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The effect of pioglitazone on the liver of streptozotocin-induced diabetic albino wistar rats

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Abstract. – *Background:* **The present study was conducted to assess the effect of Pioglitazone, an oral antidiabetic drug with selective PPAR-gamma agonist effect; in a dose of 4 mg/kg B.W. once a day orally for eight weeks on the liver of streptozotocin-induced diabetic rats.**

Material and Methods: **Sixty male adult albino Wistar rats were equally randomly into six groups (n=10). Group I: *normal control group* was received no medication. Group II: *distilled water control group*, they are non diabetic group and received distilled water once a day orally by gastric tube for 8 weeks. Group III: *citrate buffer control group*, they are non diabetic received a single intraperitoneal injection of an equivalent amount of vehicle (citrate buffer, pH 4.5) 1 ml/kg at the time of induction. Group IV: *Pioglitazone control group*, they are non diabetic received pioglitazone HCl, single dose of 4 mg/kg b.w. once a day orally by gastric tube for eight weeks. Group V: diabetic control group, they are streptozotocin-induced diabetic rats that received no medication. Group VI: *diabetic treated group*, they are streptozotocin-induced diabetic rats treated by pioglitazone for eight weeks.**

Results: **At the end of the experiment microscopic examination of the liver sections in the diabetic control group, showed mild to moderate portal inflammatory infiltrate, mostly lymphocytic as well as intralobular cell necrosis and apoptosis as well as bile stasis. These results were associated serologically with elevation of all liver parameters. Pioglitazone administration in the normal rats for eight weeks didn't show any significant difference neither serologically nor histopathologically compared with normal control group. Moreover, pioglitazone administration caused statistically significant reduction in the mean levels of liver tests, as well as fasting blood glucose of the STZ-induced diabetic rats.**

Conclusion: **There is no evidence that pioglitazone administration has a harmful effect on the liver. On the other hand, it has a potential beneficial effects on the liver during treatment of STZ-induced diabetic rats, suggesting that liver toxicity isn't a class effect of the thiazolidinediones but rather a unique effect of troglitazone.**

Key Words:

Experimental diabetes, Thiazolidinediones (TZDs), Pioglitazone and Hepatotoxicity.

Introduction

Type 2 diabetes mellitus (T2DM) increased 1.5-fold annually between 1975 and 1990[1]. According to a new study led by the World Health Organization predicted that Egypt will be among the top 10 countries with the highest prevalence of diabetes by 2030[2].

Epidemiological evidence of T2DM suggests that without an effective prevention and control programmes, the prevalence will continue to increase globally[3]. Approximately 75-80% of people with diabetes die of cardiovascular diseases. People with T2DM have a two to four times higher risk of coronary heart disease than the rest of the population, and their prognosis is poorer. The risk of cerebrovascular and peripheral vascular disease is also significantly higher[4]. It is also associated with a large number of liver disorders including elevated liver enzymes, fatty liver disease, cirrhosis, hepatocellular carcinoma, and acute liver failure[5].

Insulin resistance (IR) is a primary component in the pathophysiology of T2DM[6]. It is also plays a role in the pathogenesis of *non alcoholic fatty liver disease* (NAFLD) in rats[7]. IR may not only worsen hyperglycemia but also may trigger various metabolic disturbances

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which also increase the incidence of other cardiovascular risk factors[8]. Metabolic syndrome is considered a clustering of cardio-metabolic risk factors including central obesity, insulin resistance, high blood glucose concentrations, elevated blood pressure, and dyslipidemia, all of which increase the risk for cardiovascular disease (CVD) and T2DM[9].

Thiazolidinediones (TZDs) represent a new class of hypoglycemic agents for the treatment of T2DM that act through improvement of insulin sensitivity[10]. They increase glucose transport and decrease insulin resistance by activation of a nuclear receptor, peroxisome proliferator activated receptor-gamma (PPARγ) in adipose tissue, liver, and skeletal muscles. In parallel to their hypoglycemic action, these drugs were found to exert beneficial effects on other components of the metabolic syndrome[11]. TZD are a unique class of antidiabetic agents that exert multiple effects beyond glycemic control; they may prevent or delay premature atherosclerotic cardiovascular disease, morbidity, and death[12].

