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Investigating the Genetic Impact of Pioglitazone on Insulin Sensitivity in Human Adipocyte Stem Cells

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Abstract: ***Background: The study focuses on Pioglitazone hydrochloride (PH), a PPARγ agonist known for its impact on metabolic diseases. It activates PPARγ, enhancing insulin response in target cells. However, the precise mechanism by which it improves insulin sensitivity remains unclear. Objectives: This research aimed to decipher PH's antidiabetic effects by analyzing key genes (PPARγ, Glut 4, PI3K, TNFα, FASN, IL6) in human adipocytes, vital in diabetes and related issues like atherosclerosis. Methods: Human adipose-derived stem cells were cultured and treated with varying PH concentrations. Cytotoxicity and triglyceride levels were assessed using MTT assays and Oil red O staining. Gene expression (PPARγ, Glut 4, PI3K, TNFα, FASN, IL6) was evaluated through semiquantitative PCR and real-time PCR. Result: At 50 µM, PH exhibited 70.823% cell viability and significantly reduced lipid accumulation compared to controls. Gene expression analysis revealed heightened PPARγ, GLUT4, PI3K, FASN expression (P<0.05), and reduced TNFα and IL6 levels. Conclusively, PH upregulates PPARγ, enhancing insulin sensitivity while reducing lipids, TNFα, and IL6. Interpretation & conclusions.: This study investigates the genetic and biochemical effects of Pioglitazone hydrochloride (PH), a PPARγ agonist, on insulin resistance and metabolic disorders in human adipocyte stem cells. PH treatment enhanced insulin sensitivity by upregulating key genes (PPARγ, GLUT4, PI3K, FASN) and reducing inflammatory markers (TNFα, IL6). Cytotoxicity assays confirmed PH's safety at 50 µM, with significant improvements in lipid accumulation and gene expression. These findings highlight PH's potential as a therapeutic agent for insulin resistance and related complications, warranting further translational studies.***

Keywords: Type 2 diabetes mellitus insulin sensitivity, lipid metabolism, adipocyte differentiation, molecular signaling, PPARγ agonist Pioglitazone hydrochloride, semiquantitative PCR and real-time PCR.

1. Introduction

Type 2 diabetes mellitus (T2DM) is a major health problem worldwide, and its prevalence is increasing in both developed and developing nations. According to the current data of the International Diabetes Federation (IDF), 537 million people (20-79 years age group) have diabetes worldwide; and expected to increase to 783 million by the end of the year 2045. Approximately 193 million people are still undiagnosed [1]. According to World Health Organization (WHO) from 2019-21, the prevalence of diabetes is growing most rapidly in low- and middle-income countries such as China and India. India reached an alarming level, in which 71 million people were living with diabetes (8.7%), which will increase to 134 million by the end of 2045 [2]. Factors such as obesity, physical inactivity, unhealthy dietary habits, and genetic factors contribute significantly to the burgeoning number of cases of type 2 diabetes. Genetic predisposition adds a distinct layer to the susceptibility of Indians to type 2 diabetes mellitus. Peroxisome proliferator-activated receptor gamma (PPAR-γ) is a member of the nuclear hormone receptor superfamily of ligand-activated transcription factors that are related to insulin signaling and homeostasis. PPAR-γ is located on chromosome 3 at the p25 locus (3p25.2). The PPAR-γ gene spans nearly 150 kb and comprises 9 exons [3].

PPAR-γ plays an important role in the pathology of numerous diseases, including obesity, diabetes, and atherosclerosis. Pioglitazone hydrochloride (PH) (commonly known as ActoS) is a member of the thiazolidinedione (TZD) family that acts as an antidiabetic drug. Upon interaction with their ligands, i.e., PH, PPAR-γ translocates into the nucleus, where it dimerizes with the retinoid X receptor (RXR)[4,5]. As a result, several target genes, whose expression is either increased or decreased, play a crucial role in maintaining glucose balance within the cell. However, the molecular mechanisms of PH on PPAR-γ and its therapeutic action for the treatment of T2DM are still not very clearly understood. Since PPARγ and pioglitazone are closely involved in the regulation of dietary fat storage and catabolism, they have been established as an important therapeutic target for the treatment of type 2 diabetes and other disorders [6-8]. This study aims to explore the effects of PH on the expression patterns of target genes relevant to T2DM and its complications. By understanding these mechanisms, we hope to identify new therapeutic targets that could lead to improved treatment strategies for T2DM.

