Chapter 3

Effect of low dose of pioglitazone treatment on pro-atherogenic markers in white adipose tissue of obese diabetic *db/db* mice

3.1 Introduction

Adipose tissue is a dynamic endocrine organ that secretes a number of factors that are increasingly recognized to contribute to systemic and vascular inflammation. Several of these factors, collectively referred to as adipokines, have now been shown to regulate, directly or indirectly, a number of the processes that contribute to the development of insulin resistance, dyslipidemia and atherosclerosis. Several adipokines are preferentially expressed in visceral adipose tissue, and the secretion of proinflammatory adipokines is elevated with increasing adiposity. Adipokines play important role in the development or prevention of diabetes [Maeda et al., 2002; Yamuchi et al., 2001] and atherosclerosis [Yamuchi et al., 2003]. Not surprisingly, approaches that reduce adipose tissue depots, including surgical fat removal, exercise, and reduced caloric intake, improve proinflammatory adipokine levels and reduce the severity of their resultant pathologies. Systemic adipokine levels can also be favorably altered by treatment with several of the existing drug classes used to treat insulin resistance, hypertension, and hypercholesterolemia which are the major risk factors for cardiovascular disease.

Thiazolidinediones (TZDs) analogs, which act as PPAR gamma agonists have been established as insulin sensitizing drugs, used in therapy of type-2 diabetes. PPAR gamma is highly expressed in white adipose tissue with lower levels in skeletal muscle and liver [Tontonoz et al., 1994]. PPAR gamma activators are key promoters of adipogenesis [Tontonoz et al., 1995; Rosen et al., 1999], improve insulin sensitivity, dyslipidemia and increase bodyweight [Nolan et al., 1994; Tominaga et al., 1993; Mitra et al., 2007]. TZDs regulate various genes involved in adipocyte differentiation or lipid and glucose homeostasis. TZDs are also known to modulate various adipokines [Moore et al., 2001; Maeda et al., 2001; Choi et al., 2005].

In addition to these metabolic effects, PPAR-γ agonists are also reported to have anti-inflammatory properties [Ghanim et al., 2001; Mohanty et al., 2004,

Megawa et al., 2007]. TZDs were demonstrated to have potential anti-atherogenic properties in studies by modulating endothelial and monocyte/macrophage function and inhibiting smooth muscle cell migration and fibrinolysis [Dandona and Aljada, 2002] which is probably mediated by its anti-inflammatory action. Furthermore, there are clinical studies where TZDs reduces the cardiovascular risk in type 2 diabetic patients [Haffner et al., 2002; Koshiyama et al., 2001; Ghanim et al., 2006]. Moreover, thiazolidinediones can inhibit the expression of proinflammatory factors, especially genes regulated by nuclear factor-κB cascade and suppressors involved in the plaque rupture [Mohanty et al., 2004]. As a representative drug of thiazolidinediones, rosiglitazone also can reduce the plasma levels of C-reactive protein, matrix metalloproteinase 9 (MMP-9), and soluble CD40L [Haffner et al., 2002; Marx et al., 2003], which indicates that it may prevent atherogenesis at a certain extent. It was also been reported that rosiglitazone showed anti-inflammatory effects in human type-2 diabetes patients at 2 mg dose, since this dose of 2mg is lower than the dose routinely administered to produce anti-hyperglycemic & insulin sensitizing effects [Ghanim et al., 2006], the findings indicated two distinct roles of TZDs. It appeared that low dose of TZDs may be used for ameliorating inflammation related to obesity and higher dose may improve the metabolic syndrome.

Therefore, in the present study, we studied the low dose effect of pioglitazone and to understand the adipokine regulation (proinflammatory and anti-inflammatory adipokines) and their possible involvement in insulin resistance which is risk factor of cardiovascular risk.

3.2 Materials and Methods

3.2.1 Animals and treatments

All animal experimentations were carried out in accordance with CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on

Animals) guidelines, using Institutional Animal Ethics Committee (IAEC) approved protocols.

This study was performed in female db/db mice of 8 to 10 wk old. The animal colonies were originally procured from the Jackson Laboratory, Maine, USA. Mice were housed in individually ventilated cages and given pelleted food (Standard Rodent diet, NIN, Hyderabad, India) and water ad libitum in a temperature (25±3℃) and humidity (50–70%)-controlled environment with a 12-h/12-h dark-light cycle. The animals were randomized into different groups (n =12) based on glucose levels followed by body weight. The animals were treated orally with pioglitazone 3 and 30 mg/kg dose or vehicle (0.5% w/v methylcellulose) per day for 14 days. On day-15 serum and visceral white adipose tissue were collected from 6 animals of each group. Fat samples were flash frozen for RT-PCR and cytokine level estimation. Remaining 6 animals of each group were subjected to oral glucose tolerance test.