At a regulatory level, hepatotoxicity is the main reason for postmarketing regulatory decisions including drug withdrawal[13]. The first TZD "Troglitazone", was approved for clinical use in 1997, but withdrawn from the Japanese and the US markets in March 2000 because of a series of cases of liver failure and death[14].

Two other members of this class, rosiglitazone and pioglitazone do not appear to have the same degree of hepatotoxicity associated with their use, although a severe liver injury has been reported[15-17]. The mechanism of troglitazone-associated hepatotoxicity has not yet been elucidated[18].

Although case reports of liver injury and failure with pioglitazone and rosiglitazone have been published[17,19,20], there are clinical studies that have indicated the link between these drugs and liver failure to be very weak[16,21]. It is currently recommended that serum *alanine aminotransferase* (ALT) levels must be evaluated before the initiation of rosiglitzone and pioglitazone therapy and that therapy not be initiated if there is evidence of active liver disease or if the serum ALT level exceeds 2.5 times *upper limit of normal* (ULN)[5]. Paradoxically, Belcher and Schernthaner[22] stated that during pioglitazone treatment there is a reduction in liver enzyme levels. Although the mechanism of this effect is not clear, the results demonstrate potential beneficial effects on the liver during treatment of patients with T2DM with pioglitazone. Recently Mc Cullough[23] suggests the use of TZDs also for the *non-alcoholic steatohepatitis* (NASH).

Monthly liver function tests, at least during the first year of therapy with pioglitazone were recommended until confirmation of its hepatic safety with adequate postmarketing experience[14,24]. One might question the utility of the current monitoring program, which adds considerably to the cost and complexity of prescribing this already expensive class of medication[19,20].

Until more long-term data on safety are available, current clinical evidence suggests that rosiglitazone and pioglitazone do not share the hepatotoxic profile of troglitazone[25]. So the aim of the present work is to evaluate the effect of Pioglitazone on the liver of streptozotocin-induced diabetic rats.

Material and Methods

Animals

Sixty adult male albino rats weighing 170-200 g each were used in this study. Rats were purchased from the National Center of Research, Cairo, Egypt. Each animal was left alone in a clean polyethylene cage under hygienic conditions and acclimatized for 3 days prior to start of the experiment. They were kept on a standard rodent chow and water *ad libitum*.

Drugs

Pioglitazone HCL

It was provided from Sigma Biosciences, Egypt. Pioglitazone HCl is an odorless white crystalline powder that has a molecular formula of $C_{19}H_{20}N_2O_3S.HCl$ and a molecular weight of 392.90 Dalton. It was given orally once a day by gastric tube in a dose 4 mg/kg b.w./day, suspended in distilled water in a dose volume of 5 ml/kg b.w. (a dose equal to the maximum recommended human oral dose of 45 mg/day) for eight weeks[7,26].

Streptozotocin (STZ)

It was provided from Sigma Biosciences, Egypt as 1-gram pure white yellowish powder. Kept in cold store and refrigerator temperature (2-8°C) away from light.

Induction of Experimental Diabetes

Diabetes was induced by intraperitoneal (i.p) single injection of freshly prepared streptozotocin (STZ) at a dose of 50 mg/kg, dissolved in di-sodium citrate buffer (pH 4.5) in a dose volume of 1 ml/kg b.w. after 16h fasting. 72h after STZ injection diabetes was confirmed in rats showing blood sugar level greater than 250 mg/dl. Animals with blood glucose levels greater than 250 mg/dl were considered for further study[27].

Experimental Protocol

Animals were randomly allocated into 6 groups (ten animal each) as following:

Group 1: *Untreated control group*; non diabetic rats received no medication. So they served as normal control group.

Group 2: *Distilled water control group*; rats were given distilled water 5 ml/kg b.w. orally once a day by gastric tube for eight weeks.

Group 3: *Citrate buffer control group*; rats were injected by an equivalent amount of vehicle (citrate buffer, pH 4.5) 1 ml/kg b.w., intraperitoneally single dose at the time of induction of diabetic group.

Group 4: *Pioglitazone control group*; diabetic rats received pioglitazone HCl, single dose of 4 mg/kg b.w./day orally once a day by gastric tube for eight weeks.

Group 5: *Diabetic control group*; rats were STZ-induced diabetic rats. This group were received no medication, just distilled water in a volume dose of (5 ml/kg b.w.) given orally once a day by gastric tube for eight weeks. So they served as disease control group.