2. Materials and Methods

Chemicals and reagents

Human adipose-derived mesenchymal stem cells (MSCs) (CL007-0.5), expansion media (Al519), differentiation media (AL521), FBS (RM112), Dulbecco's Modified Eagle

Medium (DMEM) and antibiotic solution (A001A) were purchased from Himedia, India. RIPA buffer (ab156034) and pioglitazone hydrochloride (ab120794) were from Abcam, USA. Human insulin (19278) and glucose (G7021) were purchased from Sigma, USA. Other reagents, *viz.* TRIzol, MTT, Dimethyl sulfoxide (DMSO), tetramethylethylenediamine (TEMED), 30% bis-acrylamide, 10% ammonium per sulfate (APS), formalin, isopropyle alcohol, oil red stain and a few additional chemicals were purchased from different biochemical agencies.

Cell Culture of Human Adipocytes

MSCs were grown in adipocyte cell expansion media (HiMedia) supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin to prevent contamination. The cells were grown at 37°C in 5% CO_2 in a humidified chamber (HERACell-150i, Thermo Scientific, USA) and incubated with fresh medium for 24 hours. To differentiate the MSCs into mature adipocytes, 1×10^5 MSCs were evenly seeded in twelve-well culture plates and allowed to fully confluent for 48 hours. After which the media was replaced with differentiation media (Himedia, India) for 15 to 18 days. Moreover, the medium was replaced with fresh medium at regular intervals. Differentiated cells were maintained in DMEM with 5% Fetal Bovine serum (FBS) and incubated at 37°C with 5% CO2. To induce a diabetic phenotype, the cells were treated with 100 nM insulin and high glucose (4.5 g/L) for 24 hours, resulting in diabetic adipocyte cells (DACs)[9]. Following this treatment, six concentrations of pioglitazone hydrochloride (0, 6.25, 12.5, 25, 50, and 100 µM) were added to the media of each well, except one well that served as the diabetic non-treated control (DNTC).The cells were incubated for an additional five hours to allow for treatment effects. (Figure 1a, b, c, d,e and f). This work was carried out from the year 2019 to 2022 in King George;s Medical University. Ethical Waiver certificate was obtained by Institution ethical committee.

Adipocyte cell viability assay

Cell viability was assessed using the MTT assay, which measures mitochondrial reductase activity. Adipocytes were cultured in 96-well plates at a density of 5,000-10,000 cells per well in DMEM supplemented with 5% FBS. After treatment for five hours, 20 µl of MTT solution (5 mg/ml) in PBS (pH 7.4) was added to each well. The cells were incubated for one hour in the dark, and the resulting formazan crystals were dissolved in 100 µl of DMSO. The absorbance was measured at 570 nm using a microplate reader reader (Multiscan Go, 1510-03399C, Thermo Scientific, USA), and cell viability was expressed as a percentage of the control.

$$\text{Cell viability (\%)} = \frac{A_{570}^{\text{Treated with Pioglitazone Hydrochloride}}}{A_{570}^{\text{Control (Untreated)}}} \text{ X } 100$$

Differentiation assay (Triglyceride content)