3.2.2 Oral Glucose Tolerance Test

Oral glucose tolerance test was performed by oral administration of glucose solution (3 g/kg body weight) in 18 hour fasted animals after 14 days pioglitazone treatment. Blood samples were drawn through retroorbital sinus before (0 min) and 30, 60 and 120 min after glucose administration and analyzed for glucose levels.

3.2.3 RNA analysis & Quantitative Real-Time Polymerase Chain Reaction [qRT-PCR]

White adipose tissue samples were homogenized in TRIzol reagent (Invitrogen, Life Technolgies, Carsbad, CA, USA) using Mixer 301 (Retsch, Germany). Total RNA was extracted following the manufacturer's protocol. 1 µg total RNA from each sample was taken for first strand cDNA synthesis using High Capacity cDNA archive kit (Applied Biosystems, USA Part No 4322171). cDNA from each

sample was taken for Quantitative Real Time PCR using ABI-prism-7300. FAM labeled Taqman probes viz Adiponectin [Mm00456425_m1], Resistin (Mm00445641_m1), TNF alpha (Mm00443258_m1), IL-6 [Mm00446190_m1], lipoprotein lipase (LPL) [Mm01345523_m1] and fatty acid binding protein (aP2) (Mm00445880_m1) were used for PCR in the presence of Taqman Universal Mastermix (Applied Biosystems, Foster City, CA, USA). VIC labeled, housekeeping gene, Mouse beta actin (Part No: 4352341E) probe was procured from Applied Biosystems and was coamplified, in each sample with every target gene[s], for normalizing the results.

3.2.4 Biochemical measurements

Serum glucose levels were determined by GOD/POD method using biochemical kit (Ranbaxy laboratories Ltd, India). Adiponectin (B Bridge CA, USA), resistin (R&D System, Minneapolis, USA) and insulin (Linco Research Inc, USA) levels in the serum were measured by ELISA method specified by the manufacturers. For cytokine assay visceral white adipose tissues were homogenized using ice-cold TRIS buffer containing 1mM phenyl methyl sulphonyl fluoride. Circulating and adipose tissue TNF alpha and IL-6 levels were measured by ELISA method using kits from BD Biosciences, USA. The tissue TNF alpha and IL-6 levels were expressed per mg of tissue protein, measured using Biuret method (Pointe Scientific, USA).

3.2.5 Data and Statistical Analysis

All the data has been expressed as Mean ± SEM. The statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey test used to identify difference between all groups. Area under curve (AUC) was calculated using graph pad prism software (4.0).

3.3 Results

3.3.1 Effect of pioglitazone treatment on fasting serum glucose, oral glucose tolerance, and insulin

Pioglitazone (30 mg/kg) treatment for two weeks showed significant ($P<0.05$) reduction in overnight fasted glucose level (142.0 ± 6.28 mg/dl, n=6) vs control (204.1 ± 13.97 mg/dl, n=6) db/db mice. However, at 3 mg/kg dose, there was no significant change in the fasting glucose (193.08 ± 18.4 vs 204.1 ± 13.97 mg/dl, n=6) compared to the control animals (Fig 3.1A). There was a statistically significant difference between the pioglitazone groups in fasting glucose after 14 days treatment.

Area under the curve (AUC) calculation from the OGTT showed a significant ($P<0.05$) reduction in glucose at 30 mg/kg dose of pioglitazone when compared to control animals (1875 ± 123.5 vs 2541 ± 210.3, n=6). On the contrary, 3mg/kg treatment showed no significant effect (2438 ± 198.3 vs 2541 ± 210.3, n=6).

Pioglitazone treatment for 2 weeks at 30 mg/kg dose significantly ($P< 0.05$) reduced the fasted insulin level compared to vehicle control (1.19 ± 0.06 vs 1.77 ± .18 ng/ml, n=6,) at the initiation of OGTT. However, treatment at 3 mg/kg did not show any significant alteration in insulin levels compared to control (1.43 ± 0.11 vs 1.77 ± .18 ng/ml, n=6) (Fig 3.1B).

