RODENT SPECIES EFFECT OF PIOGLIAZONE ON LIVER FUNCTIONS IN NONDIABETIC AND DIABETIC ANIMALS

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Summary

In the Present study Pioglitazone was investigated on liver function in rodent species diabetic adult male Sprague-Dawley rats or Swiss mice. Pioglitazone (10 mg/kg/day, p.o) was administered for 28 days in rats injected with single dose of Streptozotocin (65 mg/kg, i.p, STZ) and Nicotinamide (110 mg/kg, i.p, NIC). STZ–NIC induced animals showed a significant ($p<0.001$) increased in the level of serum glucose, glycosylated heamoglobin (HbA1c), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma glutamyl transpeptidase (γGTP) and lipid peroxidation or malondialdehyde (MDA) in liver tissue but there was no significant changes in total bilirubin (TB) levels in rats and mice. Whereas, the endogenous antioxidants such as reduced glutathione (GSH), catalase (CAT) and superoxide dismutase (SOD) were significantly decreased as compared to control rats and mice. Treatment with Pioglitazone (10 mg/kg/day, p.o) showed a significant alteration in all the serum markers and biomarkers of oxidative stress. PIO 10mg/kg was found to elevate the condition i.e it further increases the liver toxicity produced by STZ–NIC treatment in rats but less significant changes in mice. Histopathological changes are also in correlation with biochemical changes. This study indicates that remarkable species differences have been observed in rodent models. Diabetic rats exhibit marked sensitivity versus diabetic mice exhibiting equally marked protection from Pioglitazone induced hepatotoxicity.

Keywords: Pioglitazone, Antioxidant, Hepatotoxicity, Rat, Mice

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Introduction

Recent epidemiological studies suggested that patients with diabetes are twice as likely to suffer hepatic failure compared to patients who do not have diabetes. Increased incidences of hepatotoxicity have been observed in patients with diabetes receiving drug therapies. Liver disease complication is one of the most common causes of morbidity and mortality in diabetic patients. Neither the mechanisms nor the predisposing factors underlying hepatotoxicity in patients with diabetes are clearly understood. Recently, a large prospective population indicated that diabetes is associated with increased risk of hepatocellular carcinoma and chronic liver diseases. Hepatotoxicity did not receive as much attention as other prevalent complications (i.e., cardiopathy, retinopathy and nephropathy) until hepatotoxicity of antidiabetic drugs emerged as a common clinical complication (1).

Liver is an important insulin dependent tissue which plays a pivotal role in glucose and lipid metabolism and is severely affected during diabetes (2). In diabetes the levels of hepatic enzymes increase. The level of ALT increase due to hepatocellular damage and is always accompanied by an increase in ALT and AST activity. Moreover the ALT and AST activity has been used as an indicator of liver function (3).

Hepatotoxicity of several structurally and mechanistically diverse chemicals is significantly altered in streptozotocin- or alloxan-induced type 1 diabetic rats (4-7). The majority of the studies have pointed out that hepatotoxicant-induced liver injury is potentiated in type 1 diabetic rats. Similarly, hepatotoxicity of some other compound has been shown to potentiate in type 2 diabetic rats (8). Interestingly, chemical-induced liver injury is differently modified by diabetes in murine models. In contrast to the enhanced hepatotoxicity in diabetic rats, diabetic mice (type 1 as well as type2) are protected from normally lethal hepatotoxicant challenge (9-12). The majority of the studies have pointed out that hepatotoxicant-induced liver injury is potentiated in type 1 diabetic rats. In contrast to the enhanced hepatotoxicity in diabetic rats, diabetic mice (type 1 as well as type 2) are protected from normally lethal hepatotoxicant challenge. Diabetic rats exhibit marked sensitivity versus diabetic mice exhibiting equally marked protection from drug-induced hepatotoxicity.

Pioglitazone is a PPARγ agonist that increases both insulin-stimulated glucose uptake in peripheral tissues and insulin sensitivity in hepatic and adipose tissue. PIO showed dose dependant beneficial effects in many of the pathological conditions including reduction in blood glucose lowers blood pressure and restores endothelial function in animals (13). Troglitazone which is withdrawn from the U.S. market in 2000 because of his high incidence of hepatotoxicity and drug-induced liver failure (14).