To examine the effect of PH on adipogenesis, Oil Red O method was used to stain accumulated intracellular triglycerides in differentiated adipocytes by using method of Inazawa et al, 2003[10] The cells were washed gently twice with ice-cold PBS (pH 7.4) and fixed with 10% formalin at room temperature for 1 hr in room temperature. After removal of the 10% formalin, wells were washed with 60% isopropyl alcohol for 5 min and then washed exhaustively with PBS (200 µl each well). Wells were allowed to dry completely before the addition of filtered 200 µl Oil Red O solution for 30 min at room temperature. The staining of lipid droplets in Human adipocytes were washed three times with PBS. Stained oil droplets were extracted with 200 µl100% isopropanol for 10 min to quantify intracellular lipids. The extracted dye was then immediately removed by gentle pipetting and its absorbance was measured spectrophotometrically at 540 nm using 96 well plate reader (Multiscan Go, 1510-03399C, Thermoscientific, USA). The lipid content was calculated according the following formula:

$$\text{Lipid Content (\%)} = \frac{A_{570}^{\text{Treated with Pioglitazone Hydrochloride}}}{A_{570}^{\text{Control (Untreated)}}} \text{ X } 100$$

Expression analysis

Reverse Transcriptase Polymerase Chain Reaction (rt-PCR) and semiquantitative PCR Total RNA was extracted by using TRIzol reagent (Invitrogen) and quantified via a NanoV-vis spectrophotometer (Nanodrop, Q5000, Quawell, USA) at 260 and 280 nm. First-strand cDNA was synthesized from 1µgof total RNA using cDNA Synthesis Kit "K1621" (Thermo Scientific, USA) in a gradient Master Cycler (Bio-Rad, USA). The primers and their details are given in Table 1**.** The PCR amplicons were documented in Gel Doc^{Tm} XR^{+} (Bio-Rad, USA) and analyzed via densitometry scanning using Image Lab^{Tm} software.

Real-time PCR

Quantitative real-time PCR was conducted with the same primers, and results were expressed as fold change relative to the control group using the ΔΔCt method, normalizing to GAPDH.

Statistical analysis

Data are presented as the mean ± SEM. Statistical analysis involved Student's t-test and one-way ANOVA, followed by Tukey's post hoc test. A P-value <0.05 indicated significance. Real-time PCR data were analyzed using Livak's method (ΔΔCt), with all data representing averages of at least three independent experiments.

3. Results

Adipocyte cell viability

MTT assay was done to assess the cell viability of human adipocyte cells induced T2DM by high glucose and insulin

and then treated with PH of various concentrations. All the experiments were performed in triplicate and the mean ± standard deviation (SD) was recorded.PH was non cytotoxic at concentration below 6.25 µM (Figure. 3.1) and no visible changes in cell morphology or attachment were observed at this dosage of PH. Non-cytotoxicity refers to cell viability ≥ 70% relative to untreated controls[10] and we observed 70.823 % cells viability at 50 µM. Therefore at this concentration no cytotoxicity was observed. However, Cell showed toxicity at 100 µM concentration of PH. Multiple comparative test between 6.25 µM vs 50 µM and 12.5 µM vs 50 µM were found to be significant (*P<0.05*) (Figure 3.1).

Triglyceride content

Adipose tissue plays a significant role in maintaining lipid homeostasis and energy balance by storing triglycerides (TG) or liberating free fatty acid in response to changes in energy demands[11] PH at 50 µM significantly reduced (*P<0.05*) lipid accumulation in human adipocytes compared with that in DNTCs. Multiple comparative tests also revealed significant differences among the 6.25, 12.5, 25 and 50 µM PH concentrations (Figure 2.2).

Semiquantitative polymerase chain reaction

Densitometric scanning analysis of the Glut4 gene revealed that the expression of this gene was upregulated by 1.65±0.011 in DM treated with 50 µM pioglitazone compared with that in DNTC-treated DM (0.33± 0.044). Similar results were obtained with concentrations of 6.25 µM (0.40±0.029), 12.5µM (0.65±0.031) and 25 µM (0.92±0.051). Overall, the expression of Glut 4 was significantly (*P<0.05*) greater in the treated group than in the DNTC. Similarly, compared with DNTC, PPARγ at a concentration of 50 µM had enhanced expression (2.21±0.172) (*P<0.05*) (Figure 3.2). Similarly, IP3K and FASN were significantly (*P<0.05*) greater (0.61±0.082 and 0.77±0.010, respectively) in the 50 µM treated group 0.083±0.011 and 0.01±0.01 DNTC treatment groups. In the case of TNF-α, significantly reduced expression was observed at 50 µM 0.38±0.071 compared with DNTC (1.97±0.221) (Figure 3.2). However, we also observed reduced expression of IL-6, but none of the concentrations we used were found to be significantly different (data not shown). *Asterisks* (*) (*P<0.05*) denote a significant difference in gene expression compared to that in the DNTC group.

