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EFFECTS OF METFORMIN ALONE, METFORMIN WITH PIOGLITAZONE ON 1,5ANHYDROGLUCITOL, ADIPONECTIN, GHRELIN IN NEWLY DIAGNOSED TYPE 2 DIABETIC PATIENTS

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ABSTRACT

Diabetes mellitus type 2 (T2DM) is a metabolic disorder characterized by a high blood glucose in the context of insulin resistance and relative insulin deficiency.T2DM associated with increased breakdown of lipids within fat cells, resistance to and lack of incretin, an intestinal peptide for insulin secretion ,high glucagon levels in the blood, low adiponectin hormone level(ADP) which is an amino acid collagen-like protein that is secreted by adipocytes to acts as a hormone with anti-inflammatory and insulin-sensitizing properties and to be involved in cardiovascular tone, T2DM also related to a paradoxical decrease in circulating ghrelin levels (GHRL) which is an amino acid peptide hormone that has been identified as a potent growth-hormone secretagogue. Low ghrelin levels are associated with high levels of insulin and insulin resistance. T2DM associated with reduced level of 1,5-AG, which is 1-deoxy form of glucose, is a validated marker of short-term glycemic control. Metformin is the most recommended euglycemicmonotherapy of T2DM belonging to Biguanides group .Pioglitazone is an insulin sensitizer drug belonging to Thiazolidindione group. The aim of this study was to investigate the effects of metformin alone, metformin with pioglitazone on fasting serum 1, 5AG, adiponecitin, ghrelin.Standard kits were used to measure biochemical profiles suggested in this study using double-sandwich ELISA technique.Tests were performed and interpreted following instruction outlined in each kit.The study included newly or recently diagnosed (≤ 1year) male and female patients with T2DM whose ages ranged between 25 and 70 years. The enrolled patients were divided into two groups, Group 1: consisted of 32 patients, treated by oral metformimover a period of 12 weeks, which is the period of the study.Group 2: consisted of 30 patients treated by metformin plus pioglitazone. There after each patient was submitted to have fasting serum adiponectin,ghrelin and 1,5AG,all parameters were measured initially, before any intervention, and later on at two steps, the 6th and the 12th week of the study time. After 12 weeks of treatment with metformin alone, metformin with pioglitazone there was significant improvement in 1,5AG (p 0.010, 0.017) respectively. A significant rise in ADP and GHRL level noticed in metformin treated group (p value 0.041,0.050) respectively. while metformin with pioglitazone showed more significant increase of GHRL (p 0.037) and only apparent insignificant rise in ADP level (p 0.053). As a conclusion metformin, metformin with pioglitazone associated with statistically significant improvement in 1,5AG level. Metformin associated witha significant rise in ADP and GHRL hormone level in blood . However, metformin with pioglitazone was more effective in elevation of GHRL hormone blood level.

KEY WORDS

Adiponectin, 1,5Anhydroglucitol, Ghrelin

INTRODUCTION

Type 2 DM (T2DM) is a metabolic disorder characterised by a high blood glucose in the context of insulin resistance and relative insulin deficiency[1]. Pathogenesis of T2DM ranging from predominantly insulin resistance with relative insulin deficiency to predominantly an insulin secretory defect with insulin resistance, which accounts for ~90–95% of those with diabetes [2].

Metformin has been the most recommended monotherapy of T2DM and it is termed "euglycemic" agents beyond toBiguanides group [3]. The proposed mechanisms of action include: first; reduced hepatic and renal gluconeogenesis. Second; slowing of glucose absorption from the gastrointestinal tract. Third; increased glucose to lactate conversion by enterocytes. Fourth; direct stimulation of glycolysis in tissues, with increased glucose removal from blood. Fifth; reduction of plasma glucagon levels [4].

Maida *et al* [5] have indeed recently reported that metformin acutely increases plasma levels of glucagon-like peptide 1 (GLP-1)thatis a member of the incretin family of peptide hormones augment glucose-stimulated insulin release from pancreatic β-cells, retard gastric emptying, inhibit glucagon secretion, and produce a feeling of satiety and induces islet incretin receptor gene expression through a mechanism that is dependent on peroxisome proliferator-activated receptor (PPAR)-α .

