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THE EFFECT OF ANTI-DIABETIC DRUGS ON THE INFLAMMATORY CYTOKINES IN HUMAN *IN VITRO* SUBCUTANEOUS ADIPOCYTES.

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ABSTRACT

Mesenchymal Stem Cells differentiated into subcutaneous adipocytes were used for the study of *in vitro* effects of antidiabetic drugs including metformin, pioglitazone and insulin on inflammatory cytokines in glucose induced diabetic human adipocytes. Glucose induction in adipocytes mimics the diabetic model *in vitro* by differentiating mesenchymal stem cells and confirmed by oil red O staining which stains lipid droplets in adipocytes. Adipocytes were incubated in growth media containing variable concentration of glucose (5-20mM) with different anti-diabetic drugs with variable concentrations. Cytokines *IL-1β, IL-6, IL-12, IL-18, TNF-α, Leptin, Visfatin* and *Adiponectin* were measured using ELISA. Student t-test (two - tailed) was used for statistical analysis. Variable fluctuations were observed in the present study in relation to cytokine secretions with anti-diabetic drugs. Finally, inflammation of cytokines decreases when metformin and pioglitazone were used in combination in *in-vitro* study. In conclusion, current data demonstrates that in whole human subcutaneous adipocytes metformin, pioglitazone and combination of both limits the regulation of inflammatory cytokines. However, pioglitazone and metformin in combination as well as insulin up-regulates *IL-1β, IL-6, IL-12, IL-18, TNF-α, Leptin, Visfatin* and *Adiponectin* protein secretion *in vitro* in mild diabetic status and highly up-regulate in severe diabetic status. This effect of anti-diabetic drugs on adipose tissue represents an additional mechanism through which this may induce

clinical benefits. Further studies are required to better understand the molecular pathway of this up-regulation in humans.

KEYWORDS: Adipocytes, diabetes, cytokines, metformin, pioglitazone, insulin.

INTRODUCTION: Adipose tissue is one of the largest cholesterol depots in the body.[1] Cholesterol is an essential molecule in animals, serving as a principal component of plasma membranes, an obligatory precursor for the biosynthesis of steroid hormones, bile acids, and bioactive oxysterols and also exerting regulatory functions. In whole body energy metabolism, two types of adipose tissue (white, WAT and brown, BAT) plays crucial role. WAT are specialized for energy storage due to their largest energy reserve triglyceride constitutes while BAT have a high capacity for energy dissipation by brown adipocyte-specific uncoupling protein 1 (UCP1) in the inner membrane of the abundant mitochondria through adaptive thermogenesis.[2] Obesity is characterized by increased mass of adipose tissue (WAT), large adipocytes and increased basal (spontaneous) lipolysis in visceral fat and is associated with inappropriate regulation of adipocyte lipolysis[3] and its metabolic complications such as cardiovascular diseases (CVD), insulin resistance and type 2 diabetes becomes a major health problems increasing throughout the world.[4]

Adipose tissue is a dynamic participant in endocrine physiology, serving as the source of secreted cytokines.[5, 6] The expression levels of adipose-derived cytokines are postulated to be risk factors for cardiovascular disease, diabetes, hypertension and other components of the metabolic syndrome.[6] Adipose tissue contains multiple cell types, including endothelial cells and macrophages, which have been associated with cytokine production.[7, 8] There is mounting evidence that adipocytes and their progenitors can be a major source of cytokines in addition to adipokines.[9-15]

Obesity represents an expansion of adipose tissue mass, and one explanation for obesity-related insulin resistance is the production of factors by adipose tissue that render some subjects more insulin resistant than others. Numerous adipocyte secretory products have recently been described that play a role in carbohydrate and lipid metabolism.[16-18] Likewise, obesity is associated with elevated levels of pro-inflammatory cytokines, such as interleukin 6 (IL-6), interleukin 8 (IL-8), and tumor necrosis factor-α (TNF-α).[19-22]

One such adipocyte secretory product is tumor necrosis factor (TNF)-α. A new role for TNF was proposed in 1993 with the description of TNF expression by adipose tissue and the elevated expression of TNF in obese, insulin resistant rodents and humans.[23-25] Another adipocyte secretory product that may be involved in insulin resistance is interleukin (IL)-6, which is a cytokine secreted by many cells, including adipocytes and adipose stromal cells.[26, 27] Like TNF, IL-6 inhibits the expression of LPL, but, unlike TNF, IL-6 does not stimulate lipolysis.[28, 29] IL-6 secretion is increased in the adipocytes of obese subjects[30] and may be important either as a circulating hormone or as a local regulator of insulin action. The potential mechanisms involved in visfatin's activity in adipose tissue has remained largely under-studied beyond its ability to activate components of the insulin signaling pathway, such as insulin receptor substrates (IRS)-1/2[31, 32], or PI3-kinase/Akt, by binding to the insulin receptor at a site distinct to insulin, itself.[31]

Studies have begun to highlight visfatin's regulation of central transcription factors, such as nuclear factor (NF)-kB and activator protein (AP)-1.[33, 34] This has addressed the potential for visfatin to elicit inflammatory responses[35], linked with elevated levels of pro-inflammatory factors, such as TNF-a and IL-6.[36-38] However, to date, the findings regarding visfatin's inflammatory role in the pathogenesis of T2DM, as well as the controlling mediators of visfatin regulation, remain unclear. In this study, we examined the effect of anti-diabetic drugs on the inflammatory cytokines *viz. IL-1β, IL-6, IL-12, IL-18, TNF-α, Leptin, Visfatin* and *Adiponectin* in human *in vitro* subcutaneous adipocytes.