Real-time PCR

The change in PPARγ expression was 1.94±0.05 at a 50 µM concentration of PH compared with 0.310±0.05 for DNTC. There was also a significant difference (*P<0.05*) in the expression of PPARγ between the 6.25 µM (0.64±0.05), 12.5µM (0.787±0.05), 25 µM (1.33±0.05). PH treatment groups. In the case of Glut4, there was approximately fourfold increase in expression (2.88±0.05) at 50 µM PH compared with DNTC (0.51±0.05). A significant change in FASN gene expression (*P*<0.05) was also observed, where a 1.12±0.05 increase in expression was observed in the 50 µM PH treatment group compared with the DNTC treatment group (0.41±0.05). However, decreased expression of the *TNFα* (0.88±0.05 at 50 µM vs 1.91±0.05 DNTC) *and IL-6* (1.37±.05 at 50 µM vs 0.82±0.05) genes was observed.

4. Discussion

Pioglitazone is also known as ActoS, an oral drug that reduces the amount of glucose in the blood. Pioglitazone is generally prescribed by clinicians either alone or in combination with other antidiabetic drugs. This drug belongs to the class of TZD and induces the activation of PPARγ, which alters the transcription of several genes involved in glucose and lipid metabolism and energy balance, including those that code for lipoprotein lipase (LPL) [12], fatty acid transporter protein, adipocyte fatty acid binding protein, fatty acyl-CoA synthase, malic enzyme, glucokinase, and the adipocyte fatty acid binding protein (AP2) [13]. FASN, an enzyme responsible for de novo lipogenesis, plays a pivotal role in synthesizing fatty acids from acetyl-CoA and malonyl-CoA[13]. Its expression is tightly regulated and associated with lipogenic activity in various tissues[14]. Studies suggest that pioglitazone treatment influences FASN expression. Although the exact underlying mechanisms remain unclear, it has been proposed that the activation of PPARγ indirectly enhances FASN expression, impacting lipid synthesis and metabolism.

Pioglitazone also increases the expression of the antiapoptotic enzyme or lipogenic gene FASN by activating the nuclear transcription factor PPARγ [15]. In the present study, PPARγ and Glut 4 expression increased in the PH treatment group. It has been reported that FASN expression is inversely associated with glucose levels, HOMA-IR, HbA1c levels, TG levels, BMI and the WHR, which indicates that, under insulin-resistant conditions, FASN expression is low, while FASN expression is positively correlated with adiponectin and HDL [14]. After PH treatment in vitro, we also observed that, compared with DNTC cells, diabetic cells significantly increased FASN mRNA expression (*P*<0.05) at a concentration of 50 µM.

Many studies have reported that pioglitazone is positively correlated with BMI, percentage of body fat, visceral and subcutaneous fat area, the WHR, fasting plasma insulin, hemoglobin A_{1C} (HbA_{1c}), NEFA, leptin, IL-6 and RUBP4 in visceral and subcutaneous regions. Pioglitazone increases total body water intake, thereby accounting for the increase in body weight that is attributable to an increase in adipose tissue mass [16]. There is much evidence suggesting that pioglitazone activates PPARγ, facilitates the downregulation of IRS phosphorylation at serine 307 (IRS-$serine^{307\text{-}P}$) [17,18] tumor necrosis factor alpha (TNF-α) and reduces the autophosphorylation of adjacent Janus kinase (JNK), resulting in increased tissue sensitivity to insulin[19]. Pioglitazone may improve insulin sensitivity by upregulating the expression of another important molecule, PI3K, which may induce kinase activity, and Akt leads to phosphorylation of both. This phosphorylation may promote the phosphorylation of rab, a monomeric G protein that releases Glut4 after confirmation changes due to phosphorylation [19–21]. We also found significant decreases in the levels of TNFα and IL6. Another study reported that one of the mechanisms that may be responsible for the enhancement of insulin sensitivity due to PH treatment is the decrease in proinflammatory IL6 level [22] . IL6 is a cytokine that is produced in adipose tissue as well as in skeletal muscles [23]. It is an important myokines [24], involved in the

