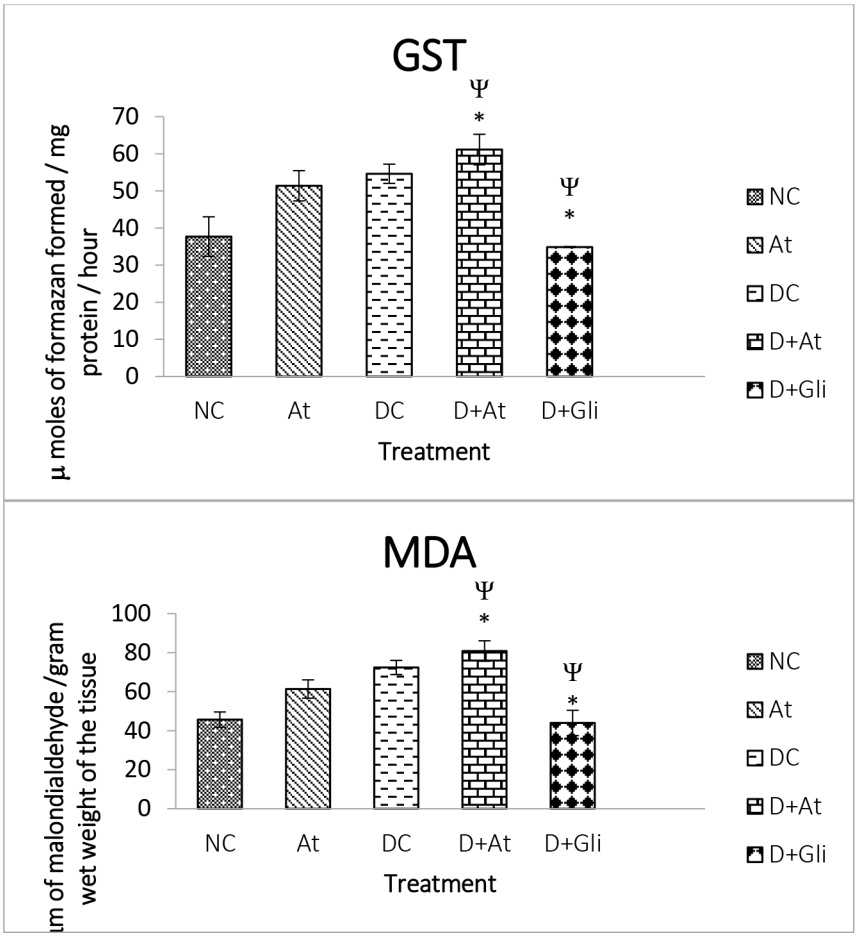


Figure 1. Continued...

Table 1. Effect of alcohol on Blood glucose levels (mg/dl) and Body weights and Insulin in diabetic rats

Groups	Blood Glucose (mg/DL)		Body Weights (Grams)		Insulin (Plasma)
	0 Day	30 Day	0 Day	30 Day	(µIU/ml)
NC	82±1.28	90±2.8	204±8.2	226±12.8	192.4±32.6
At	84±1.4	104±5.28	202±10.4	232±14.6*	86.2±10.4*
DC	224±10.6*	268±12.4*	218±10.8	142 ±8.2	66.4±4.8*
DC+At	248±16.84*	406±22.8*	220±12.6	108±12.6	42.6±12.8*
DC+Gli	256±18.28*	126±8.2*	204±14.6	194±16.4	190±8.6

All the values are ±SD of six individual observations
Values are significant compared to diabetic control ($p<0.001$)

Table 2. Effect of alcohol on Lipid metabolic profiles in diabetic rats

Groups	HDL (µg/ml)	LDL (µg/ml)	VLDL (µg/ml)	Total Cholesterol (µg/ml)	Triglyceride (mg/dl)
NC	14.82±8.2	38.46±4.2	19.4±2.6	104.2±8.2	92.46±4.2
At	12.46±21.4	84.±5.6*	25.8±4.2	186.2±12.4*	182.46±10.8*
DC	10.2±36.4*	126.84±10.2*	42.6±6.8*	234.6±10.2*	438.16±38.2*
DC+At	6.8.±24.2*	184.2±34.6*	64.8±7.4*	286.4±14.6*	438.16±38.2*
DC+Gli	12.4±6.2	42.6±2.8	22.4±1.8	118.4±6.2	118.26±8.4*

All the values are means ± SD of six individual observations.
* Significant at $p < 0.001$ with respect to normal control.