Literature survey showed that, there was no report regarding the effect of PIO on Comparison of hepatotoxicity in diabetic and non-diabetic animal and observes species different in diabetic rodent models. In light of above survey, the present study was designed to evaluate the effect of PIO on liver functions and biomarkers of oxidative stress in STZ-NIC induced diabetic in rats.

Materials and Methods

Drugs and Chemicals

Pioglitazone hydrochloride was obtained as a gift sample from Alembic Pharmaceuticals Pvt. Ltd., Baroda, India. STZ and NIC were obtained form SIGMA, St. Louis, MO, USA. All other chemicals and reagents used in the study were of analytical grade.

Experimental Animals

All experiments and protocols described in present study were approved by the Institutional Animal Ethics Committee (IAEC) of The M.S. University, Baroda. Sprague–Dawley rats (210±15gm) and Swiss mice (17±21gm) were housed in-group of 3 animals per cage and maintained under standardized laboratory conditions (12- h light/dark cycle, 24°C) and provided free access to palleted CHAKKAN diet (Nav Maharashtra Oil Mills Pvt., Pune) and purified drinking water *ad libitium*.

Experimental Induction of type 2 Diabetes in Rats and mice

Type 2 Diabetes was induced in rats or mice by a single intraperitoneal (i.p) injection of Streptozotocin (65 mg/kg) in overnight fasting rats or mice followed by the i.p administration of Nicotinamide (110 mg/kg) after 15 minutes. STZ was dissolved in citrate buffer (pH 4.5) and NIC was dissolved in normal saline. After 7 days following STZ and NIC administration, blood was collected from retro-orbital puncture and serum samples were analyzed for blood glucose (15). Animals showing fasting blood glucose higher than 250 mg/dL were considered as diabetic and used for the further study.

Experimental Protocol

Animals were divided in to following groups, each group containing six animals and the treatment period for whole study was 4 weeks.

Group 1: Nondiabetic control, received 0.5% CMC as vehicle in rats (1ml/kg/day, p.o, **ND**).

Group 2: Nondiabetic treated with PIO (10 mg/kg/day, p.o, **ND-PIO**) in rats.

Group 3: Diabetic rats control, single injection of STZ (65 mg/kg, i.p) and NIC (110 mg/kg, i.p, **DB**).

Group 4: Diabetic rats treated with PIO (10 mg/kg/day, p.o, **DB-PIO**).

Group 5: Nondiabetic control, received 0.5% CMC as vehicle in mice (1ml/kg/day, p.o, **ND**).

Group 6: Nondiabetic mice treated with PIO (10 mg/kg/day, p.o, **ND-PIO**) in mice.

Group 7: Diabetic control, single injection of STZ (65 mg/kg, i.p) and NIC (110 mg/kg, i.p, **DB-CON**) in mice.

Group 8: Diabetic mice treated with PIO (10 mg/kg/day, p.o, **DB-PIO**).

Biochemical Estimations

Characterization of Type 2 Diabetes Model

Type 2 diabetes was confirmed by measuring non fasting serum glucose (SPAN diagnostics Pvt., India) and the degree of uncontrolled diabetic (DB) state was confirmed by measuring HbA1c (Ion Exchange Resin method). After 4 weeks, diabetes was confirmed by measuring glucose and HbA1c as mentioned above.

Estimation of Serum Markers

On 4th weeks blood samples were collected from retro-orbital plexus under light ether anesthesia and centrifuged at 2500 rpm for 20 minutes to separate serum. Glucose, HbA1c, AST, ALT, ALP, γGTP and TB were estimated from serum sample using standard Diagnostic Kit. In vitro quantitative determination of the activity of AST, ALT and TB (SPAN Diagnostics Pvt., India), ALP and γGTP (Crest Biosystems, India) were done using enzymatic kit in serum.

Estimation of biomarkers of Oxidative stress

The excised liver was then weighed and homogenized in chilled Tris buffer (10 mM, pH 7.4) at a concentration of 10% (w/v). The homogenates were centrifuged at $10,000 \times g$ at $0^{o}C$ for 20 min using Remi C-24 high speed cooling centrifuge. The clear supernatant was used for the assay of following antioxidant parameters. The levels of Lipid peroxidation (LPO) formation and the activities of endogenous antioxidant enzymes such as catalase (CAT), reduced glutathione (GSH) and superoxide dismutase (SOD) were estimated by the method of Slater and Sawyer (16), Hugo Aebi as given by Hugo (17), Moron et al (18) and Mishra and Fridovich (19).