regulation of insulin sensitivity. It has been previously documented that IL6 may inhibit the insulinsignaling pathway by upregulating the expression of the cytokine signaling-3 (factor SOCS3), a marker of the activation of IL6 signaling [25]. In turn, SOCS3, as well as SOCS1, may lead to impaired IRS1 and IRS2 tyrosine phosphorylation, which results in the upregulation of IRS1 at residue 307 and the downregulation of IRS2 at tyrosines 608/612. This phosphorylation of IRS2 proteins provides docking sites for further downstream signaling molecules, including the p85 subunit of PI3K. This binding induces the activation of the p110 catalytic subunit of PI3K. The p110 subunit acts as a key mediator required for insulin-induced physiological responses. Activated PI3K also catalyzes the upregulation of phosphatidylinositide 3,4-biphosphate and phosphatidylinositide 3,4,5-triphosphate, leading to the binding and activation of further downstream kinases, including phosphoinositide-dependent serine/threonine kinase 1, Akt and PKCλ/ζ (protein kinase C), all of which are known to play important roles in improving insulin sensitivity [26,27].In conclusion, we demonstrated that activation by the PPARγ agonist PH improves insulin sensitivity by increasing the expression of PI3K, which increases the kinase activity of PI3K, increasing the phosphorylation of rab protein, which is covalently bound to the Glut4 protein. This phosphorylation of rab leads to a change in the confirmation of Glut4 release in the membrane, leading to improved insulin sensitivity [28,29]. Therefore, PH represents an effective tool for targeting several important genes that may lead to therapeutic approaches for the regulation of insulin resistance and therefore can prevent and/or delay diabetes progression. Our findings suggest that strong molecular interactions occur between these signaling molecules, which can be used as targets for the treatment of T2DM. However, additional detailed studies are needed to determine the translation level to better understand the molecular mechanism through which PH promotes PPARγ-mediated insulin sensitivity.

Conflict of interest or competing interests

The authors declare that they have no conflicts of interest or competing interests.

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Availability of data and materials

The data supporting the findings of this study are available from the corresponding authors upon request.

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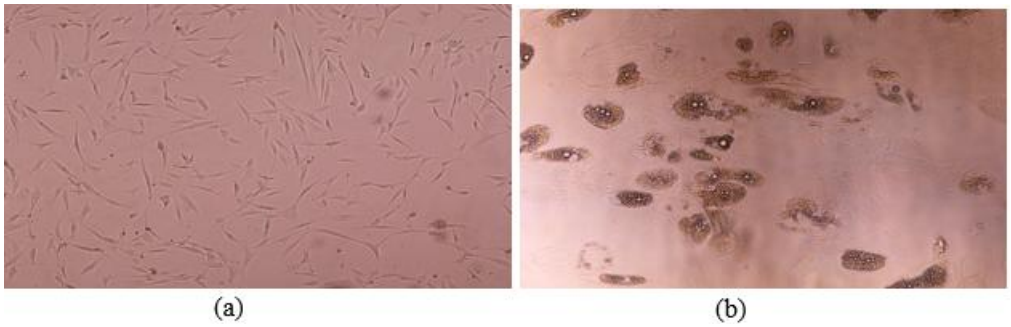
Table 1: Primer sequences and product sizes of the respective genes