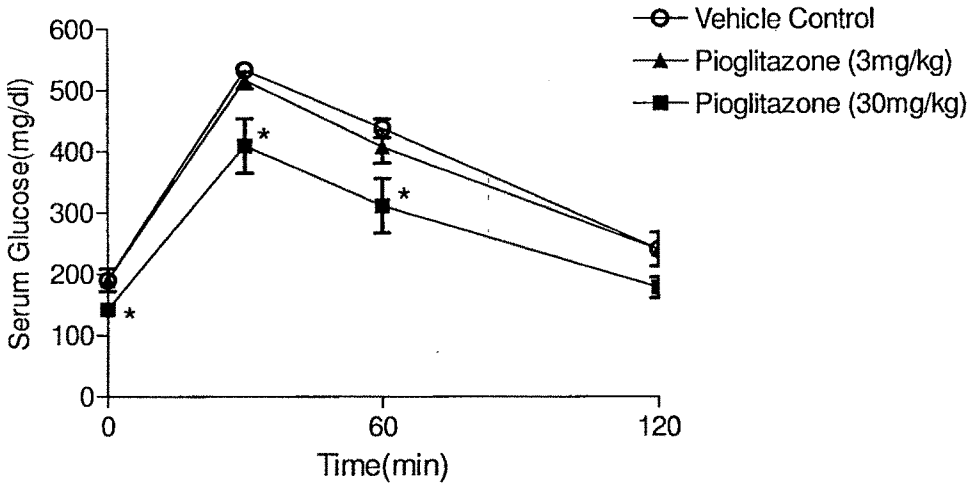

Fig 3.1A.

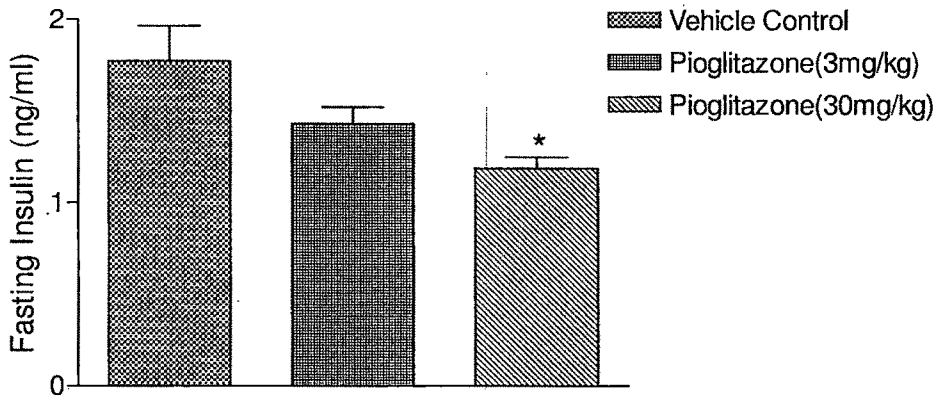

Fig. 3.1B

Fig. 3.1: Effect of Pioglitazone treatment on serial glucose increase depicted as line graph after glucose load (A) and fasting serum insulin (B) of db/db mice. Serum insulin level of different groups were represented in the bar diagram. Values are Mean ± SEM (n = 6 mice/group). * $P < 0.05$, as compared to vehicle-treated control db/db mice. # $P < 0.05$ for fasting glucose when compared between pioglitazone 3mg/kg vs 30mg/kg treatment.

3.3.2 Effect of pioglitazone treatment on body weight gain in db/db mice

Treatment with therapeutic dose (30mg/kg) of pioglitazone caused significant ($P<0.05$) body weight gain as compared to control animals (Table 3.1). However, no significant effect was found at 3mg/kg dose.

Table 3.1 Table depicts the change in body weight of different groups in the *db/db* mice treated with pioglitazone for 14 days.

Groups	Initial body weight (g)	Body weight (g) on the day 14	Change in body weight
Vehicle Control	39.4 ± 1.1	42.3 ± 0.9	2.9 ± 0.9
Pioglitazone(3mg/kg)	40.2 ± 0.9	43.6 ± 1.9	3.4 ± 1.4
Pioglitazone(30mg/kg)	38 ± 1.5	45.4 ± 1.7	7.4 ± 1.3 *

Values are described as mean ± SEM. * $P < 0.05$ as compared to vehicle control.

3.3.3 Effect of pioglitazone treatment on WAT mRNA expression or serum levels of metabolic biomarkers (aP2, LPL and adiponectin)

At 30mg/kg dose of pioglitazone, aP2, LPL and adiponectin WAT mRNA expressions were increased significantly ($P<0.01$) when compared to vehicle treated animals (Fig. 3.2, 3.3, and 3.4A). At this dose significant ($P<0.01$) increase in serum adiponectin level (48.6 ± 3.07 vs 23.2 ± 0.66, µg/ml, n=6) was observed which correlated well with the mRNA expression (Fig. 3.4B). No significant changes in mRNA expression or serum levels were observed in above mentioned variables at 3mg/kg dose. LPL, aP2 mRNA expression and serum adiponectin between the two dose groups were significantly different.