Histopathology of liver

For light microscopic evaluation, liver tissues of each group were fixed in 10% phosphate buffered formalin. Paraffin-embedded specimens were cut into 6 mm-thick sections and stained with hematoxylin and eosin (H&E). The liver tissues were examined under a light microscope (Olympus Bioxl) for the presence of tubular changes and interstitial inflammatory cell infiltration by an observer blinded to the animal treatment group.

Statistical Analysis

All of the data are expressed as mean ± SEM. Statistical significance between more than two groups was tested using one-way ANOVA followed by the Bonferroni multiple comparisons test or unpaired two-tailed student's t-test as appropriate using a computer-based fitting program (Prism, Graphpad 5). Differences were considered to be statistically significant when $p < 0.05$.

Results

Characterization of Type 2 Diabetes.

As shown in Table 1 showed a significant ($P<0.01$, respective control) decrease in body weight levels in STZ-NIC treated mice (DB group) as compared to ND group. Treatment with PIO (10 mg/kg, p.o) showed a significant ($P<0.01$) increase in body weight as compared to control nondiabetic (ND) and DB animals. Table 1 showed a significant ($P<0.001$) increase in serum glucose and HbA1c levels in STZ-NIC treated rats or mice (DB group) as compared to ND animals. The levels of glucose and HbA1c was significant ($P<0.001$) decreased after treatment with PIO (10 mg/kg, p.o) as compared to DB rats or mice.

Table 1. Effect of Pioglitazone (10 mg/kg/day, p.o) on changes in body weight, serum glucose and HbA1c level in nondiabetic and diabetic rats and mice.

Group		Body weight (gm)	Glucose (mg/dl)	HbA1c (%)
Rats	**ND-CON**	248.33±5.95	101.0±15.13	5.45±0.37
	ND-PIO	300.00±12.87 $^{\$\$\$}$	60.75±16.93	4.023±0.25
	D-CON	224.83±8.52	406.8±15.93$^{\$\$\$}$	11.18±0.52$^{\$\$\$}$
	D-PIO	265.50±8.11***	117.8±15.11***	5.77±0.2576***
Mice	**ND-CON**	32.83±1.42	99.71±10.03	5.06±0.31
	ND-PIO	39.17±1.44$^{\$}$	65.69±8.88	4.23±0.57
	D-CON	25.00±1.06$^{\$\$}$	288.4±9.76$^{\$\$\$}$	9.55±0.42$^{\$\$\$}$
	D-PIO	33.00±0.96**	165.1±8.44***	6.84±0.51***

Values are expressed as mean ± SEM for six animals in the group. $^{\$}P<0.05$, $^{\$\$}P<0.01$, $^{\$\$\$}P<0.001$, considered statistically significant as compared to ND-CON group. $^{*}P<0.05$, $^{**}P<0.001$; $^{***}P<0.001$ considered statistically significant as compared to D-CON group.

