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Biochemical Efficacy of Various Anti-Diabetic Drugs on A Combination of High Fat Diet Fed And Low Dose of Streptozotocin Treated Rats As A Model for Type 2 Diabetes

Biochemistry

KEYWORDS : T2DM, Insulin Resistance, Combination therapy, HFD.

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ABSTRACT

The present study was aimed to explore the biochemical effect of Metformin, Pioglitazone and Sitagliptin as a combination therapy in diabetic male albino rats induced by feeding high-fat diet followed by intra- peritoneal injection of a single low dose of streptozotocin (25 mg/kgb.w) to investigate the glycemic condition, lipids profile, hormones and safety of drugs on different tissues. Ninety male albino rats were included in this study, from which 15 were randomly selected as the normal control group (group A) and the remaining 75 were considered as T2DM model group. Group A was fed with commercial balanced diets and the T2DM model group, with high fat diets. After 6-weeks feeding, the T2DM was injected i.p. with low dose of streptozotocin (STZ) (25 mg kg−1) then randomly allocated into four groups according to the drug treatment, Diabetic (group B), Janumet (group C), Janumet plus Pioglitazone (group D), and Metformin plus Pioglitazone group (group E). the obtained results revealed that Diabetic rats were showing highly significant increase in plasma glucose level, HbA1C, insulin resistance (IR) with hyperinsulinemia as well as hyperglucagonemia, Total cholesterol (TC), Triacylglycerol (TG), Low-density lipoproteins (LDL-C), and Thyroid stimulating hormone (TSH) also significantly elevated while High-density lipoprotein-cholesterol (HDL-C) and Amylase significant decrease. On the other hand, body weight was showed a different behavioral pattern, where it was detectably significant decrease in the end of experiment. Treatment with combination of Metformin, pioglitazone and Sitagliptin improved glucose hemostasis, lipid profile, and insulin resistance beside their safety on kidney, liver, Pancreas and thyroid tissues.

INTRODUCTION:

Diabetes mellitus is one of the most common endocrine disorders arises when insufficient Insulin is produced or when the available insulin does not function correctly (Garg et al., 2014). Prevalence and incidence of type 2 diabetes mellitus (T2DM) is rapidly increasing worldwide; it is predicted, according to the latest estimates of the World Health Organization (WHO), that diabetes will be the 7th leading cause of death in 2030 (WHO, 2013) due to macro and microvascular complications, which results in severe illness and premature death, with elevated personal and economic costs (Stumvoll et al., 2005).

The common features of T2DM are insulin resistance followed by a defect in insulin secretion, which lead to hyperglycaemia; (Virally et al., 2007). Obesity (particularly visceral fat obesity) is accompanied by a decrease in muscle mass, induces insulin resistance, and is closely associated with the rapid increase the risk of developing diabetes (Kohei, 2010).In fact, degeneration of Langerhans islets with β-cell loss is secondary to insulin resistance and is regarded as the most important lesion for progression of the disease (Marchetti et al., 2010; Kahn, 2000).

Based on these assumptions, it is becoming clear that T2DM management by using pharmacological agents, must envision not only glycaemic control but also, and particularly, the mechanisms behind progression of pancreatic deterioration and underlying evolutional complications. In fact, T2DM therapeutics should be able to preserve β-cell mass as the main stay of disease control, by addressing the mechanisms implicated in diabetic pathogenesis, including apoptosis, inflammation or even an added capacity for cell proliferation.

Metformin, a biguanide agent acts primarily as an insulin sensitizer. Its primary clinical site of action is in the liver, improving hepatic insulin sensitivity and as a result, decreasing hepatic gluconeogenesis. Metformin also has significant effects on peripheral insulin sensitivity, primarily at muscle and modestly at adipocyte by phosphorylation and activation of AMP-activated protein kinase (Kirpichnikov et al., 2002).

Pioglitazone, a member of the thiazolidinedione class, is a potent peroxisome proliferator activated receptor (PPAR)-gamma receptor agonist. PPAR-gamma is a ligand co-activated transcription factor involved in glucose and lipid metabolism. PPAR active drugs increase intracellular fatty acid oxidation, reducing intracellular lipid and diacyl glycerol levels, improving insulin signaling. This effect may be enhanced by or due to an increase in circulating adiponectin, an adipocytokine which increases peripheral fat oxidation and decreases vascular inflammatory responses (Phillips et al., 2003).