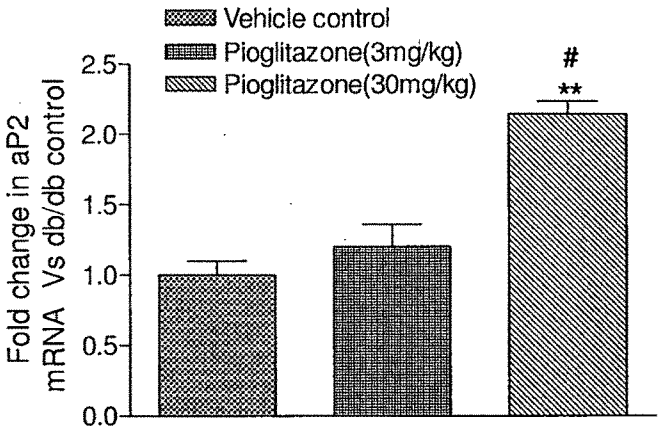

Fig. 3.2: Effect of pioglitazone on the expression aP2 mRNA in WAT of db/db mice. Fold change in treatment groups against the vehicle control db/db is represented in the bar diagram. Each value represents mean ± SEM (*n* = 6 mice/group). ** P < 0.01 as compared to vehicle-treated control db/db mice. # P < 0.01 when compared with pioglitazone 3mg/kg vs 30mg/kg treated db/db mice

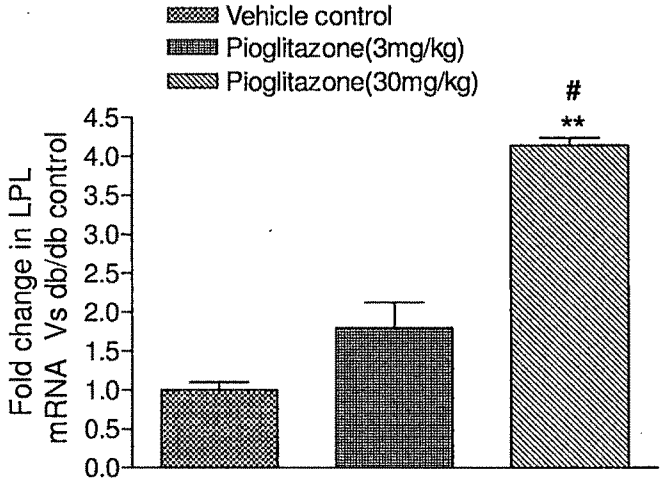

Fig. 3.3: Effect of pioglitazone on the expression LPL mRNA in WAT of db/db mice. Fold change in treatment groups against the vehicle control db/db is represented in the bar diagram. Each value represents mean ± SEM (n = 6 mice/group). ** P < 0.01 as compared to vehicle-treated control db/db mice. # P < 0.01 when compared with pioglitazone 3mg/kg vs 30mg/kg treated db/db mice