Effect of PIO on serum marker enzymes

Figure 1 showed a significant (P<0.001) increase in serum AST and ALT levels in STZ-NIC treated rats or mice (DB-CON) as compared to ND-CON rats and mice. Treatment with PIO (10 mg/kg, p.o) for 4 weeks, showed further significant (P<0.001) increase in serum AST and ALT level as compared to DB-CON and ND-CON rats group. Whereas Treatment with PIO (10 mg/kg, p.o) for 4 weeks, showed further significant (P<0.01, P<0.05, respective control) increase in serum AST and ALT level as compared to DB-CON and ND-CON mice group.
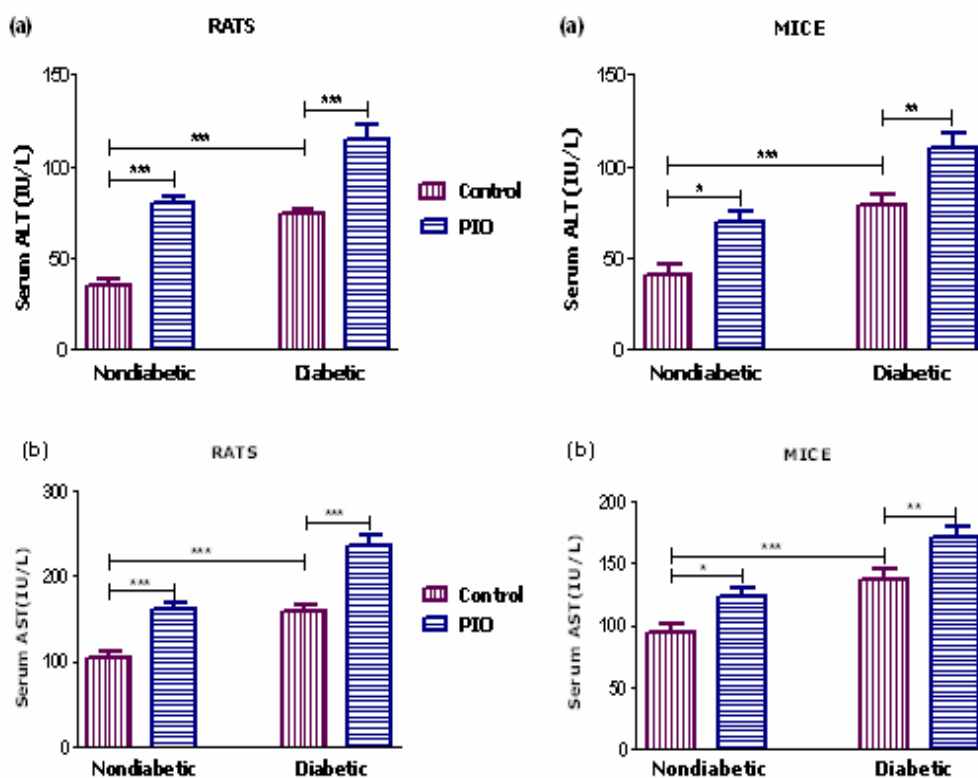

Figure1. Effect of Pioglitazone (10 mg/kg/day, p.o) on serum ALT (a) and AST (b) level in nondiabetic and diabetic rats and mice. Values are expressed as mean ± SEM for six animals in the group. [*] P<0.05, [**]P<0.001; [***]P<0.001 considered statistically significant as compared to Control group.

Administration of STZ-NIC alone significantly (P<0.01) increases ALP and γGTP levels as compared to control rats or mice but there was no significant changes in the levels of TB. As shown in table 2, treatment with PIO (10 mg/kg, p.o) showed a significant (P<0.001) increase in ALP, γGTP and TB as compared to control ND and DB rats. Whereas, treatment with PIO

(10 mg/kg, p.o) for 4 weeks showed significant (P<0.05) increase changes in the serum levels ALP, γGTP and TB level as compared to control ND and DB mice.
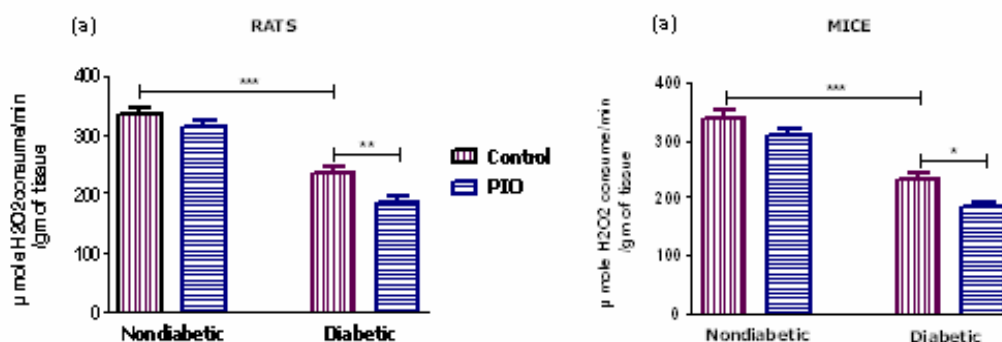
Table 2. Effect of Pioglitazone (10 mg/kg/day, p.o) on changes in ALP, γGTP and Total bilirubin level in nondiabetic and diabetic rats and mice**.**