Enhancing the incretin effect is now a possible therapeutic target in T2DM, using GLP-1 analogues or DPP-4 inhibitors. Sitagliptin belongs to a class of oral antidiabetic drugs, the gliptins, which inhibit the enzyme DPP-4 that degrades incretins, prolonging the physiological actions of GLP-1 (Seshadri and Kirubha, 2009). GLP-1, a prominent

active compound of the incretin family, modulates many processes in pancreatic islet: it potentiates insulin synthesis and secretion (Holst et al., 2009), inhibits glucagon secretion (Toft et al., 2001), increases islet cell proliferation, and decreases cell apoptosis (Li et al., 2003). Studies previously also shown that Sitagliptin is able to ameliorate glucose dysmetabolism, insulin resistance, inflammation and oxidative stress in an animal model of T2DM (Mega et al., 2011).

Hence, there exists a continued quest among the investigators for establishing the ideal animal model for type 2 diabetes. Present study was used high fat diet (HFD) fed rat model followed by a low dose streptozotocin (STZ) injection as previous studies have reported that the rats fed with HFD are already mildly hyperglycemic, obesity, hyperinsulinemia, and insulin resistance (Shafrir, 2003) and more susceptible to develop significant hyperglycemia and hyperlipidemia with low doses of STZ, which mimics the human type-2 diabetes (Bansal et al., 2012).So the main objectives of this study was to develop a rat model that replicates the natural history and metabolic characteristics of human type 2 diabetes and investigation of various anti-diabetic compounds as (Metformin-Pioglitazone and Sitagliptin) in combination with each other on glucose hemostasis, lipid profile, and insulin resistance.

MATERIALS AND METHODS

Chemicals:

Streptozotocin purchased from MP Biomedicals, LLC (29525 Fountain PKWY, Solon, OH 44139), Metformin HCl (Cidophage, 500mg) was Kindly provided by CID Company, Cairo, Egypt, Pioglitazone HCl (Glustazone, 30 mg) was provided by Chemipharm Pharmaceutical Industries S.A.E. 6TH October, Egypt, Sitagliptin / Metformin HCl (Janumet, 50mg/500mg) purchased from Merck Sharp and Dohme Ltd, Hertford Road, Hoddesdon, United Kingdom.

Animals:

90 healthy male Sprague-Dawley rats about 90-100g body weight were purchased from lab. Animal House, faculty of Vet. Medicine. Suez Canal University. All animals were conditioned at room temperature (22-25o C) at a natural photoperiod for two week before experiment execution. A commercial balanced diet and tap water ad libitum were provided.

Development of HFD-fed and STZ-treated Type 2 Diabetic rats:

The rats were allocated into two dietary regimens consisting of 15 and 75 rats by feeding either commercial balanced diet or HFD (58% fat, 25% protein and 17% carbohydrate, as a percentage of total kcal) ad libitum, respectively, for the initial period of 6 weeks (Reed et al.,2000). The composition (Table A) and preparation of HFD as were described elsewhere (Srinivasan et al., 2005).

Table (A): Composition of HFD

Ingredients	Diet (g/kg)
Powdered NPD	365
Lard	310
Casein	250
Cholesterol	10
Vitamin and mineral mix	60
dl-Methionine	03
Yeast powder	01
Sodium chloride	01

After the 6 weeks of dietary manipulation, All animals were fasted overnight, HFD-fed rats (75 rats) was injected intraperitoneally (i.p.) with low dose of STZ (25 mg/ kg b.w) (Srinivasan et al., 2005; Latt et al., 2013).STZ was dissolved in a citrate buffer (pH 4.4) with a concentration of 15 mg/ml. and the animals fed commercial balanced diet (15 rats) were injected with vehicle buffer only (0.2 ml citrate buffer/rat/i.p.) (Rachel et al., 2012).

Confirmation of Diabetes:

One week after the STZ injection, all animals were fasted overnight and blood glucose level was measured by using a portable glucometer (Contour TS-Bayer-Switzerland) in the blood collected from eye canthus. Animals with fasting blood glucose (FBG) level >200 mg/dl were considered as diabetic and selected for further pharmacological studies. The rats were allowed to continue to feed on commercial balanced diets until the end of the study (Srinivasan et al., 2005).

Experimental Design:

The duration of experiment was 8 weeks from drugs administration. Rats were randomly divided into 5 groups as the following:

- Group (A):-Served as control group and received commercial balanced diet all over the experimental period (8 weeks).

- Group (B):- Received HFD for 6 weeks and injected STZ (25 mg/ kg b.w /i.p.) and served as control Diabetic group all over the experimental period (8 weeks).