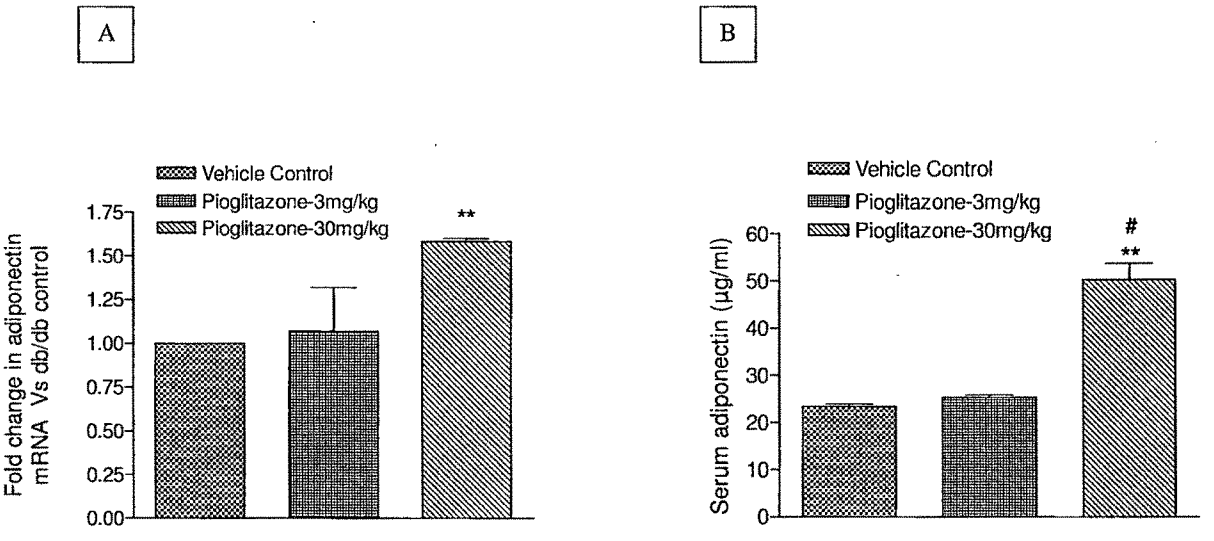

Fig. 3.4: Effect of pioglitazone on the expression adiponectin mRNA in WAT (A) and serum adiponectin (B) in db/db mice. Fold change in mRNA in WAT and serum adiponectin level of different groups were represented in the bar diagram. Each value represents mean ± SEM (n = 6 mice/group). ** P < 0.01, as compared to vehicle-treated control db/db mice. # P < 0.01 for serum adiponectin when compared between pioglitazone 3mg/kg vs 30mg/kg treatments.

3.3.4 Effect of pioglitazone treatment on WAT mRNA expression and/or protein levels of inflammatory biomarkers (TNF alpha, IL-6, SOCS-3 and resistin)

Two-week treatment with 3 mg/kg dose of pioglitazone significantly (P<0.05) decreased transcripts of TNF alpha, IL-6 and SOCS-3 in WAT when compared to control animals (Fig. 3.5A, 3.6A, 3.8). Serum TNF alpha and IL-6 levels were below the detectable limits of the assays used. When adipose tissue levels of TNF alpha and IL-6 were measured, 3 mg/kg pioglitazone treatment, caused significant reduction in TNF alpha (43.12 ± 2.95 vs 56.23 ± 4.78, pg/mg protein, n=6, P<0.05), but slightly reduced IL-6 level (24.9 ± 3.2 vs 32.6 ± 2.91, pg/mg protein, n=6) which was not significant when compared to control group (Fig.

3.5B, 3.6B). Resistin mRNA was found to decrease significantly ($P<0.05$) at 3 mg/kg dose group (Fig. 3.7A). Significant lowering of serum resistin level was observed at 3 mg/kg dose of pioglitazone (7.40 ± 0.34 vs 11.45 ± 0.85, ng/ml, n=6, $P< 0.05$) which was in accordance with the tissue mRNA expression (Fig. 3.7B).

Pioglitazone 30 mg/kg treatment decreased WAT TNF alpha, IL-6 and SOCS-3 mRNA expression significantly when compared to control animals. Interestingly reduction in TNF alpha expression was found to be saturated at low dose and there was no significant difference between the two doses tested (Fig. 3.5A, 3.5B). There was significant reduction in IL-6 mRNA in both the doses whereas IL-6 protein levels decreased only at the highest dose. However, there was no statistical difference between the two dose groups of pioglitazone either in mRNA or protein levels (Fig. 3.6A, 3.6B).