Zhou *et al* [6] reported that the activation of Adenosinemonophosphate AMP-activated protein kinase (AMPK) by metformin in the liver, and probably in other tissue provides a unified explanation for the pleiotropic beneficial effects of this drug.

Pioglitazone acts to decrease insulin resistance. Their primary action is the regulation of genes involved in glucose and lipid metabolism and adipocyte differentiation [7].Pioglitazone is legends of peroxisome proliferator-activated gamma receptor (PPAR-γ), a part of the steroid and thyroid super-family of nuclear receptors [8]. In persons with diabetes, a major site of action of pioglitazone is adipose tissue, mediated through PPAR alpha, where the drug promotes glucose uptake and utilization by increasing glucose co-transporters 1 and 4 and modulates synthesis of lipid hormones or cytokines and other proteins involved in energy regulation. Also it regulates adipocyte apoptosis and differentiation. In lipid tissues, pioglitazone, also lower free fatty acids, enhanced insulin signaling, reduced tumor necrosis factor alpha (TNF alpha) and remodeling of adipose tissue. Together, these can increase glucose uptake and utilization in the peripheral organs and decrease gluconeogenesis in the liver, thereby reducing insulin resistance [9].

1,5Anhydroglucitol (1,5AG) was first discovered in the plant family *Polygala senega*in 1888. The presence of the compound in human blood [10] and cerebrospinal fluid [11] was established in 1972 and 1973, respectively.1,5-AG, is 1-deoxy form of glucose, is a majormetabolically inert circulating polyol arising primarily from ingestion and excreted competitively with glucose [12].Research studies have shown that 1,5AG originates mostly from foods with a mean daily intake of ~4.4 mg/day. The rate of intake is matched by the rate of daily excretion. A bodily pool of about 500–1000 mg of 1,5AG is constantly maintained [13] and this body pool may originate from an accumulation of small amounts of retained dietary 1,5AG or from biosynthesis . Dietary variation does not appreciably affect the efficacy of such measurements because the content of 1,5AG is similar in various starches, meats, seafood, vegetables, fruits and beverages. Only raw soybeans have been demonstrated to have

significantly enriched levels of 1,5AG, although processed soybeans (*e.g.,* soy sauce) have 1,5AG content essentially equivalent to all other starches [13].1,5AG is a validated marker of short-term glycemic control [12].

1,5AG levels in blood respond within 24 h as a result of glucose's competitive inhibition of 1,5AG reabsorption in the kidney tubule, since reabsorption of filtered 1,5AG in the proximal tubule is competitively inhibited by glucose ,so it is an indicator to identify rapid changes in hyperglycemia [14], when glucose levels rise, even transiently, urinary loss of 1,5AG occurs, and circulating levels fall [15].

1,5AG levels were significantly correlated with fasting plasma glucose, fructoseamine level and HbA1c were it being a useful adjunct to glycatedhaemoglobin for blood glucose monitoring in patients with diabetes [13], but1,5AG level was highly specific and a decreased level indicated diabetes mellitus because of its high sensitivity. According to the selectivity index (sensitivity value times specificity value), 1,5AG determinations were superior to HbA1c measurements for diabetes screening [15].

Adiponectin(ADP) is a 244–amino acid collagen-like protein that is solely secreted by adipocytes to acts as a hormone with anti-inflammatory and insulin-sensitizing properties [16] and to be involved in cardiovascular tone [17].

Obesity caused down-regulation of adiponectin which is the mechanism whereby obesity could cause cardiovascular diseases, insulin resistance and diabetes [16].The high molecular weight oligomer of ADP has been implicated as the major active form responsible for the insulin-sensitizing effects of adiponectin in the liver and peripherally than total adiponectin levels [18].