Primer	Sequencs	MER	Product Sze
GAPDH	(F) 5'TCACCAGGGCTGCCATTTGC3'	20	496 bp
	(R) 5'GACTCCACGACATACTCAGC 3'	20	
PPAR-γ	(F) 5' ATCTTTCAGGGCTGCCAGTT 3'	20	200 bp
	(R) 5' AAGCCTTGGCCCTCGGATAT 3'	20	
GLUT 4	(F) 5' GATGAGAACGACTGAGGGGC 3'	20	200 bp
	(R) 5' GTCACACGAGGGGAATGAGG 3'	20	
TNF-α	(F) 5' TCAGAGGGCCTGTACCTCAT 3'	20	200 bp
	(R) 5' ATGGGCTCATACCAGGGCTT 3'	20	
IL6	(F) 5' CTTCGGTCCAGTTGCCTTCT3'	20	180 bp
	(R) 5' CTCAGGGCTGAGATGCCG3'	18	
IP3K	(F) 5' ACTGGGAAATGACCCTGCC 3'	19	370 bp
	(R) 5' GGAAGCCCGTTGGGGAC 3'	17	
FASN	(F) 5' ACGTGGGCATCAACTCCTTT 3'	20	251 bp
	(R) 5' GTAGCCACGGAAGGGCAT 3'	18	



(a) (b)

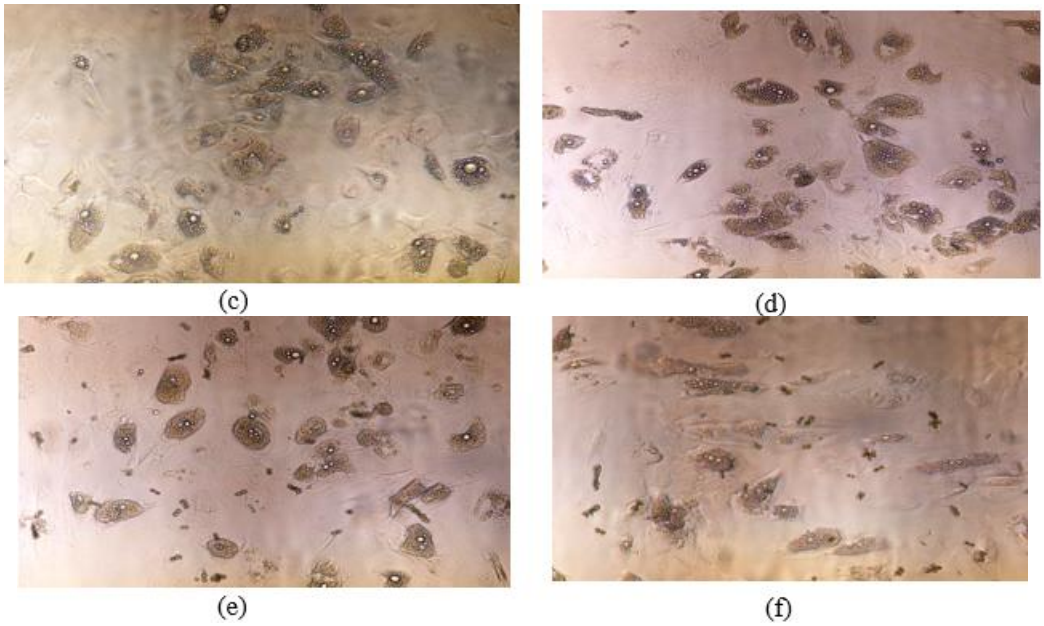


Figure 1: (a): Human mesenchymal stem cells at 5X; (b): differentiated and diabetic nontreated control adipocyte cells (10X); (c): treated with 6.25 µM (10X); (d): 12.5 µM (10X); (e): 25 µM (10X); and (f): 50 µM pioglitazone hydrochloride (10X).