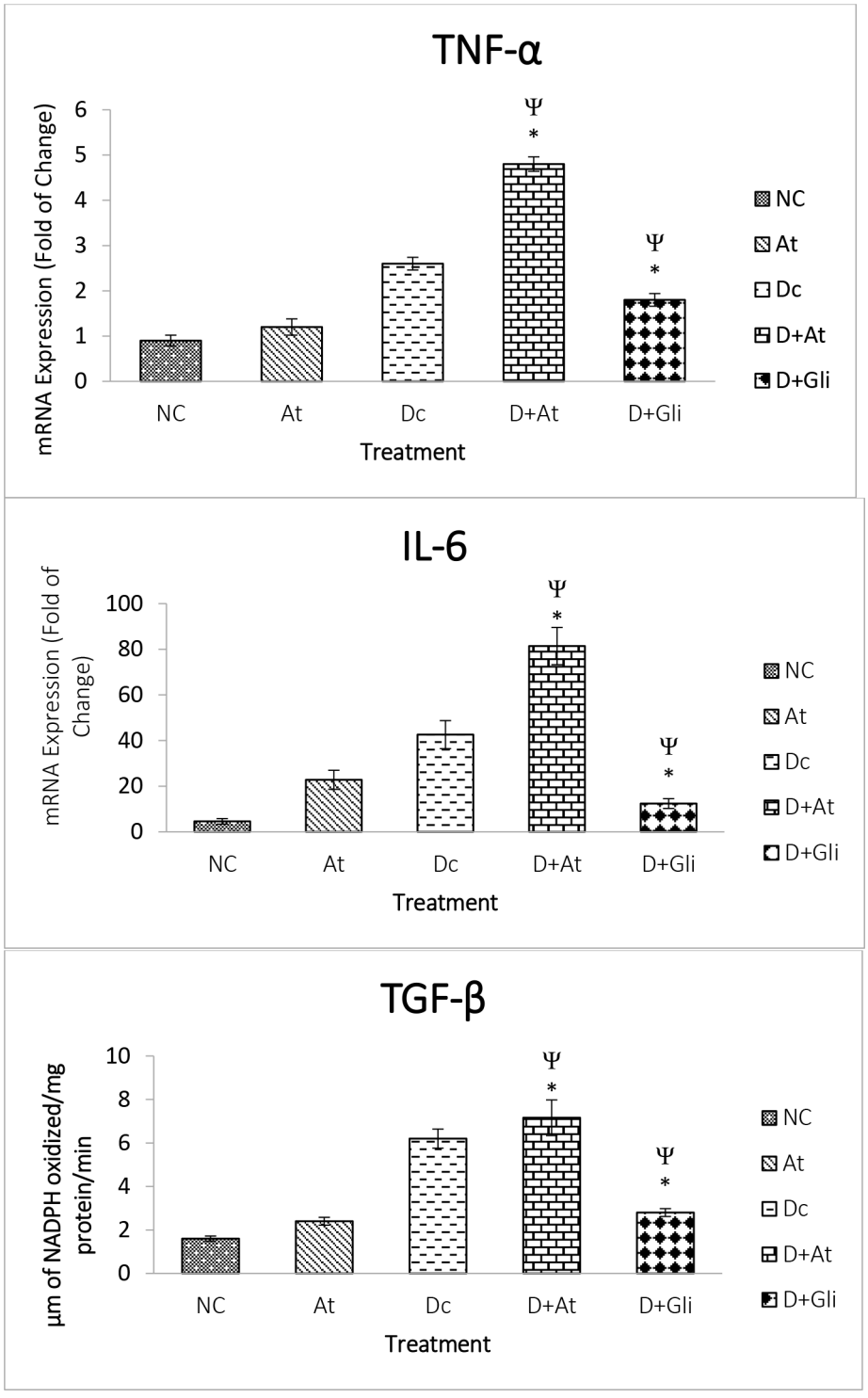


Figure 2. Effect of alcohol on gene markers in diabetic rats.