Adiponectin may decrease the risk of T2DM, including suppression of hepatic gluconeogenesis, stimulation of fatty acid oxidation in the liver, stimulation of fatty acid oxidation and glucose uptake in skeletal muscle, and stimulation of insulin secretion [16][19] . These effects may be partly mediated by stimulatory effects of adiponectin on signaling pathways for 5' AMP–activated protein kinase and PPAR-α.

Adiponectin receptors have been cloned in the skeletal muscle (AdipoR1) and liver (AdipoR2), which appear to comprise a novel cell-surface receptor family. AdipoR1 and AdipoR2 have been shown to mediate AMP-activated protein kinase, peroxisome proliferator-Activated receptor-alpha (PPAR-α) ligand activities, glucose uptake and fatty-acid oxidation by adiponectin [20].

In vascular endothelium, adiponectin decreases monocyte adhesion to endothelium, suppresses macrophage–to–foam cell transformation, and inhibits vascular smooth muscle cell proliferation and migration [16].

Based on the observation that various drug classes exert beneficial effects on insulin resistance partly by increasing plasma adiponectin levels, it could be hypothesized that substances that enhance or mimic adiponectin to activate its receptors and/or post receptor signaling pathway may be a promising therapeutic strategy in the prevention and treatment of diabetes [21].

Ghrelin is a 28 amino acid peptide hormone that has been identified as a potent growth-hormone secretagogue [22]. It is secreted by many tissues, but its main source is the gastric mucosa [23][24].

Ghrelin is a target for post-translational modifications, which results in two different forms in plasma and stomach: acylated ghrelin (AG) form (with *n*-octanoyl modification at Ser3) and desacyl-ghrelin (DG) form (without acylation) [25].A relative excess of AG

compared to DG has been reported in insulin resistance and related conditions [24]. Ghrelin is a gut hormone that is secreted to the blood stream .In all vertebrate species, ghrelin is mainly produced in the stomach [26].

The GHS-R is a G protein-coupled receptor that binds ghrelin and acts on the pituitary gland and hypothalamus to stimulate (GH) release [27]. G-protein ($G\alpha_{i2}$-subunit) mediated signaling is important for the action of ghrelin to suppress glucose-induced cytosolic Ca^{2+} concentration $[Ca^{2+}]_i$ increase and insulin release and that theghrelin-induced attenuation of $[Ca^{2+}]$ and activation of voltage-dependent K^+ Channel [28].

Reduction of electrical activity in β-cells of the pancreas is another possible mechanism of action for ghrelin. Low ghrelin levels are associated with high levels of insulin and insulin resistance [29].Insulin is shown to inhibit ghrelin secretion in healthy normal-weight and overweight persons [30]. Insulin is associated negatively with total ghrelin and DG concentrations while AG had positive association [31].Physiological increases in insulin levels may play a key role in regulating postprandial plasma ghrelin concentrations, since meal-induced ghrelin suppression is absent in severe insulin deficiency [32].

The main physiological functions of ghrelinare regulation of pituitary hormone secretionand energy homeostasis,regulation of gastrointestinal motility and secretion,regulation of glucose homeostasisand cardiovascular functionby promoting vascular endothelial cell function. The ability of ghrelin to promote cardiac microvascular endothelial cell (CMEC) proliferation, migration and nitric oxide (NO) secretion underlie this effect [33]. Also it increases cardiac index and stroke volume, decreases blood pressure and prevents left ventricular remodeling [34].

PATIENTS AND METHODS

This study is a randomized, single blinded interventional ,dose escalation study of 12 weeks treatment duration., comparative and prospective study.The study was conducted on adult patients with T2DM attending a Diabetic and Endocrine Diseases Clinic over a period of one year.

Study concept and design were approved by Ethical Committee at AL-Nahrain College of Medicine in AL-Nahrain University-Iraq.

The study included newly or recently diagnosed (≤ 1year) male and female patients with T2DM whose ages ranged between 25 and 70 years.

The study excluded patients who were known to have hepato-biliary disease, hypothyroidism, chronic kidney disease andnephrotic syndrome. Cigarette smoking, the use of any glucose altering medications, such as oral contraceptive, diuretics, steroids and neuroleptics during the last month. In addition, pregnant or lactating women, patients with hematological abnormalities.