MATERIALS AND METHODS

Cell culture: Wharton's Jelly Mesenchymal Stem Cells (Himedia) were cultured for 48 h in Dulbecco's modified Eagle's medium/ Ham's F12 (1:1) supplemented with 10% fetal calf serum containing antibiotics and normal glucose levels (5 mM). These cells are isolated from human umbilical cords collected post-partum. Wharton's jelly is the gelatinous connective tissue from umbilical cord and is a rich source of multipotent stem cells. Cells isolated from Wharton's Jelly are known to differentiate in to adipogenic, osteogenic, chondrogenic, cardiomyogenic lineages and dopaminergic neurons. Proliferatium medium is composed of DMEM/F-12 medium (1:1, v/v), HEPES, FBS and antibiotics. Adipocyte differentiation medium was used in differentiation phase which is optimized for adipogenic differentiation of actively proliferating human mesenchymal stem cells in vitro. Factors that potentiate adipogenic differentiation through activation of factors/regulatory enzymes like C/EBP and

peroxisome proliferator-activated receptor- γ (PPAR- γ) can be invoked by supplementing the medium with growth factors. During maintenance of adipocytes biotin and panthothenate were added. Adipocytes were maintained till the accumulation of lipid droplets. At 7 days, a vast majority of cells (more than 90%) had accumulated lipid droplets.

Oil Red O staining and cytokine estimations: Cells were stained with Oil Red O. Oil Red O belongs to a family of lipophilic or fatty acid soluble dyes. These dyes are used to demonstrate triglycerides, lipids and lipoproteins. Oil-Red-O is generally used to detect presence of fat globules i.e. identification of adipocytes within tissue or adipocytic differentiation of cells. Cultured cells were fixed in a 10% solution of formaldehyde in phosphate-buffered saline for 5min at room temperature, washed with 60% isopropanol and stained with Oil Red O solution (in 60% isopropranol) for 10 min followed by repeated washing with water (4 times with 10 ml). Stained cells were immediately viewed under phase contrast inverted microscope and images captured using a mounted digital camera. Adipocytes were incubated in growth media containg variable concentration of glucose (5-20mM) with different anti-diabetic drugs {Metformin hydrochloride, pioglitazone hydrochloride (Sigma Aldrich, USA) and insulin (HiMedia, India)} with variable concentrations as shown in **Figure 1**. Adipocytes were centrifuged at 300g for 2 min. Supernatant was used for enzyme linked immunosorbent assays (ELISA) for *IL-1β, IL-6, IL-12, IL-18, TNF-α, Leptin, Visfatin* and *Adiponectin* in human *in vitro* subcutaneous adipocytes were performed using a commercially available kit from invitrogen (USA) according to the manufacturer's instructions. All readings were recorded in triplicates.

Statistical analysis: The values were plotted using Microsoft excel. Student t-test (two - tailed) was used for statistical analysis. Differences were considered significant when P values were ≤ 0.05. Results were expressed as mean $\pm$ standard deviation.

RESULTS

Cytokine levels of *IL-1β* in untreated and treated adipocytes of variable concentration of glucose (5-20mM) with different anti-diabetic drugs have been shown in **Figure 1**. Inflammatory cytokines have been increased according to the increase in glucose concentrations including *IL-1β, IL-6, IL-12, IL-18, TNF-α, Leptin, Visfatin* and *Adiponectin* which mimics the diabetic model. However, anti-diabetic drug when included in the cells for maintaining nurture in milieu of cell we found that metformin alone (50mg/ml to 200mg/ml) did not show any significant observation in *IL-1β* level. During statistical calculation 15mM

showed significant association (P = 0.020) **(Table 1)**. Similarly pioglitazone (5ml/ml to 20mg/ml) also not showed any change at 5mM and 10mM glucose adipocytes however, at 15mM and 20mM the *IL-1β* level was suddenly decreased (P = 0.028 and 0.002 respectively) **(Table 1)**. While in combination of both metformin and pioglitazone in combination we found sudden decrease in *IL-1β* level and showed significant decrease in 5mM glucose concentration (P = 0.012) and highly significant decrease in 20mM glucose concentration (P <0.001) **(Table 1)**. Apart from this when insulin in alone is used then sudden increase in *IL-1β* level was observed and did not show any significant association except at 20mM glucose concentration (P <0.001) **(Table 1)**. However, in case of level of *IL-6* increase as the concentration of glucose increases from 5mM to 20 mM but anti diabetic drug effect was observed in all cases including metformin, pioglitazone, both in combination and as well as in insulin which decreases the level of IL-6 **(Figure 2)**. No significant was observed in case of metformin while in pioglitazone significant association was observed in 10mM and 15mM glucose concentration (P = 0.013 and 0.002 respectively) **(Table 1)**. 20mM glucose concentration showed highly significant association with pioglitazone (P <0.001) **(Table 1)**. Moreover, in combination of both the drugs and insulin alone showed highly significant observation in all the glucose concentrations (5mM, 10mM, 15mM and 20mM) (P <0.001) except insulin at 5mM (P = 0.008) **(Table 1)**. The change in level of *IL-12* have been shown in **Figure 3** and showed that the level is almost similar in low concentration of glucose while it increases as increase in glucose concentration *viz.* 15mM and 20mM. Sudden decrease in level were observed in metformin, pioglitazone and in combination of both while in insulin the level increases with the increase in insulin level **(Figure 3)**. 15mM of glucose with 150mg/ml of metformin, 5mM of glucose with 5mg/ml of pioglitazone and 15mM of glucose with 15mg/ml of pioglitazone showed significant association (P = 0.007, 0.027 and 0.009 respectively) **(Table1)**. However, metformin and pioglitazone and insulin in 20mM of glucose showed highly significant association in our study (P <0.001) **(Table 1)**. Similar variations have been observed in level of *IL-18* **(Figure 4)**. Significant association were observed in 15mM glucose with metformin 150mg/ml, 20mM glucose with 20mg/ml pioglitazone and 5mM glucose with 50mg/ml metformin + 5mg/ml pioglitazone (P = 0.020, 0.002, 0.003 respectively) **(Table 1)**. In 20mM glucose both drugs in combination and insulin alone showed highly significant association **(Table 1)**. *TNF-α* level also showed similar variation as seen in *IL-12* and *IL-18* **(Figure 5)**. Significant associations were observed in 15mM glucose with metformin and pioglitazone (P = 0.015 and 0.020) while in 20mM glucose with pioglitazone, both metformin and pioglitazone incombination and insulin