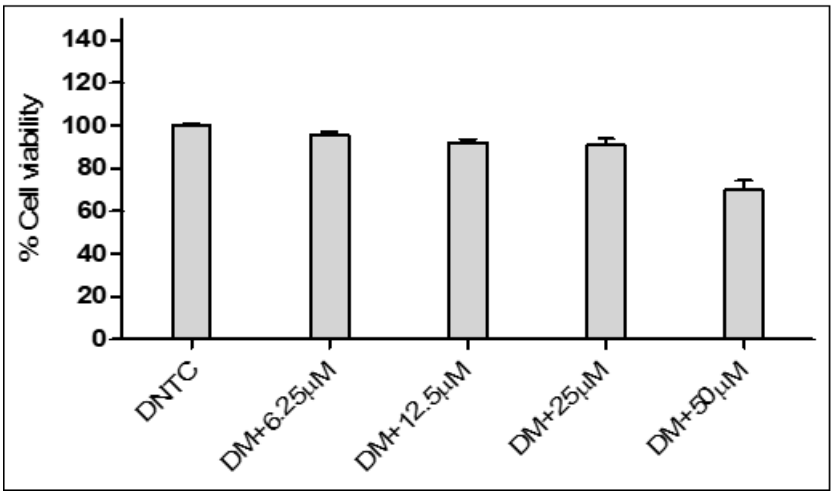


Figure 2.1

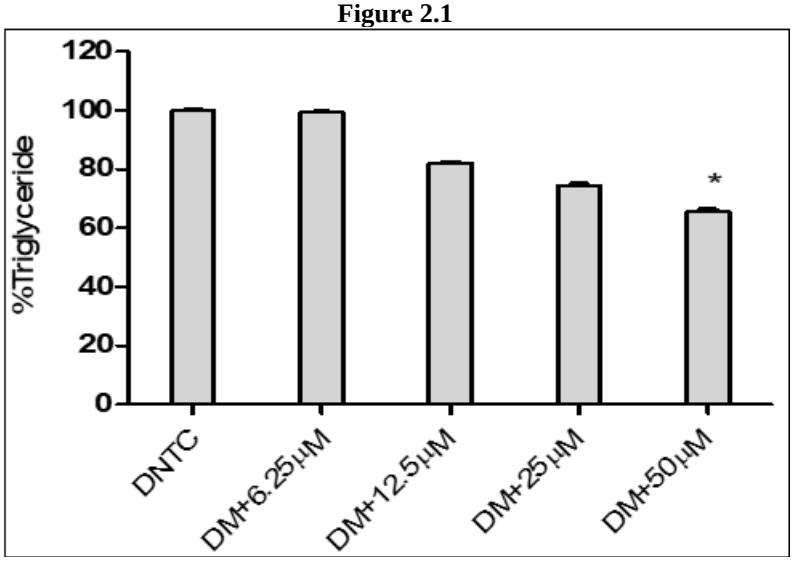


Figure 2.2

Figure 2.1 Effect of cell viability determined via the MTT assay and **Figure 2.2** Relative lipid content quantified via Oil Red O staining in human adipocytes. The results are presented as the mean value ± SEM. of five replicate measurements. Bar graphs represent the relative amounts of accumulated lipids in cultured human adipocytes after treatment with various concentrations of pioglitazone hydrochloride.