Group 6: *Diabetic treated group*: rats were STZ-induced diabetic rats treated by pioglitazone HCl, in a dose of 4 mg/kg b.w./day orally single dose by gastric tube for eight weeks.

Biochemical Analysis

Three blood sample were obtained throughout the experimental study, the first at zero time, second sample was obtained 72 hrs after induction of diabetes for verify success of induction, the last sample was obtained at the end of experiment. All samples were taken after overnight fasting from the tail vein, serum samples were separated and tested for fasting blood glucose (FBG) while liver injury was assessed by serum ALT, AST, ALP and GGT. All parameters were measured spectrophotometrically (with the Hitachi 912, Roche Diagnostics Co., Mannheim, Germany).

In H&E-stained slides, apoptotic cells were identified using morphologic characteristics including cell shrinkage, nuclear condensation or fragmentation and formation of apoptotic bodies, as well as eosinophilic cytoplasm or lacking cellular structures[29].

Drachenberg et al[30] showed that hematoxylin-eosin (H&E) histostaining can replace the TUNEL assay with almost the same efficiency of apoptosis detection for biopsy samples.

Histological Techniques

At the end of the experiment, the animals were sacrificed by decapitation under ether anesthesia. The liver of each rat was dissected out, fixed immediately in 10% neutral buffered formalin solution, then processed to prepare 5 μm thick paraffin sections suitable for performance of histological techniques. For histological techniques, sections were stained with Hematoxylin & Eosin (H&E), for general architecture of the liver tissue and with Masson's trichrome stain to detect liver fibrosis[(28)].

Statistical Analysis

Data were collected, tabulated and the results were evaluated using SPSS version 15. Means ± SD were used to describe the data. Student's t-test was used to test for statistical differences between two groups. *P* value ≤0.05 was considered as statistically significant. We used ANOVA test to test the significance of the difference between quantitative variables.

Results

Of the 60 rats included at the beginning of the study, five rats in model group and one in the diabetic treated group were dead. No death occurred in the non diabetic control groups.

Histopathological Studies

Control Groups and Diabetic Treated Group

Microscopic examination with H&E and Masson's trichrome staining of the liver sections of all control groups (1, 2, 3 and 4) *i.e*. (normal control group, distilled water group, buffer citrate group and pioglitazone control group respectively) as well as diabetic group treated with pioglitazone, revealed that the sections were morphologically normal. Figure 1 showed a normal lobular architecture with central veins and radiating hepatic cords with irregular sinusoids; Figure 2 showed a normal distribution of collagen with a thin rim around central vein.

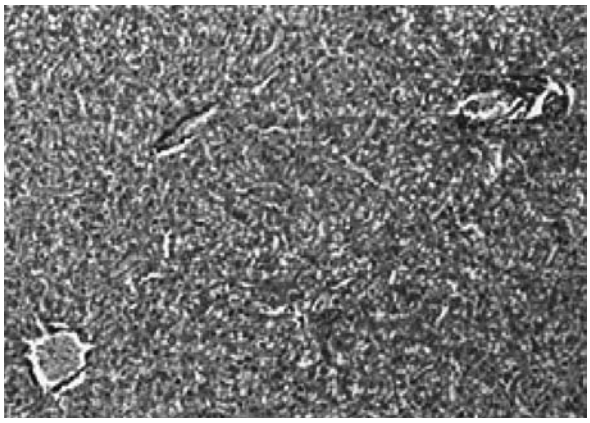

Figure 2. Liver tissue from control group showed normal lobular architecture and a normal distribution of collagen with a thin rim around central veins. [Masson × 100].

Diabetic Control Group

Microscopic examination of the liver sections with H&E staining, showed a moderate portal inflammatory infiltrate, mostly lymphocytic as well as intralobular cell necrosis and apoptosis (Figure 3) as well as bile stasis (Figure 4).

Serological Markers

After 72 Hours from STZ Induction of Diabetes

As shown in Table.I, fasting blood glucose was estimated to be significantly higher in diabetic group after 72 hours of induction of diabetes with STZ when compared to buffer citrate control group (304.7±52.3 versus 152±13.2, *p*-value <0.05). Induction of diabetes with STZ resulted also in a statistically significant elevation of all liver biochemical markers: AST, ALT, ALP, and GGT (*p*-value <0.05) (Table I).