showed significant association (P = 0.034, 0.003 and 0.002 respectively) **(Table 1)**. Moreover, levels of leptin, visfatin and adiponectin showed different patterns **(Figure 6, 7 & 8)**. In all three cases level initially decreases in 5mM glucose and 10 mM glucose then increases in higher glucose concentrations of 15mM and 20mM with respect to drugs treatments viz. metformin, pioglitazone, metformin + pioglitazone and insulin. Leptin, visfatin and adiponectin levels increases in insulin treatment as compared to untreated controls adipocytes. Leptin showed significant association with 15mM glucose with 150mg/ml metformin, 5mM, 15mM and 20mM glucose with 5, 15 and 20mg/ml pioglitazone respectively (P = 0.007, 0.032, 0.010 and 0.045 respectively) **(Table 1)**. While in 20mM glucose with both drugsin combination and with insulin showed highly significant association (P <0.001) **(Table 1)**. Visfatin level showed significant association in 15mM glucose with 150mg/ml metformin and 5mM glucose with 50mg/ml metformin + 5mg/ml pioglitazone (P = 0.011 and 0.008). In 20mM glucose all drugs and their combinations showed highly significant association except with 200mg/ml metformin (P <0.001) **(Table 1)**. Moreover, in adiponectin level 15mM glucose with all drugs showed significant association and also in 20mM glucose except with metformin. 5mM glucose with pioglitazone 5mg/ml alone showed significant association **(Table 1)**. Finally, inflammation of cytokines decreases when metformin and pioglitazone were used in combination in *in-vitro* study.

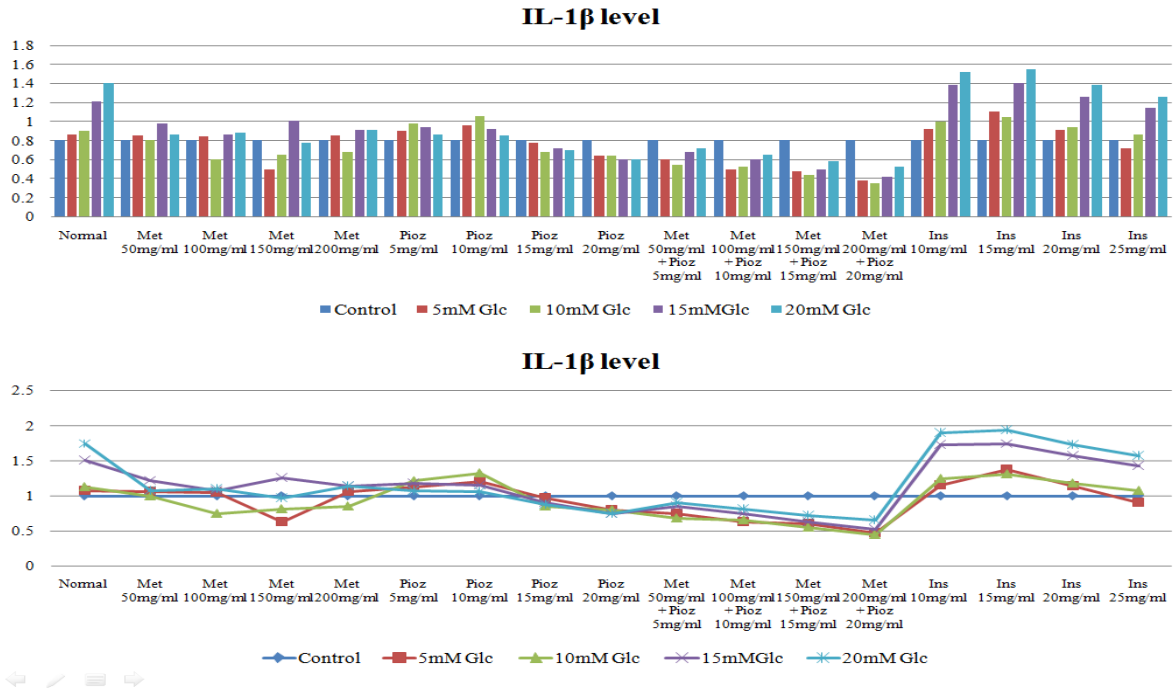

Figure 1: Cytokine level of *IL-1β* in untreated and treated adipocytes of variable concentration of glucose (5-20mM) with different anti-diabetic drugs.