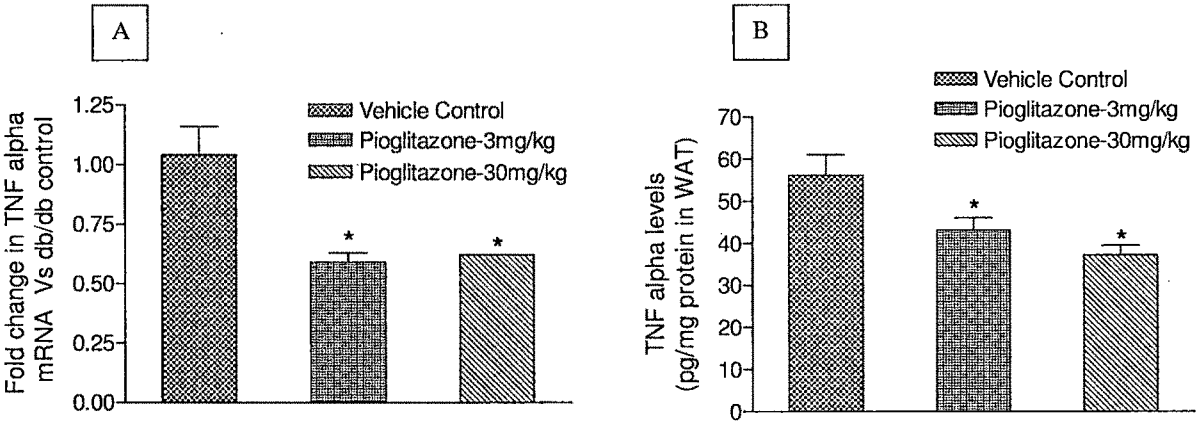

Fig. 3.5: Effect of pioglitazone on the expression TNF alpha mRNA in WAT (A) and tissue TNF alpha levels (B) in db/db mice. Fold change in mRNA in WAT and TNF alpha levels of different groups were represented in the bar diagram. Each value represents mean ± SEM (n = 6 mice/group). * $P < 0.05$, as compared to vehicle-treated control db/db mice.

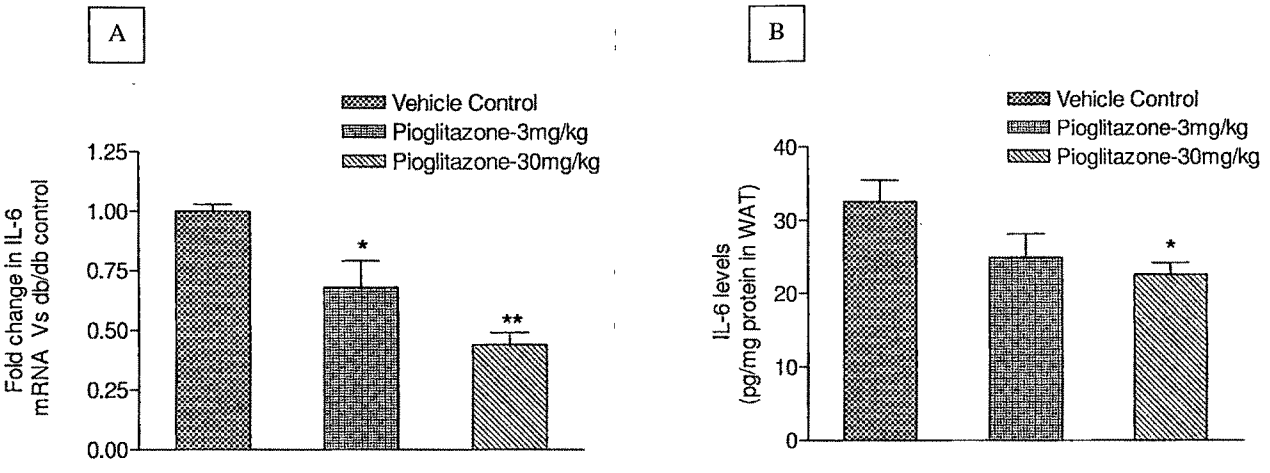

Fig. 3.6: Effect of pioglitazone on the expression IL-6 mRNA in WAT (A) and IL-6 tissue levels (B) in db/db mice. Fold change in mRNA and IL-6 level levels of different groups were represented in the bar diagram. Each value represents mean ± SEM (n = 6 mice/group). * P < 0.05 and ** P < 0.01, as compared to vehicle-treated control db/db mice.