At the End of Experiment (After Eight Weeks of Treatment)

No statistically significant difference existed in the four control groups (normal control, distilled water control, buffer citrate control and Pioglitazone control). All the laboratory markers were found to be significantly higher in diabetic control group compared with normal control group. Pioglitazone treatment resulted in a statistically significant reduction of the entire laboratory markers when compared with the mean val-

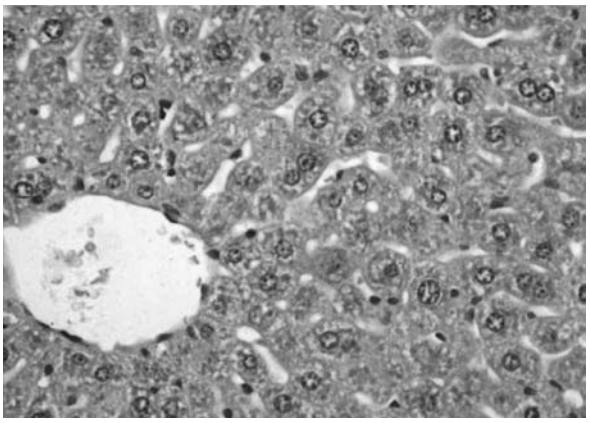

Figure 1. Section of the liver of a rat from the normal control group showing normal central vein and radiating hepatic cords. [H&E × 400].

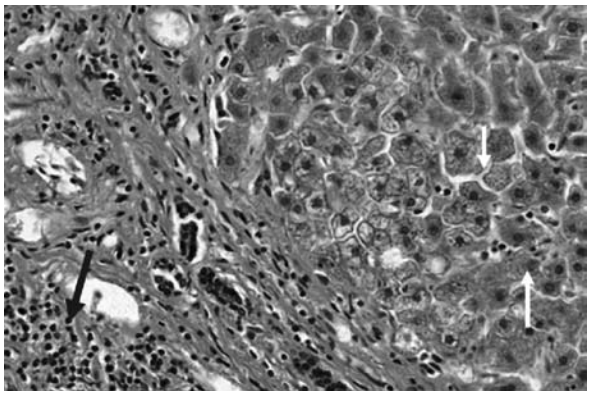

Figure 3. Section in the liver of the diabetic untreated case showing moderate portal inflammatory infiltrate, mostly lymphocytic (black arrow) as well as intralobular cell necrosis and apoptosis (white arrow). [H&E × 400].

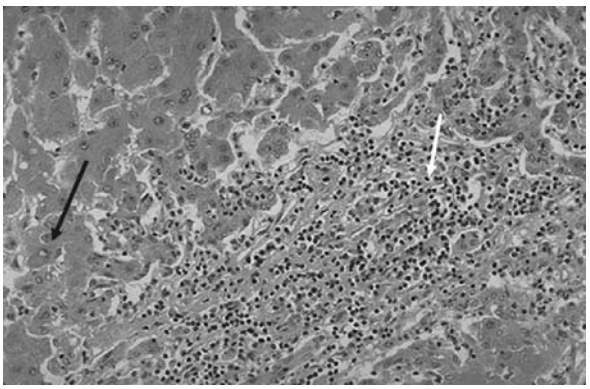

Figure 4. Section in the liver of the diabetic untreated case showing moderate portal inflammatory infiltrate, mostly lymphocytic (white arrow) as well as bile stasis (black arrow). [H&E × 400].

ues of the diabetic control group that didn't receive pioglitazone (Table II).

Discussion

Diabetes is associated with a number of clinical complications and it is the sixth leading cause of death in the U.S.[31,32]. The morbidity of the diabetes is uptrend in the world. Diabetes and its chronic complications lead to a considerable reduction in the quality of life and conspicuous increase in mortality[33].

Hepatotoxicity accompanied with diabetes did not receive much attention as other prevalent complications (i.e., cardiopathy, retinopathy, nephropathy) until the hepatotoxicity of the antidiabetic drugs emerged as a common clinical complication[32]. According to European Medicines Agency (EMEA)[34], one of the most frequent reasons for the withdrawal from the market of an approved drug during the last decade is the liver toxicity.