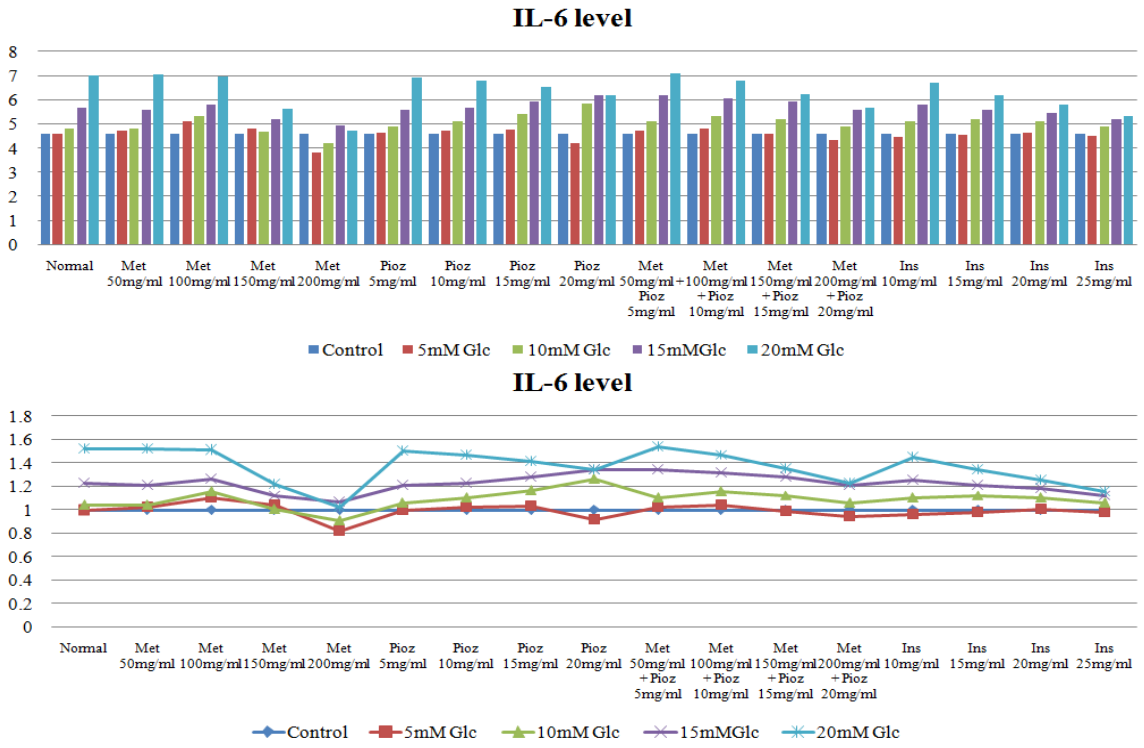

Figure 2: Cytokine level of *IL-6* in untreated and treated adipocytes of variable concentration of glucose (5-20mM) with different anti-diabetic drugs.

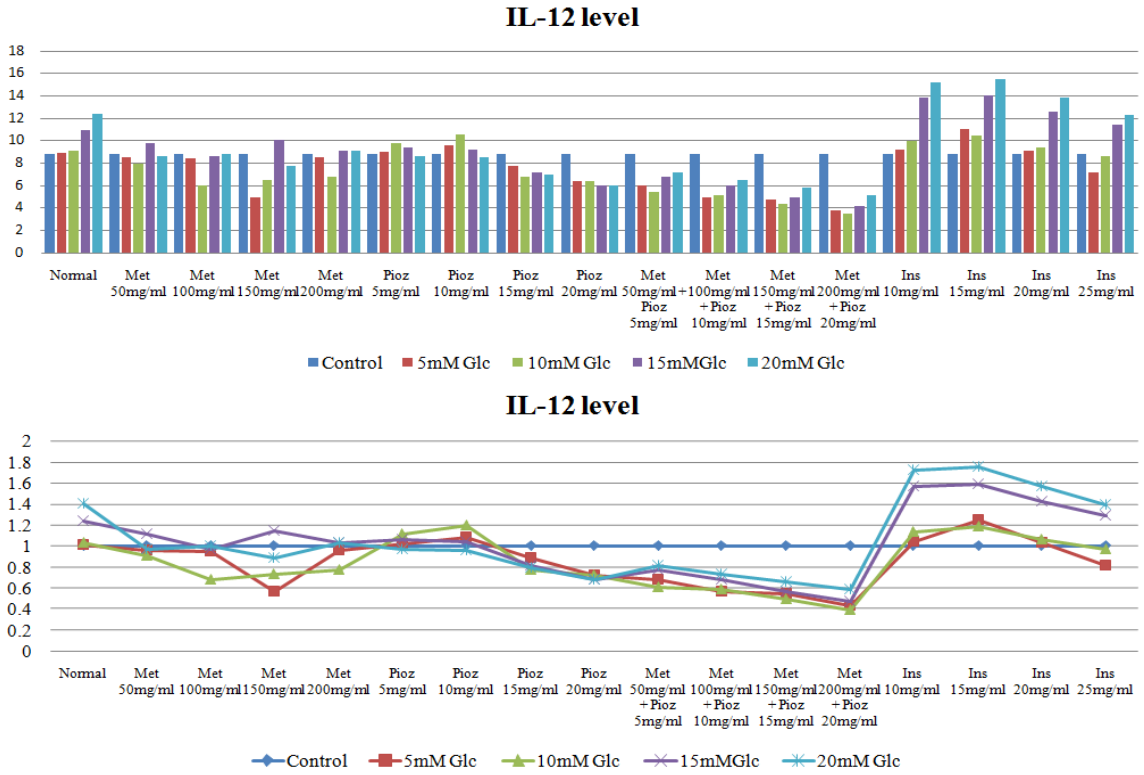

Figure 3: Cytokine level of *IL-12* in untreated and treated adipocytes of variable concentration of glucose (5-20mM) with different anti-diabetic drugs.