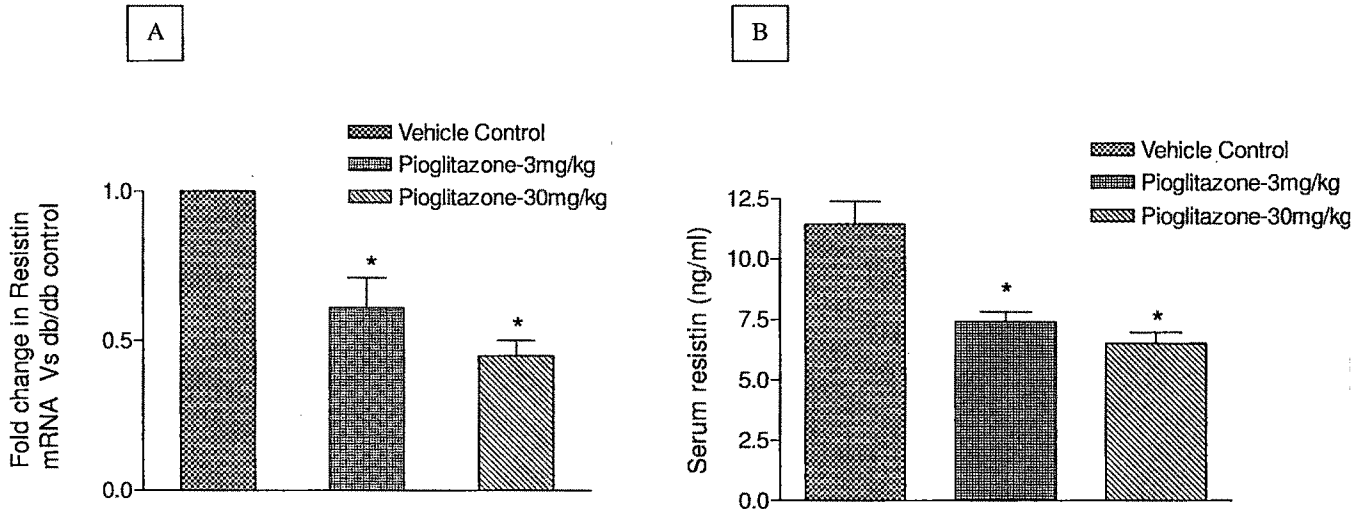

Fig. 3.7: Effect of pioglitazone on the expression resistin mRNA in WAT (A) and serum levels (B) in db/db mice. Fold change in mRNA and serum resistin level were represented in the bar diagram. Each value represents mean ± SEM (n = 6 mice/group). * P < 0.05, as compared to vehicle-treated control db/db mice.

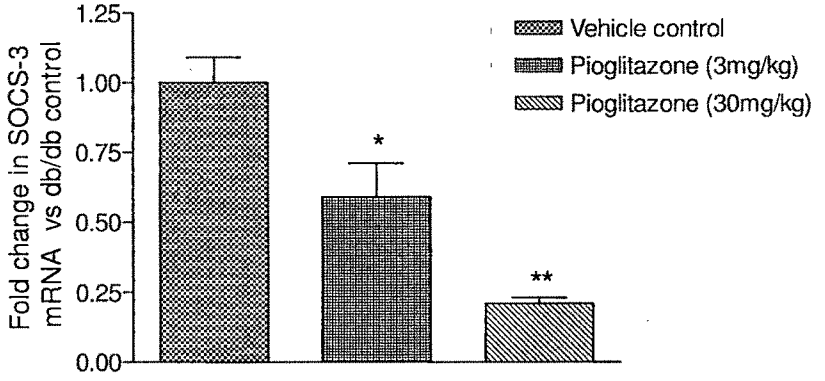

Fig. 3.8: Effect of pioglitazone on the expression SOCS-3 mRNA in WAT in db/db mice. Fold change in mRNA level were represented in the bar diagram. Each value represents mean ± SEM (n = 6 mice/group). * $P < 0.05$, ** $P < 0.01$ as compared to vehicle-treated control db/db mice.

3.4 Discussion:

At therapeutic dose of pioglitazone demonstrated PPAR gamma related changes by lowering the fasting glucose levels in db/db mice. Amelioration of hyperglycemia in the presence of reduced plasma insulin levels, suggests that insulin sensitivity has been improved in pioglitazone treated db/db mice at therapeutic dose. Our finding is supported by the fact that, rosiglitazone significantly ameliorated hyperglycemia and hyperinsulinemia in diet induced obese mice [Yamauchi et al., 2001]. Along with the changes in glucose and insulin, there were also significant upregulation in some PPAR gamma related metabolic markers expression e.g. LPL, aP2 and adiponectin in the WAT of db/db mice. In the present study we observed pioglitazone treatment at the therapeutic dose resulted in significant body weight gain in the db/db mice. This could be explained by the parallel increase in expression of the adipogenesis marker aP2 and LPL through PPAR gamma in the WAT. The increase of LPL synthesis in WAT may in part explain the increased adiposity observed during long-term treatment with TZDs [de Souza et al., 2001; King et al., 2002]. This