Troglitazone, the first approved TZDs, was withdrawn from the market following 94 reported cases of liver failure. Rosiglitazone and pioglitazone, so-called second- generation TZDs, were introduced into the market by the time troglitazone was withdrawn. These two newer drugs in the TZD class have a much larger margin of safety for liver toxicity[33]. In post-marketing experience with pioglitazone, reports of hepatotoxicity and hepatic failure have been received. Case reports of patients who experienced elevated liver function tests while receiving pioglitazone have been published, including one report of fulminant hepatic failure with pioglitazone[20,35,36]. However, it is somewhat debatable whether a true class effect exists[37].

The present research was conducted to study the effect of "pioglitazone" on the liver of streptozotocin-induced diabetic rats. As a general rule, clinically significant drug-induced liver injury is often defined as elevations in liver enzymes (AST or ALT) more than three times the upper limit of normal [ULN] at any time after starting a new drug, and considered as a serious liver injury and the implicated drug should be discontinued[38,39]. These criteria are now popularly described as Hy's Rule for monitoring drug hepatotoxicity[40].

In the present study, induction of diabetes by STZ injection was confirmed after 72 hours by fasting blood glucose level that was estimated to be significantly higher in diabetic group compared to buffer citrate control group**.** The clinical manifestations (polyphagia, polyuria and polydipsia accompanied by weight loss were seen in adult rats within three days of STZ injection ensured induction of diabetes. The STZ is believed

Table I. Comparison mean values of biochemical tests in post-induction time among diabetic group and buffer citrate control group. Data are means ± SD.

Test	Diabetic group (post STZ induction) (n = 14)	Buffer citrate control group (n = 10)
FBG	304.7 ± 52.3*	152 ± 13.2
ALT	91.6 ± 11.8*	53.4 ± 9.8
AST	175.7 ± 57.9*	126.9 ± 12.4
ALP	180.2 ± 22.3*	111.7 ± 19.1
GGT	7.9 ± 0.8*	5.7 ± 0.5

*Statistically significant difference (p-value for t-test <0.05).

Table II. Mean biochemical tests among all groups at the end of the experiment. Data are means ± SD.

Test	Normal control group (n = 10)	Distilled water control group (n = 10)	Buffer citrate control group (n = 10)	Pioglitazone control (n = 10)	Diabetic control (n = 5)	Diabetic treated (n = 9)
FBG	132.5 ± 12.6#	134.4 ± 15.1#	142 ± 11.7#	123.6 ± 7.63#	326.0 ± 40.52*	163.1 ± 38.45*#
ALT	45.2 ± 8.34#	47.6 ± 7.72#	49.4 ± 10.1#	42.2 ± 4.21#	70.4 ± 6.35*	53.56 ± 8.16*#
AST	112.9 ± 15.3#	116.5 ± 14.98#	119.6 ± 11.9#	109.1 ± 11.86#	163.2 ± 12.87*	137.33 ± 23.18*#
ALP	89.3 ± 18.6#	104.5 ± 30.36#	98.5 ± 20.6#	83.0 ± 13.56#	196.4 ± 59.7*	113.44 ± 30.72#
GGT	5.1 ± 0.68#	5.5 ± 0.97#	5.7 ± 0.9#	5.0 ± 0.67#	7.6 ± 1.34 *	6.11 ± 1.05*#

*Statistically significant difference versus normal control group (p-value <0.05)
#Statistically significant difference versus diabetic control group (p-value <0.05).

to induce diabetes within 3 days by destroying the beta cells of pancreas[41].

In the present study, induction of diabetes mellitus by streptozotocin resulted in significant increase in all liver parameters compared with citrate buffer control group after 3 days of induction before starting of any treatment. This effect was similar to those of other studies, who demonstrated that the STZ transient toxic effect on liver function disappeared by 15-30 days[42,43]. This indicates that all biochemical and histopathological changes at the end of our experiment were due to the effect of diabetes mellitus on liver rather than to STZ injection.