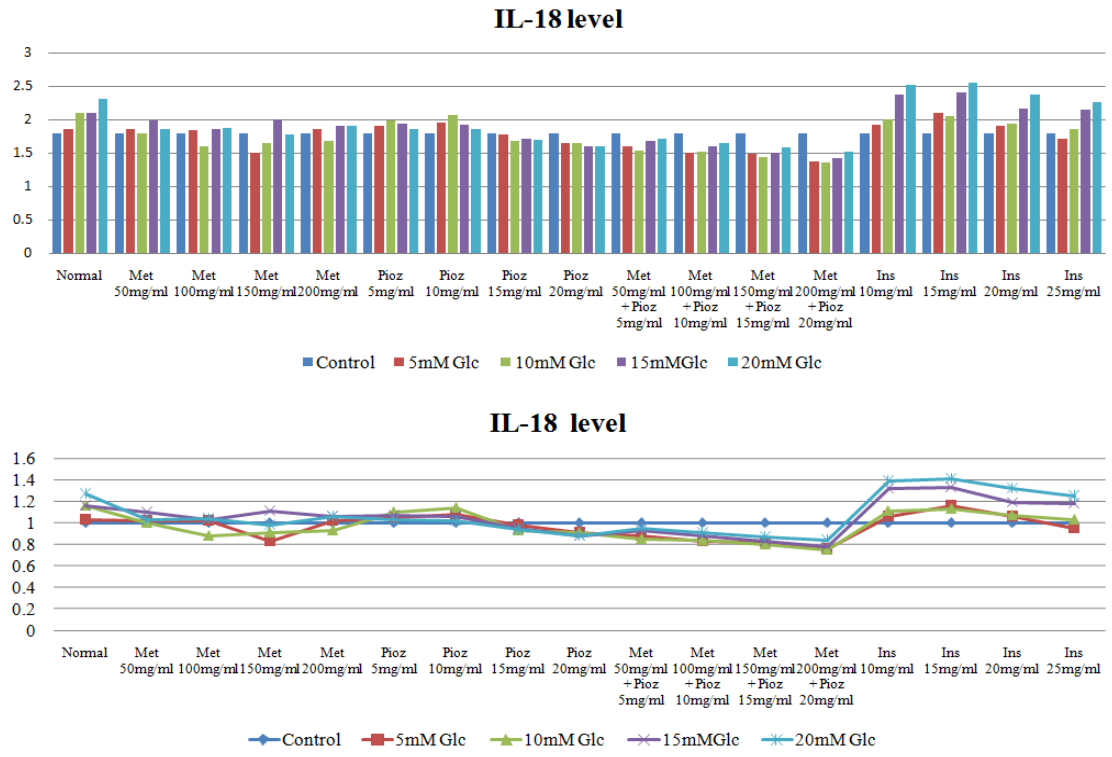

Figure 4: Cytokine level of *IL-18* in untreated and treated adipocytes of variable concentration of glucose (5-20mM) with different anti-diabetic drugs.

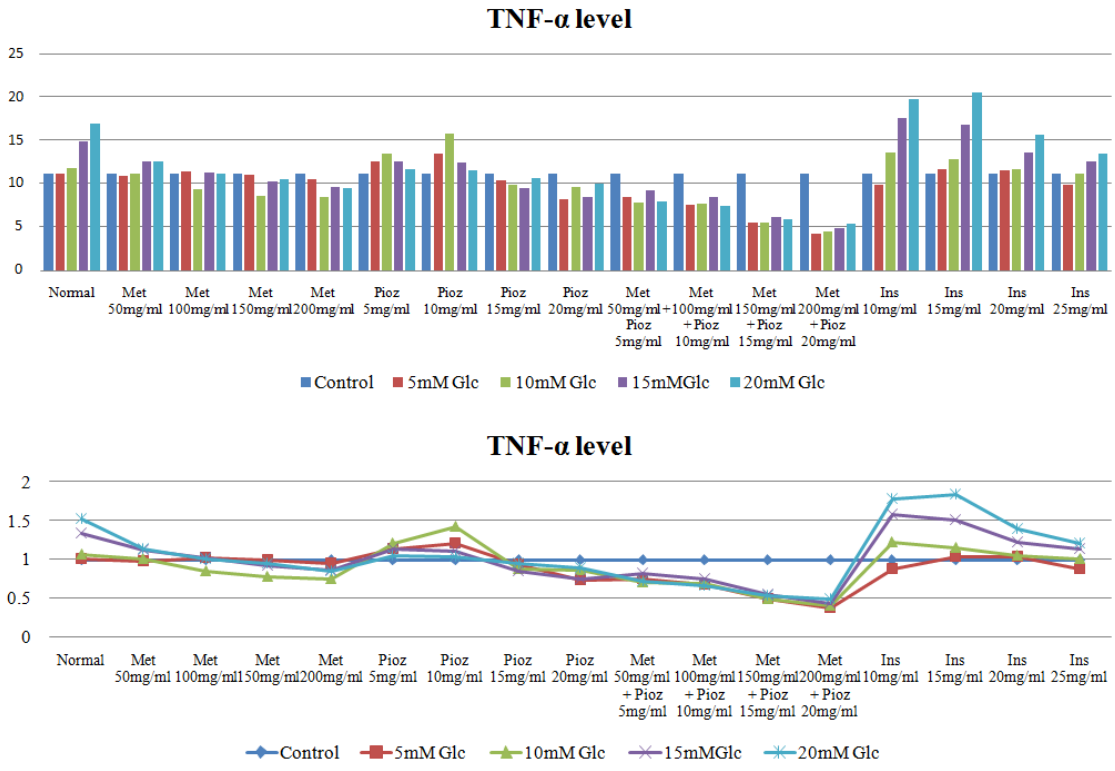

Figure 5: Cytokine level of *TNF-α* in untreated and treated adipocytes of variable concentration of glucose (5-20mM) with different anti-diabetic drugs.