observation were supported by reports from Yamauchi and coworkers that rosiglitazone increased body weight in *db/db* mice & increased expression of adiponectin levels in differentiated 3T3L1 adipocytes in vitro [Yamuchi et al., 2001]. In our current study the observed elevation of adiponectin could be explained as adiponectin is mainly expressed in adipocytes and is known to be affected by adipogenic differentiation. Therefore our results indicated that therapeutic dose of pioglitazone produced PPAR gamma related changes in glucose level and modulation of metabolic biomarkers in WAT.However, treatment with low (3mg/kg) dose of pioglitazone did not alter serum glucose level or other PPAR-related metabolic biomarkers (LPL, aP2, and adiponectin) in WAT indicating the absence of metabolic effects of PPAR gamma. Similar observation was made by Srinivasan *et al.* who demonstrated that 3mg/kg dose of pioglitazone was not sufficient to achieve anti-hyperglycemic and insulin sensitizing effect in high-fat fed rats [Srinivasan et al., 2004]. Interestingly significant reduction in expression of TNF alpha, IL-6 and resistin mRNA in WAT were found at this dose. TNF-alpha and IL-6 are inflammatory cytokine released in greater quantities by obese humans and patients with insulin resistance, not only initiates but also propagates atherosclerotic lesion formation. TNF-alpha activates the transcription factor nuclear factor-kB (NF-kB), which accelerates experimental atherogenesis, in part by inducing the expression of VCAM-1, ICAM-1, MCP-1, and E-selectin in aortic endothelial and vascular smooth muscle cells [Ouchi et al., 1999]. Further, antiatherosclerotic effect of systemic TNF alpha inhibition is now well established [Branen et al., 2004; Canault et al., 2004]. So the inhibition of TNF alpha production might play a role in the anti-atherosclerotic effects of pioglitazone. IL-6 is also known to promote the release of endothelial adhesion molecules and chemokines [Yudkin et al., 1999]. Adding to the possible link between adipokines and cardiovascular risk, it was recently shown that resistin is secreted primarily by the macrophage in the human rather than the adipocyte as in rodents. Resistin is a proinflammatory cytokine [Kaser et al., 2003; Lehrke et al., 2004; Lu et al., 2002; Bokarewa et al., 2005] and increases the expression of plasminogen activator inhibitor-1 which is also

involved in atherosclerosis [Jung et al., 2006]. Further, potential role of resistin in atherosclerosis has been documented [Jung et al., 2006; Reilly et al., 2005]. The anti-inflammatory effects of low-dose pioglitazone thus include several mediators of inflammation, which might indicate a reduced cardiovascular disease (CVD) risk. It is therefore possible that, pioglitazone and other TZDs may be used as anti-inflammatory and anti-atherogenic drugs because atherosclerosis is a chronic inflammation of the arterial wall.

Reduction in TNF alpha and IL-6 expression and their corresponding protein levels in WAT at low dose emphasized the anti-inflammatory effect of pioglitazone rather than the metabolic effect. Several reports have indicated that TZDs may produce anti-inflammatory effects through NF-kB which is a molecular target for PPAR-γ agonists [Ghanim et al., 2006; Chawla et al., 2001; Diep et al., 2004]. TNF alpha IL-6 and resistin are the important mediators of inflammation in preadipocyte and inhibits adipocyte differentiation in vitro [Lacasa et al., 2006; Kim et al., 2001]. It is also reported that proinflammatory cytokine/chemokine in preadipocytes suppress PPAR gamma and insulin responsiveness in human adipocytes [Chung et al., 2006]. Low-grade systemic inflammation is associated with increase in proinflammatory cytokines in obese condition [Lion et al., 2003; Stuart et al., 2003]. This is supported by the fact that increased expression of TNF alpha, IL-6 and SOCS-3 was observed in WAT of db/db mice compared to C57BL/6J mice. Furthermore, I have demonstrated in our previous chapter both obesity and lipopolysaccharide (LPS)-induced endotoxemia increased in suppressor of cytokine signaling (SOCS-3) proteins in WAT suggest that SOCS-3 is an important signaling and its role in inflammation [Ueki et al., 2004]. Inhibition of TNF alpha, IL-6, SOCS-3 and resistin expression observed at sub-therapeutic dose of pioglitazone indicated that the drug may first correct the proinflammatory state of adipose tissue and may prime these tissues for insulin sensitizing and adipogenic effects as indicated by enhanced expression of adiponectin, LPL and aP2 at the therapeutic dose. This is supported by reports that early anti-inflammatory role of pioglitazone or rosiglitazone in human diabetic

patients is followed by delayed anti-diabetic effect [Forst et al., 2008; Mohanty et al., 2004].

In conclusion, our results indicate that low dose of pioglitazone produced anti-inflammatory effect by suppressing TNF alpha and IL-6 in WAT without altering metabolic parameters. Thus the anti-inflammatory effect may precede insulin-sensitizing effects observed at high dose in diabetic db/db mice. Furthermore, atherogenic role of resistin and antiatherogenic action of adiponectin and direct reciprocal effects of resistin and adiponectin on vascular inflammation has been reported (Kawanami et al., 2004). In our present study, increase in adiponectin and fall in resistin concentration may imply pioglitazone can be used as an anti-atherogenic drug in future.