Group		ALP (IU/L)	γGTP (IU/L)	TB (IU/L)
Rats	**ND**	138.2±7.43	75.24±4.45	0.719±0.041
	ND-PIO	305.4±6.29[$$$]	143.2±4.07[$$$]	1.288±0.051[$$$]
	D-CON	180.8±6.12[$$]	107.7±7.53[$$]	0.891±0.056
	D-PIO	358.5±7.85[***]	168.1±2.973[***]	1.901±0.059[***]
Mice	**ND-CON**	115.3±9.49	48.50±6.23	0.565±0.058
	ND-PIO	152.2±6.76[$]	74.13±5.51[$]	0.968±0.099[$]
	D-CON	159.4±6.41[$$]	81.14±5.66[$$]	0.776±0.056
	D-PIO	195.2±9.91[*]	106.7±5.06[*]	1.155±0.118[*]

Values are expressed as mean ± SEM for six animals in the group. [$]P<0.05, [$$]P<0.01, [$$$]P<0.001 considered statistically significant as compared to ND-CON group; [*] P<0.05, [**] P<0.001, [***]P<0.001 considered statistically significant as compared to D-CON group.

Effect of PIO on Biomarkers of oxidative stress

MDA level was significantly (p<0.001) increased and the levels of GSH, CAT and SOD were significantly (p<0.001) decreased in STZ-NIC treated rats or mice when compared to control group. Treatment with PIO (10 mg/kg) again significantly (p<0.01) increased MDA and decreased the levels of GSH (p<0.001), CAT (p<0.01) and SOD (p<0.01) as compared to control DB rats (Fig. 2). Whereas, treatment with PIO (10 mg/kg) for 4 weeks, showed significantly (p<0.01) increased MDA and decreased the levels of GSH (p<0.05), CAT (p<0.05) and SOD (p<0.05) changes in the tissue levels as compared to control DB mice.
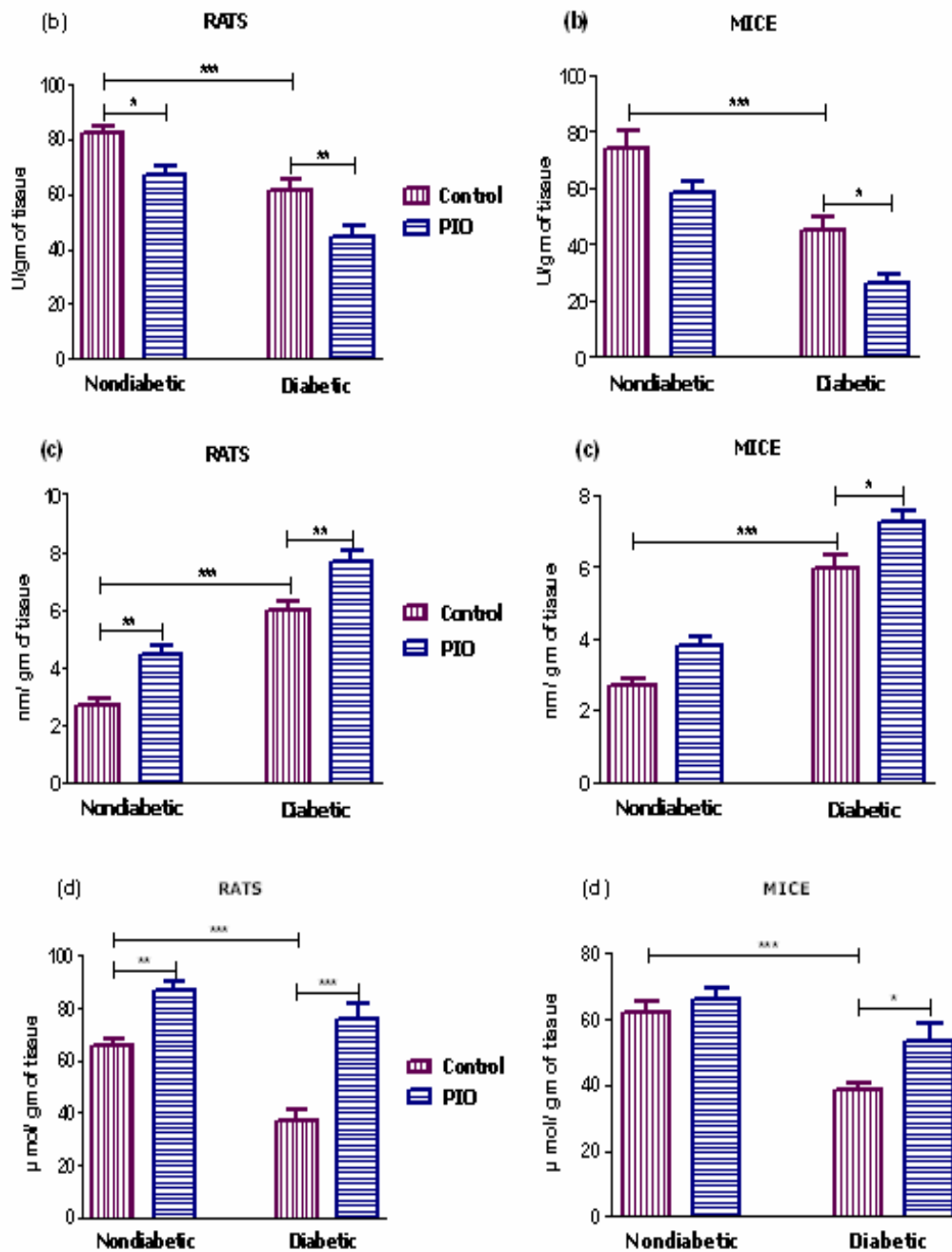
Figure 2. Effect of Metformin (50 mg/kg/day, p.o) and Pioglitazone (10 mg/kg/day, p.o) on CAT (a), SOD (b), MDA (c) and GSH (d) level in nondiabetic and diabetic rats and mice. (a) Catalase (CAT), b) Superoxide dismutase (SOD), c) Lipid Peroxidase (LPO) and d) reduced glutathione (GSH) levels in rats subjected to after 4 weeks, Values are expressed as mean ± SEM for six animals in the group. [*]P<0.05, [**]P<0.001, [***]P<0.001 considered statistically significant as compared to Control group.