To have an idea about the normal values of study and in order to assess how much the drugs used in the study were able to normalize the abnormal parameters, other 30, apparently healthy, volunteers whose age matched enrolled patients were involved. Their data were obtained, manipulated, tabulated and analyzed in the same way as the study patients.

All the enrolled participants (n. 92) were informed about the aim of the study and an oral consent was obtained from each of them. Thereafter, the patients were divided into two groups as follow:

Group 1: consisted of 32 patients, treated by oral *metformim alone* (merk-Germany), 500 mg b.d initially and the dose was adjusted according to the HbA1c and FSG readings over a period of 12 weeks, which is the period of the study.

Group 2: consisted of 30 patients treated by *metformin plus pioglitazone* (SPECIFAR PHARMACEUTICALS, Athens-Greece). Pioglitazone dose was adjusted according to glycemic response, either 15mg bid. or 30mg once daily.

There after each patient was submitted to havefasting serum adiponectin , fasting serum ghrelin, fasting serum 1,5AG, were measured. Standard kits were used to measure biochemical profiles suggested in this study using double-sandwich ELISA technique, tests were performed and interpreted following instruction outlined in each kit.

An initial physical examination was conducted andall the above parameters were assessed initially, before any intervention, and later on at two steps, the 6th and the 12th week of the study time. The data were recorded in specially preformed case record.

Statistical Analysis After data gathering, the data were tabulated, organized and introduced into a computer file. The statistical analysis was carried out using Statistical Package for the Social Science (SPSS); version 21. Descriptive statistic; mean ±standard deviation (±SD), was used to describe numerical values [35]. The differences between the means were considered significant at the 5% confidence level and the level of significance was set at $p<0.05$, $p<0.01$ and $p<0.001$ as significant, highly significant and very highly significant respectively.

The inferential statistics; one way analysis of variance (ANOVA) followed by T test comparison *t*-test for one sample was used to compare between parameters within treatment groups and to compare the same parameter with its analogue in other treatment groups. Independent *t*-test was used to compare between the results of studied parameters obtained at the 12th week from treatment for each group with their corresponding at the controls to identify which parameter approaches more the normal value.

RESULTS

The patients were 92; 63 males and 29 females. Their ages ranged between 33 and 70 years with a mean age ±SD (49.5±7.93) for females and (46.5±11.57) for males. The BMI on initial visit in general was (33.51±5.85).

After 12 weeks of treatment with metformin alone, there was significant improvement in 1,5AG with a mean±SD before and after treatment was (7.4±1.5→ 10.5±0.7). A significant rise in ADP and GHRL, with mean±SD was (9.0±3.6→18.3±4.3) for ADP and (104.9 ± 28.5→252.9 ±37) for GHRL (Table .1)

Table 1 : Effect of treatment by metformin on HbA1c,1-5 AG, ADP, GHRL from baseline and at the 6th and 12th week of treatment

Drug	Duration	1-5AG	ADP	GHRL
Met.	**Base line**	7.4 ± 1.5	9.0 ± 3.6	104.9 ± 28.5
	Week 6	9.2 ± 1.5	12.1 ± 3.9	172.1 ± 33.4
	Week 12	10.5 ± 0.7	18.3 ± 4.3	252.9 ± 37.7
***p* value (t-test)**		0.010	0.041	0.050

On comparing the results of metformin at the 12th week with the control group, the parameters didn't approach the control group values (Table. 2)

Table .2 Comparison between metformin group and *control group* in regard to 1-5 AG, ADP, GHRL at the 12th week of treatment.

Drug	1-5AG	ADP	GHRL
Met.	10.5 ±0.7	18.3 ± 4.3	252.9 ± 37.7
Control	25.9 ±8.6	23.8 ± 4.3	305.9 ± 52.9
***p* value (independent Samples t-test)**	0.000	0.000	0.000

Using metformin with pioglitazone for 12 weeks showed a significant improvement in 1,5AG with a mean±SD before and after treatment was (7.3±1.8→11.5±1.3).A significant increase of GHRL (106.3±21.8→213.9±33.2)was noticed with an apparent increase in ADP (8.7±1.1→21.4±3.6) but this increase was statistically insignificant (Table. 3).