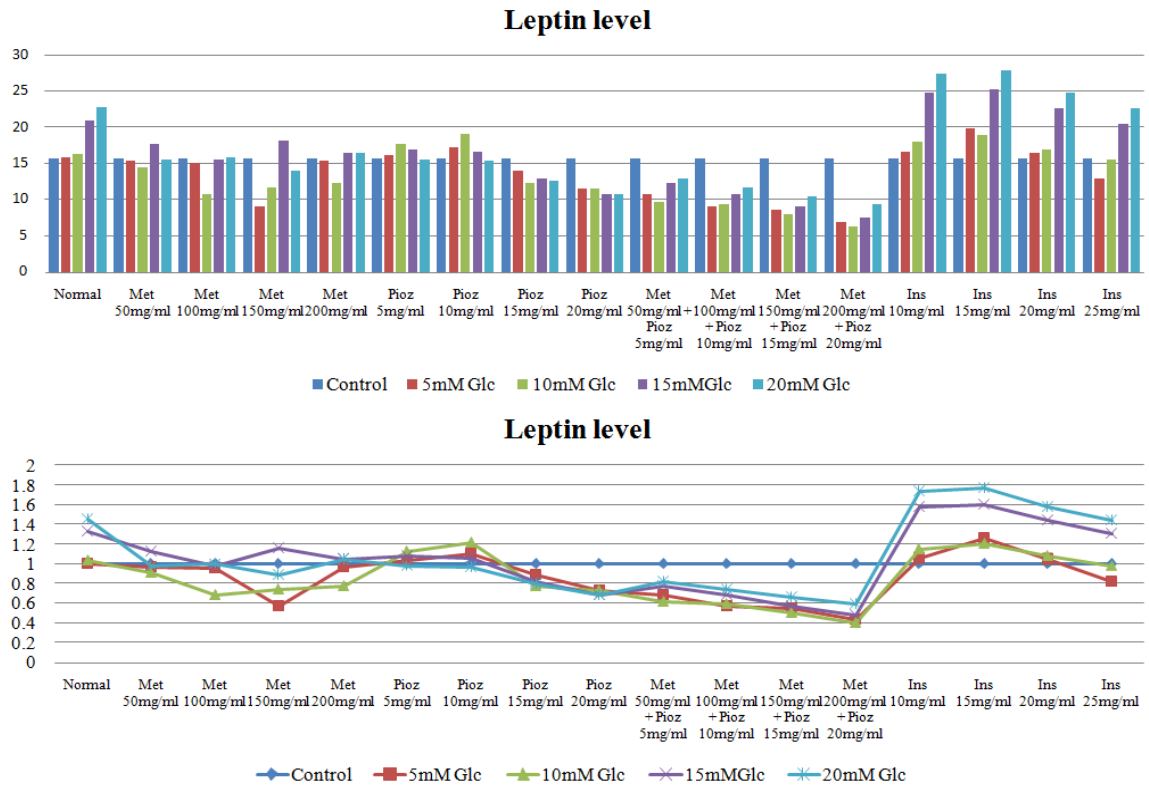

Figure 6: Cytokine level of *Leptin* in untreated and treated adipocytes of variable concentration of glucose (5-20mM) with different anti-diabetic drugs.