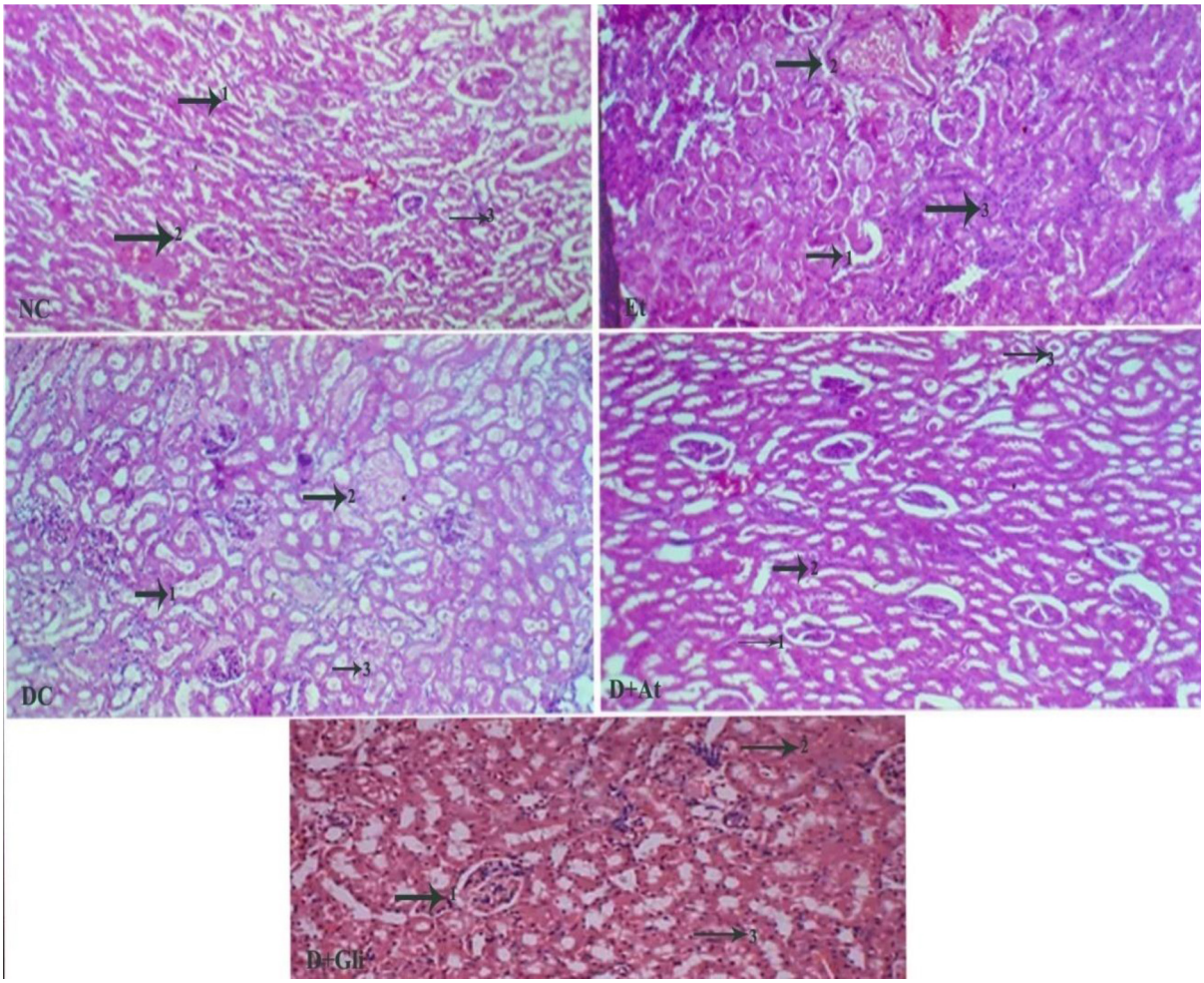


Figure 3. Effect of alcohol on Kidney tissue during diabetic condition.

Photo micrograph of Normal control (NC) kidney showing normal architecture with normal bowmen's capsule. Photo micrograph of Alcohol treated (At) kidney showing **1.** Necrosis of the Renal cells **2.** Congestion of blood vessels, **3.** Degeneration of renal parenchyma. Photo micro graph of Diabetic Control (DC) kidney showing **1.** Tubular degeneration, **2.** Focal necrosis of tubules, **3.** Degeneration of tubules. D. Photo micro graph of Diabetic +Alcohol treated (D+At) rat kidney showing **1.** Hyaline casts, **2.** Dilatation of Bowmen's capsule. Diabetic + Glibenclamide supplementation (D+Glb) showing **1.** Regenerated of bowmens capsule, **2.** Regenerated of renal tubules, **3.** Regenerated renal cells (10X).