Table .3 Effect of treatment by metformin with pioglitazone on 1-5 AG, ADP, GHRL from baseline and at the 6th and 12th week of treatment.

Drug	Duration	1-5AG	ADP	GHRL
Met + Pioglitazone	**Base line**	7.3±1.8	8.7 ± 1.1	106.3 ± 21.8
	Week 6	9.0 ± 1.4	15.6±2.8	150.3±27.1
	Week 12	11.5 ± 1.3	21.4 ± 3.6	213.9±33.2
***p* value (t-test)**		0.017	0.053	0.037

On comparing effect of therapy by metformin with pioglitazone after 12 weeks of treatment with the control group values, it was shown that the parameters didn't approach the control group values (Table . 4).

Table .4 Comparison between metformin with pioglitazone group and *control group* in regard 1-5 AG, ADP, GHRL at the 12th week of treatment.

Drug	1-5AG	ADP	GHRL
Met + Pioglitazone	11.5±1.3	21.4 ±3.6	213.9±33.2
Control	25.9±8.6	23.8±4.3	305.9±52.9
***p* value (independent Samples t-test)**	0.000	0.025	0.000

Comparison between the effects of metformin, metformin with pioglitazone on 1,5AG during the study period

The effects of metformin alone, metformin with pioglitazone on 1,5AG showed statistically significant improvement (Table.5)

Table.5 Effect of treatment by metformin, metformin with pioglitazone on 1-5AG from baseline and at the 6th and 12th week of treatment.

Drug	Duration	Met.	Met +Pioglitazone	*p* value (t-test)
1,5AG	**Base line**	7.4 ±1.5	7.3 ± 1.8	0.000
	Week 6	9.2±1.5	9.0 ±1.4	0.000
	Week 12	10.5 ±0.7	11.5 ±1.3	0.001
p- value (t-test)		0.010	0.017	(F test) 0.000

Comparison between the effects of metformin, metformin and pioglitazone on ADP levels during the study period.

Metformin with pioglitazone caused apparent increase in ADP but it was statistically insignificant (Table .6)

Table .6 Effect of treatment by metformin, metformin with pioglitazone on ADP at the 6th and 12th week of treatment.

Drug	Duration	Met.	Met + Pioglitazone	*p* value(t-test)
ADP	**Base line**	9.0 ±3.6	8.7±1.1	0.001
	Week 6	12.1 ±3.9	15.6±2.8	0.007
	Week 12	18.3±4.3	21.4 ± 3.6	0.003
***p* value (t-test)**		0.041	0.053	(F test) 0.001

Comparison between the effects of metformin, metformin with pioglitazone on GHRL levels during the study period.

Metformin with pioglitazone caused significant increase in GHRL level after 12 weeks of treatment (Table .7)

Table .7 Effect of treatment with metformin alone, metformin and pioglitazone and metformin and flax seed oil on GHRL from baseline and at the 6th and 12th week of treatment.

Drug	Duration	Met.	Met +Pioglitazone	*p* value (t-test)
GHRL	**Base line**	104.9 ±28.5	106.3 ± 21.8	0.001
	Week 6	172.1 ± 33.4	150.3 ± 27.1	0.002
	Week 12	252.9 ±37.7	213.9 ± 33.2	0.003
p- value (t-test)		0.050	0.037	(F test) 0.000

DISCUSSION

Effects of metformin on study parameters

No studies about the effects of metformin on 1,5AG to be compared with current results, this registers originality of our research in this regard.

In this study, there was a significant rise in ADP after treatment with metformin for 12 weeks (*p* 0.041). Another study byAdamia *et al* [36], reported that the results of investigations of ADP after Metformin 6 months therapy shown that circulating ADP levels were significantly increased (*p* 0,008). Fujita *et al* [37] showed that after 4 weeks treatment with metformin, serum ADP levels were not significantly elevated in metformin-treated patients, that's mean 4 weeks may be not enough for

metformin to exert a change in ADP serum level.