Effect of PIO on Histopathological changes

Liver sections were examined by light microscopy (Fig. 3) for necrosis and inflammatory cell infiltration. Livers from untreated ND and DB rats or mice were indistinguishable indicating that diabetes alone has no discremible effects in the liver. PIO (10 mg/kg) administration caused significant effects in the livers of both ND and DB rats. In the liver of ND group, rats at 4 weeks after PIO (10 mg/kg) administration showed swollen hepatocytes with inflammatory cell infiltration in centrilobular area were minimally evident. In contrast, in the liver of DB rats at 4 weeks after PIO (10 mg/kg) administration showed vacuolar degeneration and centrilobular necrosis of hepatocytes and mild inflammation. Liver section from DB and ND mice after treatment with Pioglitazone showed similar change and characterized by presence of swollen hepatocytes with inflammatory cell infiltration in centrilobular area (Fig. 3).
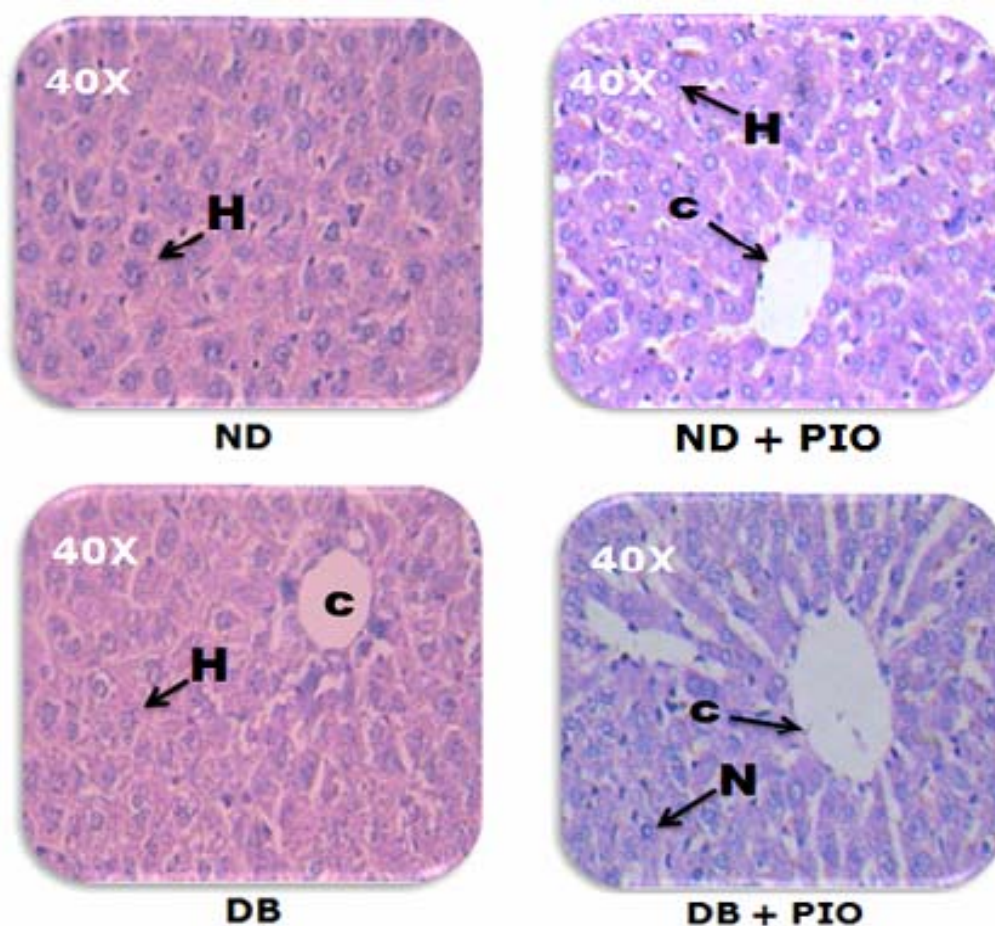
A)   RATS
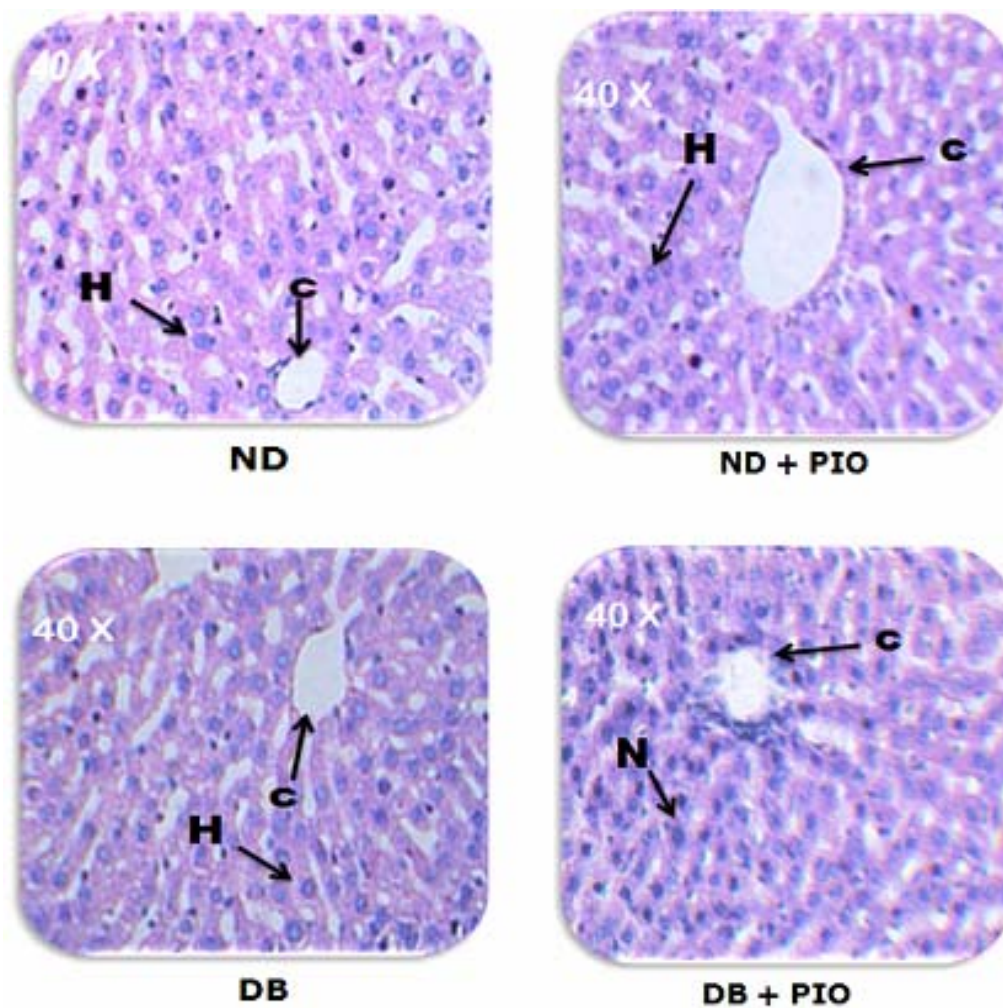
B)   **MICE**