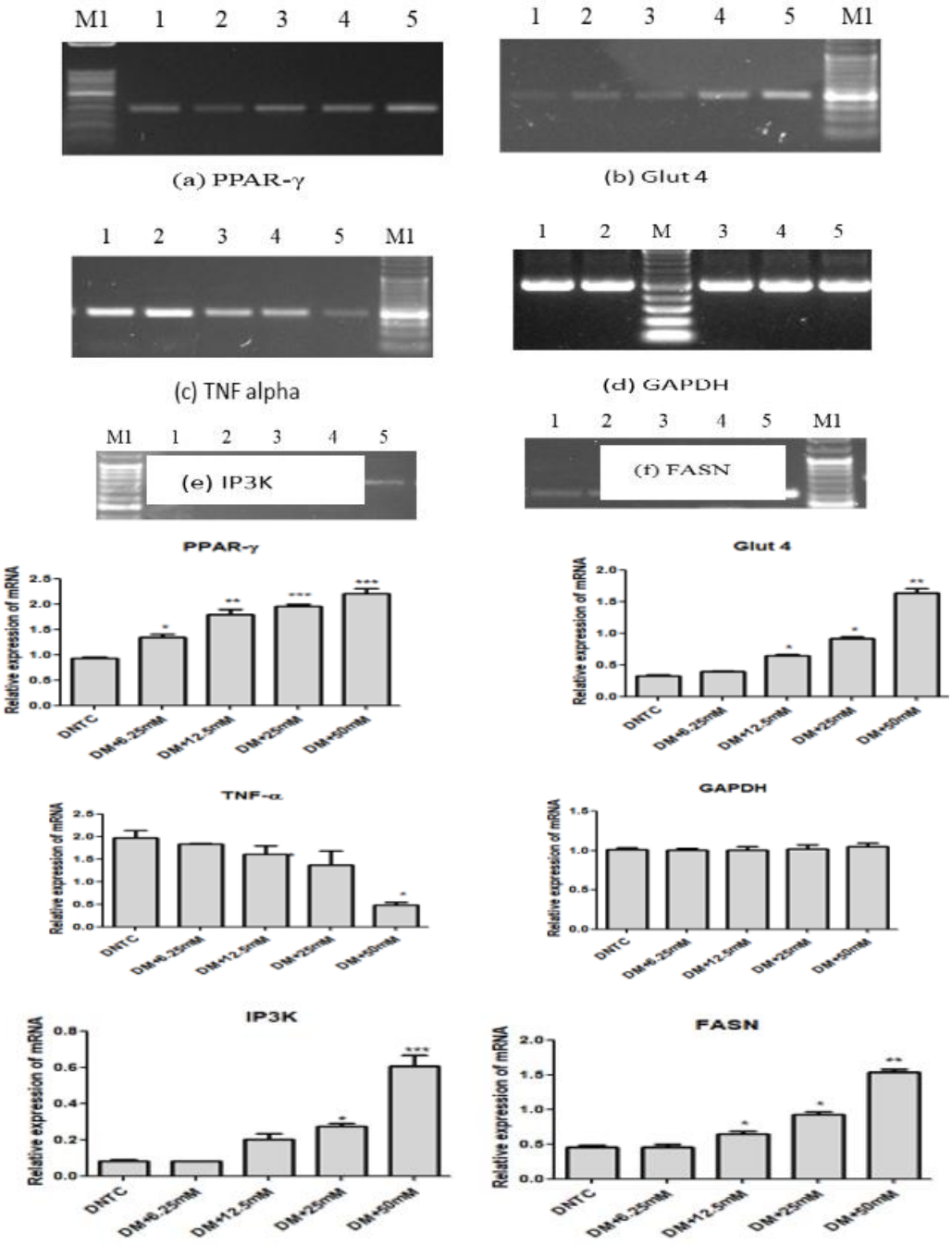


Figure 3.1: Gel images of the respective genes (a)PPAR-γ, (b) Glut4, (c) TNFalpha (d) GAPDH, (e) IP3K, and (f) FASN transcripts after treatment with different concentrations of pioglitazone hydrochloride in human adipocytes. Lane 1: DNTC, Lane 2: DM+treated with 6.25 µM, Lane 3: 12.5 µM, Lane 4: 25 µM and Lane 5: 50 µM. The expression of the transcripts was determined by reverse transcription polymerase chain reaction (PCR) and real-time PCR and the products were checked on 1.8% agarose gels, stained with ethidium bromide. M1; 50 bp, M; 100 bp ladder. For the control we used GAPDH. **Figure 3.2** Densitometry gel scanning analysis Bars represent the mean ± SEM of three independent experiments$P<0.05$ compared with DNTC. *Asterisks* (*) ($P<0.05$) denote a significant difference in gene expression compared to that in the DNTC group