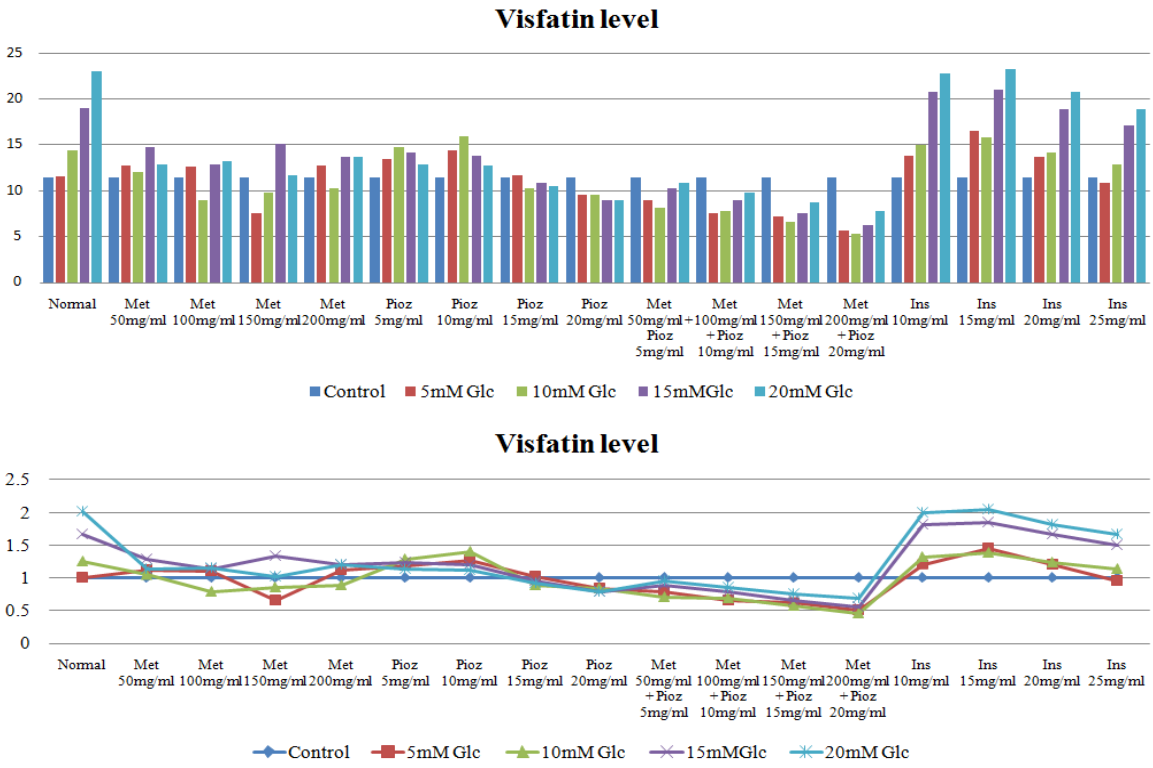

Figure 7: Cytokine level of *Visfatin* in untreated and treated adipocytes of variable concentration of glucose (5-20mM) with different anti-diabetic drugs.

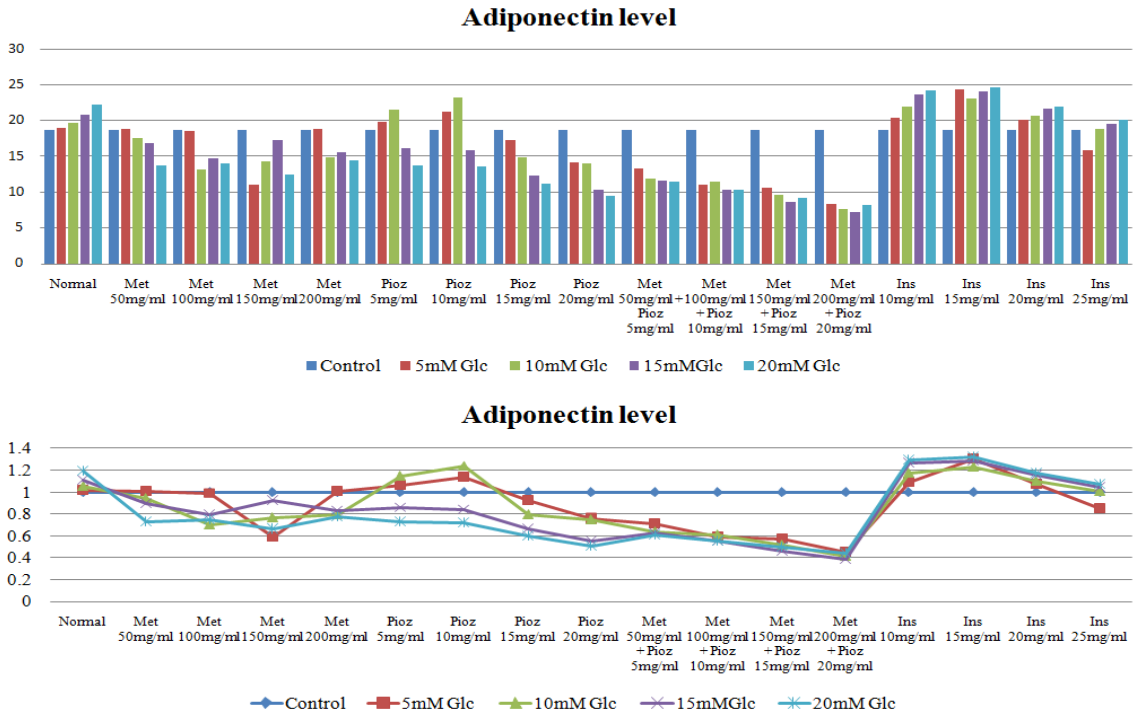

Figure 8: Cytokine level of *Adiponectin* in untreated and treated adipocytes of variable concentration of glucose (5-20mM) with different anti-diabetic drugs.

Table 1: Cytokine levels (*IL-1β, IL-6, IL-12, IL-18, TNF-α, Leptin, Visfatin* and *Adiponectin)* in untreated and treated adipocytes of variable concentration of glucose (5-20mM) with different anti-diabetic drugs showing P-values with change in fold of levels.