Figure 3. Effect of Pioglitazone (10 mg/kg/day, p.o) on Liver tissues in nondiabetic (ND) and diabetic (ND) rats (A) and mice (B). **C** = Central vein, **N** = Hepatocytes Necrosis, **H** = Normal Hepatocytes

Discussion

The present study was undertaken with the objective of exploring the hepatic function of PIO in STZ-NIC induced diabetic rats or mice. Recent studies have suggested that prevalence of type 2 diabetes is rapidly increasing. Peroxisome proliferator-activated receptors are nuclear transcription factors that play a role in insulin sensitivity (20).

In STZ-NIC induced diabetes, the characteristic loss of body weight caused by an increase in muscle wasting (21).

In the present study treatment PIO (10 mg/kg) showed significant increase in body weight which may be because of formation of oedema in the tissue. In the present study, an increase in the levels of serum glucose and HbA1c in STZ-NIC treated rats or mice confirmed the induction of diabetes mellitus. STZ causes diabetes by the rapid depletion of β-cells and thereby brings about an eduction in insulin release. HbA1c level has been reported to be increased in patients with diabetes mellitus (21). It was reported that during diabetes mellitus, the excess of glucose present in the blood reacts with hemoglobin to form HbA1c (22).The level of HbA1c is always monitered as a reliable index of glycemic control in diabetes (23). Elevated levels of HbA1c observed in our study reveal that diabetes animals had prior high blood glucose level. Significant decrease was observed in the glucose and HbA1c level in diabetic rats or mice after treatment with PIO (10 mg/kg) when compared with DB-CON rats or mice at the end of experimental period.

In STZ induced animals a change in the serum enzymes is directly related to changes in the metabolic functions of AST, ALT, ALP and γ- GTP (24-26). It has been reported that the increased levels of transaminases under insulin deficiency (27) were responsible for the increased gluconeogenesis and ketogenesis during diabetes. The increased levels of serum AST, ALT ALP have already been reported to be associated to liver dysfunction and leakage of these enzymes to the liver cytosol in to the blood stream in diabetes (28). Enhanced in the activity of AST, ALT, ALP and γ- GTP in PIO treated diabetic rats or mice indicates the alleviating role of the PIO against STZ–NIC induced hepatocellular necrotic changes.

Oxidative stress originating from improper control of the reduction of O_2 is believed to play a role in the tissue and cellular damage caused by a variety of conditions in diabetes (29). The effects of thiazolidinediones on oxidative stress are difficult to predict (30). Previous studies have proved that, thiazolidinedione exposure increase oxidative stress (31). SOD and CAT are considered as primary enzymes since they are involved in the direct elimination of reactive oxygen species (32). SOD is an important defense enzyme, which catalyzes the dismutation of superoxide radicals (33) and CAT is a hemoprotein, which catalyzes the reduction of hydrogen peroxides and protects tissues from highly reactive hydroxyl radicals (34). The reduced activity of SOD and CAT in the liver observed in diabetes may pose deleterious effects as the result of the accumulation of superoxide anion radicals and hydrogen peroxide (35). GSH, the most important biomolecule protecting against chemical induced toxicity, participates in the elimination of reactive intermediates by reduction of hydroperoxide in the presence of glutathione peroxidase (36, 37). In our study, the activity of endogenous antioxidants was significantly changed with PIO (10 mg/kg). Treatment with PIO further decreases the levels of endogenous antioxidants and increases the level of lipid peroxidation.

This study concluded that PIO at 10 mg/kg may show some liver damage in STZ-NIC induced diabetic rats. Whereas, with high doses and chronic treatment it showed further liver damage. Diabetic rats exhibit marked sensitivity versus diabetic mice exhibiting equally marked protection from Pioglitazone induced hepatotoxicity. While the rat diabetic models appear to be suitable, the diabetic mouse models might not be suitable in preclinical testing for potential hepatotoxic effects of Pioglitazone because regardless of type 2 diabetes, mice are resistant to chronic Pioglitazone induced toxicities. Diabetic mice appear to be less susceptible to show hepatotoxicity at same doses compared to rats, suggest that for study as hepatotoxicity mice may not be a preferred species to study long term toxicity specifically related to liver function in diabetic condition.

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