Table 3. Effect of alcohol on ALT, AST and ALP in diabetic rats

Groups	ALT	ALT	ALP
	(IU/L)	(IU/L)	(IU/L)
NC	40.42±8.2	38.46±4.2	134.2±10.4
At	258.6±21.46	224.±5.6*	286.4±32.6
DC	288.2±36.4	186.84±30.2*	312.7±40.2
DC+At	326.8.±24.2	314.±34.6	348.6±42.64
DC+Gli	61±6.4*	49±2.8*	146.8±8.2

All the values are means ± SD of six individual observations.
* Significant at $p < 0.001$ with respect to normal control.

3.6 Impact of Alcohol on Histopathology of Kidney Tissues in Diabetic Rats

In alcoholic rats, diabetic rats, we observed that renal tissue is damaged. Whereas in alcohol treated diabetic rats, there was more degeneration of renal cells, glomeruli. This shows that alcohol intoxication leads to renal organ damage (Figure 3).

4. Discussion

Alcohol abuse and alcohol intoxication leads to organ damage in human, and it causes liver cirrhosis, renal damage and neuronal injury.

In the current study, the blood glucose levels are elevated and insulin levels are depleted in diabetic animals due to oxidative stress. However, in alcohol ingested rats and D+At groups, the levels of blood glucose are increased, and insulin levels are decreased. Upregulation of blood glucose and downregulation of insulin levels are observed in alcohol administered diabetic subjects. Lee *et al.*[12] reported elevated levels of blood glucose in diabetic conditions which received alcohol. Funayama *et al.*[13] also reported that elevated blood glucose levels and depleted insulin levels were observed in D+At subjects. Hence, in our study, we reported the upregulation of blood glucose and downregulation of insulin in diabetic rats which received alcohol. This condition may be due to oxidative stress (Table 1).

The body weights are reduced in the alcohol, diabetic groups. However, the body weights are further decreased in alcohol treated diabetic rats (Table 1). The insulin levels are decreased in alcoholic rats, diabetic rats. However, diabetic rats which received alcohol showed depleted levels than diabetic control rats (Table 1).

GPx and GR play a major role in the cellular defence against oxidative stress. In our study, we reported depletion of GPx and GR activity in alcoholic, diabetic rats. GPx is responsible for metabolising lipid peroxides. GR helps to restore the reduced GSH from oxidised GSSG by the activation of GPx. Decreased GPx activity in alcoholic and diabetic rats due to the action of GPx on lipid hydroperoxidases[14]. Depletion of GR activity in alcoholic, diabetic rats, due to the production of toxic free radicals in these groups. Hence, to overcome this free radical stress condition, GR acts on H_2O_2 hence, GR activity was reduced in diabetic condition[15]. Whereas in the D+At group, GPx and GR activities are decreased more than alcohol, diabetic groups. This may be due to the production of more toxic H_2O_2 and lipid peroxides. So GPx and GR act against these free radicals. Hence, GPx and GR activities were depleted more in alcohol administered diabetic rats (Figure 1).

In this study, GSH level was decreased and GST activity was increased in alcoholic and diabetic rats. The detoxification pathway of ROS involves the oxidation of GSH to GSSH, which results in a decrease in GSH levels. Sekhar *et al.*[16] reported that GST activity increased in alcohol treated rats. Increased GST activity suggests its activation during oxidative stress condition[17]. In this study, we reported depleted GSH and elevated GST in alcoholic and diabetic rats. The ROS generated during the oxidative stress condition leads to downregulation of GSH and upregulation of GST. In the alcohol administered diabetic rats, we reported a very low level of GSH and higher levels of GST. This could be due to the action of GSH and GST on the toxic substances which are produced in diabetic rats which received alcohol (Figure 1).

In this study, MDA levels are increased significantly in alcoholic and diabetic rats. During a diabetic condition, glucose metabolism is altered, so this leads to the production of reactive oxygen species, which further aggravates lipid peroxidation and tissue membrane damage[18]. Lipid peroxidation has been elevated in alcohol intoxicated and diabetic rats due to the production of toxic free radicals[19]. During a diabetic condition, lipid peroxides are produced more and also increased oxidative stress[20]. Hence, in the current study, malondialdehyde (MDA) levels are increased in the kidneys of alcohol treated and diabetic rats, indicating excessive formation of free radicals resulting in renal tissue damage. In alcohol administered diabetic rats, MDA levels are increased than alcohol, diabetic rats. The enhanced free radical generation and lipid production products increase the MDA in alcohol administered diabetic rats. Hence, in D+At rats, we observed higher levels of MDA (Figure 1).