On contrary, Megan V. Cannon *et al* [38], observed a significant lowering of ADP levels following 4 months treatment with metformin which was in disagreement with our study.

In this study, there was only apparent rise in GHRL level after 12 weeks of treatment with metformin (*p* 0.050). In a study done by Shaker *et al* [39] serum levels of ghrelin, after three months treatment with metformin, showed a significant elevation. Doogue*et al* [40], as well, noticed an increase in ghrelin concentrations after 6 weeks of treatment with metformin (*P* 0.003) in patients with T2DM. It is likely, in his idea, that the ghrelin response was secondary to improved glycaemic control and weight reduction induced by metformin as GHRL correlates inversely with BMI [40].

Contradicting to these results, Gagnon *et al* [41] reported a decrease in circulating levels of the orexigenic hormone ghrelin after 6 months treatment, were they thought that metformin directly inhibit stomach ghrelin production and ↑secretion of AMPK. This occurs through activation of AMPK activator that significantly inhibited ghrelin secretion.

Effect of metformin with pioglitazone on study parameters

The complimentary mechanisms of action of a combination of pioglitazone hydrochloride and metformin may have clinically beneficial effects in the treatment of patients with T2DM. Bailey *et al* [42] found that when the patients become uncontrolled on submaximal doses of metformin, increasing the metformin dose or adding a second agent are alternative management strategies for restoring glycemic control.

No available data regarding the relation between pioglitazone and fasting serum 1,5AG, so our article is original in this regard.

There was apparent increase in ADP (*p* 0.053) which was statistically insignificant in our study.

A study performed by Sharma *et al* [43] showed that 12-week treatment with metformin and pioglitazone significantly increased ADP concentrations (*p*< 0.001). Petra B. Musholt *et al* [44] had mentioned that following oral antidiabetic drugs treatment period with pioglitazone and metformin., there was a significant improvement of ADP levels (*p* 0.017), both were in dis-agreement with us .

Kaku K [45] noticed that during a 12-weeks treatment with metformin 500 or 850 mg/day and pioglitazone 15 mg/day then increased to 30 mg/day for a further 16 weeks, adiponectin increased significantly, also this result in dis-agreement with us.

In this study, both drugs also caused a significant increase of GHRL which was not detected in case of treatment by metformin monotherapy (*p* value 0.037).

this is mainly attributed to pioglitazone action as a legends of peroxisome proliferator-activated gamma receptor (PPAR-γ), which are a nuclear receptors complex that modulate the expression of the genes involved in lipid and glucose metabolism, insulin signal transduction and adipocyte and other tissue differentiation thus pioglitazone improves glycemic control in people with Type 2 diabetes by improving insulin sensitivity and promotes glucose uptake and utilization by increasing glucose transporters 1 and 4 and modulates synthesis of lipid hormones or cytokines and other proteins involved in energy expenditure .this improvement in glycemic control allow increment in GHRL level in addition to the mechanisms about metformin effect regarding weight loss that is associated with elevation in GHRL level [9].

Studies about the action of the pioglitazone on ghrelin levels are scarce [46]. Kadoglou *et al*

[47] had noticed that after 14 weeks of combination treatment there was considerable increase in fasting plasma GHRL (p< .05). Taslimi *et al* [48] study reported that metformin and pioglitazone caused significant reduction in fasting ghrelin levels (p<0.05).

Kaku K [45] after a 12-week treatment with metformin 500 or 850 mg/day and pioglitazone 15 mg/day for 12 weeks then increased to 30 mg/day for a further 16 weeks noticed that combination therapy was associated with significantly increased ghrelin level.

CONCLUSION

Metformin, metformin with pioglitazone associated with statistically significant improvement in 1,5AG level. Metformin associated with a significant rise in ADP and GHRL hormone level in blood . However, metformin with pioglitazone was more effective in elevation of GHRL hormone blood level.

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