In the present study, at the end of the experiment, no significant differences existed between serum FBG, ALT, AST, ALP and GGT levels in rats that had received pioglitazone alone, citrate buffer, and distilled water in comparison to the normal control group. Also there were no changes in the histopathological results between such groups, indicating that neither pioglitazone nor buffer citrate or oral distilled water alone had a significant effect on these parameters. On the other hand, the untreated diabetic group exhibited a statistically significant rise in serum level of FBG, associated with increase in all liver enzymes. These findings were confirmed by the histopathological features that exhibited mild to moderate portal inflammatory infiltrate, mostly lymphocytic as well as intralobular cell necrosis and apoptosis as well as bile stasis in the diabetic untreated group, indicating the relation between diabetes and the incidence of hepatotoxicity. These results agree with Vagula and Devi[32] who emphasized that diabetic patients are twice as likely to suffer hepatic failure compared to patients who don't have diabetes.

In the present study, treatment by pioglitazone resulted in highly significant reduction in FBG in comparison with diabetic untreated group. These findings could be attributed to PPARγ agonists that lead to (1) improved hepatic insulin sensitivity during the post absorptive state, resulting in decreased hepatic glucose production; (2) improved muscle insulin sensitivity under conditions of hyperinsulinemia, resulting in increased tissue glucose uptake; (3) improved adipose tissue insulin sensitivity resulting in decreased FFA release; and (4) increased circulating levels of adiponectin[44].

According to the liver enzymes, pioglitazone treatment significantly ameliorated the significant increase of all liver parameters that observed in the diabetic untreated group whereas pioglitazone administration alone didn't elevate the liver enzymes. These data reaffirm the results from the pre-marketing clinical trials in which hepatotoxicity, a precursor to liver failure, was not found to be a significant problem in the pioglitazone treatment groups[15,45]. Other studies have reported the lack of evidence of link between pioglitazone and liver failure[21].

The effects of TZDs in reducing ALT in patients with type 2 diabetes mellitus is believed to arise from improvement in the metabolism of serum free fatty acids and reduction of fat accumulation in the liver[46].

The results of this study also agree with the results obtained by Bajaj et al[47] who emphasized that, pioglitazone treatment for 6 months significantly improved multiple metabolic and histological abnormalities compared with diet alone. Moreover, the results of Home and Pacini[48] support these observations.

The results of liver testing from a large number of diabetic individuals confirm that those

with Type 2 diabetes have higher than expected values of liver tests. Treatment with pioglitazone caused decreases in values of liver tests in a greater number of patients than with metformin or gliclazide[49].

In the present study, results showed that there is no histological abnormalities detected neither in the diabetic group treated with pioglitazone nor in the pioglitazone control group. These results support the hepatic safety of pioglitazone during treatment and indicated that pioglitazone may greatly ameliorated hepatocyte degeneration, necrosis and infiltration of inflammatory cells associated with development of diabetes.

These findings agree with Yuan et al[50], who showed that treatment with pioglitazone ameliorated hepatocyte degeneration, necrosis and infiltration of inflammatory cells significantly compared with model group. Liver functions were also improved apparently. These data demonstrated that PPARγ agonists also had anti-inflammatory effects, and subsequently retarded the progression of hepatic fibrosis in rats.

The absence of an increased risk of hepatic abnormalities has also been found in randomized controlled clinical trials of pioglitazone compared with placebo[51] or conventional oral antidiabetic agents[22].

The mechanism for these effects of pioglitazone is unclear. Reductions of liver enzymes occurred relatively early in the treatment, before maximum effects on glucose levels were seen, and therefore effects on other metabolic variables could be involved. The studies showed that decreases in free fatty acids (FFA) produced by pioglitazone and not by other hypoglycaemic agents occur with a similar time course as decreases in liver enzymes. This could result in a reduction of hepatic fat and lead to improvement of enzymes. However, there was no correlation between the extent of change in FFA and extent of decrease of liver enzymes, making this direct effect unlikely[52]. Since insulin resistance in itself is proposed to lead to fatty changes in the liver, a correlation between improvement in insulin sensitivity and reduction in liver enzymes might have been expected[53]. Alternatively, other effects of pioglitazone may play a role. Patients with diabetes have increased levels of lipid peroxidation products that was ameliorated by a pioglitazone treatment[54]. The various anti-inflammatory effects of pioglitazone, may also play a key element[55].

In conclusion, there is no evidence that pioglitazone administration has a harmful effect on the liver. Conversely, it has potential beneficial effects on the liver during treatment of STZ-induced diabetic rats, suggesting that liver toxicity isn't a class effect of the TZDs but rather a unique effect of troglitazone.

Further studies with more investigations are mandated to confirm the results of our study.

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