	Cytokine	*Metformin*	*Pioglitazone*	*Metformin+ Pioglitazone*	*Insulin*
5mM Glucose	***IL-1β***	0.783	0.212	**0.012**	0.172
	IL-6	0.986	0.418	**<0.001**	**0.008**
	IL-12	0.228	**0.027**	0.056	0.516
	IL-18	0.783	0.715	**0.003**	0.148
	TNF-α	0.489	0.112	0.546	0.449
	Leptin	0.254	**0.032**	0.059	0.444
	Visfatin	0.977	0.740	**0.008**	0.127
	Adiponectin	0.379	**0.050**	0.155	0.097
10mM Glucose	***IL-1β***	0.627	0.547	0.476	0.570
	IL-6	0.812	**0.013**	**<0.001**	**<0.001**
	IL-12	0.439	0.762	0.784	0.790
	IL-18	0.627	0.370	0.542	0.619
	TNF-α	0.941	0.428	0.707	0.432
	Leptin	0.500	0.833	0.976	0.892
	Visfatin	0.396	0.256	0.286	0.388
	Adiponectin	0.764	0.999	0.079	0.068
15mM Glucose	***IL-1β***	**0.020**	**0.028**	0.421	0.875
	IL-6	0.960	**0.002**	**<0.001**	**<0.001**

	IL-12	**0.007**	**0.009**	0.098	0.316
	IL-18	**0.020**	0.150	0.271	0.752
	TNF-α	**0.015**	**0.020**	0.203	0.288
	Leptin	**0.007**	**0.010**	0.164	0.382
	Visfatin	**0.011**	0.094	0.669	0.829
	Adiponectin	**0.009**	**0.012**	**0.019**	**0.037**
20mM Glucose	***IL-1β***	0.133	**0.002**	**<0.001**	**<0.001**
	IL-6	0.090	**<0.001**	**<0.001**	**<0.001**
	IL-12	0.655	0.063	**<0.001**	**<0.001**
	IL-18	0.133	**0.002**	**<0.001**	**<0.001**
	TNF-α	0.454	**0.034**	**0.003**	**0.002**
	Leptin	0.585	**0.045**	**<0.001**	**<0.001**
	Visfatin	0.101	**<0.001**	**<0.001**	**<0.001**
	Adiponectin	0.379	**0.021**	**0.005**	**0.001**

DISCUSSION

In the present study, we assessed the effects of anti-diabetic drugs viz. metformin, pioglitazone and insulin on adipose tissue in relation to inflammatory effects. Glucose introduction to the adipocytes mimics the diabetic model *in vitro*. In subcutaneous adipocytes, we observed a stimulating effect of metformin on *IL-1β, IL-6, IL-12, IL-18, TNF-α, Leptin, Visfatin* and *Adiponectin* secretion. The data suggests that subcutaneous adipocytes has a greater sensitivity to metformin action. This finding is consistent with the demonstration that subcutaneous adipocytes is the only fat depot capable to secrete *IL-1β, IL-6, IL-12, IL-18, TNF-α, Leptin, Visfatin* and *Adiponectin* under treatment with another insulin-sensitising agent, pioglitazone.[39] The lack of intracellular adiponectin accumulation suggests that metformin is able to up-regulate adiponectin release by modulating posttranslational events. Supporting this hypothesis, metformin potentiates rosiglitazone-induced decrease in endoplasmic reticulum retention of ERp44 protein[40] that, together with the release protein Erol-1α, modulates intracellular adiponectin content and its secretion.[41, 42]

In contrast to what is observed in subcutaneous adiposytes, metformin did not modify adiponectin secretion in adipocytes differentiated in vitro. Thus, it could be hypothesised that the non-adipose components of subcutaneous adiposytes (i.e. preadipocytes, fibroblasts, infiltrating macrophages, lymphocytes, endothelial cells, etc.) participate in the regulation of metformin effects on adiponectin production and secretion. This hypothesis agrees with the inability of metformin therapy to modify in vivo adiponectin content and secretion in a model of isolated adipocytes of diabetic patients.[43] The possible role of the 'non-adipose components' in the modulation of adiponectin synthesis in subcutaneous adiposytes is also supported by our in vitro results. This finding adds evidence to the anti-inflammatory

properties of metformin that have been demonstrated in vitro in human vascular smooth muscle cells, macrophages and endothelial cells, and in vivo in diabetic and non-diabetic subjects.[44-47] Interestingly, addition of metformin to lifestyle intervention provided more metabolic benefits than lifestyle intervention and placebo. It could therefore hypothesised that metformin merely influences tissue availability of adiponectin through a decrease in the local inflammatory state. The discrepancy observed in different experimental conditions between adiponectin expression and release as well as serum levels demonstrates that the molecular mechanisms by which adiponectin is synthesized and secreted are complex and still far from being understood.[48-50] Pioglitazone increased the gene expression of several factors involved in mitochondrial biogenesis. Pioglitazone increased the expression of genes required for fatty acid oxidation, including PPAR-γ, medium-chain acyl-CoA dehydrogenase, carnitine palmitoylotransferase I and malonyl-CoA decarboxylase. The expression of homolog implicated in the regulation of fatty acid oxidation tended to be higher after pioglitazone treatment.[51] However, in the present report it has been shown that pioglitazone inclusion down regulate the inflammatory cytokine secretions. Moreover, in insulin addition as reported in previous reports on the effects of insulin on the metabolism of human adipose tissue the concentrations of glucose used in the incubation media have varied over a wide range. Whereas high levels of glucose do not appear to depress the insulin-induced acceleration of glucose uptake by adipose tissue. In our result we have investigated the inflammatory cytokine secretions has increased by the uptake of high glucose with insulin in adipocytes. In conclusion, current data demonstrates that in whole human subcutaneous adipocytes metformin, pioglitazone and combination of both limits the regulation of inflammatory cytokines. However, pioglitazone and metformin in combination as well as insulin up-regulates *IL-1β, IL-6, IL-12, IL-18, TNF-α, Leptin, Visfatin* and *Adiponectin* protein secretion in vitro in mild diabetic status and highly up-regulate in severe diabetic status. This effect of anti-diabetic drugs on adipose tissue represents an additional mechanism through which this may induce clinical benefits. Further studies are required to better understand the molecular pathway of this up-regulation in humans.

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