TGF-β, IL-6 and TNF-α levels are up-regulated in alcoholic and diabetic rats. Whereas in alcohol administered diabetic rats, expression of TNF-α, TGF-β and IL-6 are more elevated than in alcohol and diabetic rats. This is very strong evidence that alcohol alters tissue damage in diabetic conditions. TNF-α is a multifunctional cytokine, and it is involved in multiple metabolic disturbances. Interleukin-6 (IL-6) regulates immune responses. Transforming growth factor beta (TGF-β) is a multifunctional cytokine. In our study, the mRNA expression of TNF–α, IL-6 and TGF-β levels are significantly elevated, due to oxidative stress induced by alcohol and diabetes. The increase in TNF–α, IL-6 and TGF-β gene markers in alcoholic rats and diabetic rats is due to the activation of the p38 MAPK and NF-κB signalling pathways during oxidative stress conditions. Whereas, in alcohol administered diabetic rats, these gene expression markers are further upregulated in alcohol treated diabetic rats. These results might be due to the indecisive effects of IL-6, TNF–α and TGF-β, which may act as both pro- and anti-inflammatory cytokines. Inflammatory disorders and alterations in glucose metabolism lead to upregulation of gene markers in alcohol treated diabetic rats[21,22] (Figure 2).

In this study, we observed a significant increase in VLDL, LDL, total cholesterol and triglyceride levels in alcohol diabetic rats than normal control rats. But HDL levels are decreased in alcohol, diabetic groups. In D+At subjects and diabetic conditions, hypertriglyceridemia, fatty liver development and increased production of lipoproteins are observed. Whereas with alcohol supplementation in diabetic rats, we witnessed further elevation in LDL, total cholesterol and triglyceride. The HDL levels are significantly decreased in alcoholic, diabetic control groups compared to the normal control group ($p<0.05$). However, with alcohol supplementation in diabetic rats, HDL levels are significantly depleted than the alcohol and diabetic groups. This condition may be due to the alterations in lipid metabolic profiles. Hence, all the lipid metabolic profiles are altered in alcohol administered diabetic rats (Table 2).

The serum ALP, ALT, and AST activities are represented in Table 1. The ALT, ALP and AST are upregulated significantly in alcohol, diabetic control groups compared to the normal group ($p<0.05$). Whereas ALT, AST and ALP levels were significantly elevated in the alcohol treated diabetic group than the alcohol and diabetic groups ($p<0.05$). The liver is a vital metabolic organ in animals, which plays an important role in glucose metabolism. In alcoholic rats, diabetic rats, glucose metabolism and lipid metabolism are altered due to oxidative stress, so ALT, ALP and AST levels are increased. Our results demonstrated that the ALT, ALP and AST levels are elevated in alcohol alcohol-supplemented diabetic group (Table 2). In a diabetic condition, alcohol intoxication may cause hepatic injuries, which may be associated with an elevation of serum ALT, AST and ALP. Our histopathological studies also proves that alcohol treatment during diabetes causes hepatic tissue damage. Our results indicated that AST, ALT, and ALP activities are considered reliable indices of hepatotoxicity (Table 3).

In the current study, renal tubules are degenerated, and congestion of blood vessels is reported in the kidneys of alcoholic rats. In alcoholic rats and diabetic rats, we observed blood congestion, dilation of cysts and degeneration of glomeruli. Whereas in alcohol treated diabetic rats, we reported hyaline casts, dilatation of the bowmen's capsule, cystic dilatation of tubules and focal necrosis of tubules. Our histopathological studies also prove that during alcohol administration in diabetic subjects, oxidative stress is more, and also the production of free radicals. Hence, renal tissue was more damaged. Thus, histopathological studies support the notion that consumption of alcohol at 2 g/kg produced significant depletion of antioxidant enzymes, also altered lipid profile and gene markers by causing renal and hepatic tissue damage in alcohol treated diabetic rats (Figure 3).

5. Conclusions

There are very few studies on the impact of alcohol on diabetic rats, and also with reference to antioxidant enzymes, lipid profiles and gene markers. Hence, the current study was designed to determine the impact of alcohol during diabetic conditions. In our study, we observed depleted activities of all antioxidant enzymes, altered lipid profiles and gene markers in alcohol administered diabetic rats. We conclude that alcohol administration in diabetic conditions appears as an oxidative stress aggravating factor. So, consumption of alcohol in a diabetic condition is unsafe.

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