- Group (C):- Received HFD for 6 weeks and injected STZ (25 mg/ kg b.w /i.p.) then received janumet (Sitagliptin 50mg/ Metformin HCl 500mg) /kg b.w. (Hanaa and salwa, 2013) for 8 weeks

- Group (D):- Received HFD for 6 weeks and injected STZ (25 mg/ kg b.w /i.p.) then received janumet (Sitagliptin 50mg/ Metformin HCl500mg) /kg b.w. (Hanaa and salwa, 2013) and pioglitazone 10mg/kg b.w. (Srinivasan et al., 2005) for 8 weeks

- Group (E):- Received HFD for 6 weeks and injected STZ (25 mg/ kg b.w /i.p.) then received Metformin/500mg/kg b.w. (Rachel et al., 2012) and pioglitazone 10mg/kg b.w. (Srinivasan et al., 2005) for 8 weeks.

Table (B) Experimental Design:

Groups	No. of rats	Treatment
A control	15	Commercial balanced diet
B Diabetic	21	HFD+STZ
C janumet	18	HFD+ STZ+janumet
D Jan + Pio	18	HFD+ STZ+janumet+pioglitazone
E Met+ Pio	18	HFD+ STZ+Metformin+pioglitazone

Drug administration

Metformin, Pioglitazone and Sitagliptin was dissolved in distilled water for oral administration into fasting rats by oral gavage once daily (Lauro et al., 2012).

Body weight measurement

Body weight was measured weekly for adjustment dose of drugs given and taken the average B.W at 4 weeks and at 8 weeks

Collection of blood serum:

Blood samples are collected at the end of 4 and 8 weeks from antidiabetic drugs administration from all groups af-

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ter overnight fasting from the medial canthus of eye using micro-hematocrit tubes. Blood was divided into 2 tubes; the first tubes contain EDTA as anticoagulant. This tube was used for estimation of glycated hemoglobin (HbA1C). The second portion of blood was taken into a clean and dry screw capped centrifuge tubes and left to clot at room temperature, then centrifuged at 3000 r.p.m for 15 minutes to separate clear serum samples for determination of different biochemical parameters.

Measurement of glycosylated hemoglobin (HbA1c), C-Peptide and glucose.
Glucose levels were determined using glucose oxidase were determined by commercial kits (Roche Diagnostics, Germany) acc. to Tietz ,2006.

Glycosylated hemoglobin was determined using fully automated auto-analyzer Cobas C311 (Roche Diagnostics, Germany). C-Peptide is estimated by using Abnova C-Peptide ELISA Kit, Catalog Number KA1259 acc. to Bonger and Garcia, 1984.

Determination of Lipid profile in Serum
TG, TC and HDL-C of serum were determined using kits from Roche Diagnostics, Germany.TG was determined using the method of Fossati and Prencipe (1982). TC was determined according to the method of Allain et al. (1974). The assay of HDL-C was carried out by the method of Lopez-Virella et al. (1977) and LDL-C was calculated according to the method of Friedewald et al., (1972).

Biochemical analyses of Serum urea, creatinine and SGPT
Were determined in the serum using (Roche Diagnostics, Germany) commercial kits. Serum blood urea nitrogen level was determined according to (Rock et al., 1987). Serum Creatinine level was estimated according to (Jaffé et al., 1886). Level of (SGPT) was determined according to (Reitman and Frankel 1957).

Analysis of pancreatic amylase and lipase.
Lipase was determined by enzymatic colorimetric assay according to Borgström, 1977; Gargouriet al., 1983 while amylase was determined by enzymatic colorimetric assay acc. to Lorentz, 1998; Junge et al 2003.

Hormonal Analysis
-Insulin is estimated by using The ALPCO Rat Insulin ELISA, Catalog Number: 80-INSRT-E01, E10 acc.to Finlay and Dillard, 2007

-Glucagon is estimated by using The ALPCO Rat Insulin ELISA, Catalog Number: 48-GLUHU-E01 acc.to Nishino et al., 1981

-TSH estimated Acc. to Tietz, 1995.

Calculation of Homeostasis Model Assessment Estimated Insulin Resistance (HOMA-IR):

HOMA-IR was calculated according to (Matthews et al., 1985) formula: HOMA-IR = fasting insulin (U/L) × fasting glucose (mg/dL)/405.

Statistical Analysis:
The obtained data were subjected to statistical analysis using SPSS version 16 program for analysis of data. The level of statistical significance was taken as $p<0.05$.

RESULTS
The obtained results presented in table (1) showed variation in body weight between two periods as at 4 weeks, Diabetic group show significant increase in body weight if compared with control group while at 8 weeks Diabetic group show significant reduction in body weight than control group. Also, significant increase in Fasting glucose, HbA1c , C-peptide levels as well as HOMA-IR in Diabetic rats when compared to normal control rats while treated groups show significant improvement in Body weight,glucose,HbA1c, C-Peptide and HOMA-IR. Table (2) showed significant increase in TG, TC and LDL-C with significant decrease in HDL-C levels in Diabetic groups and In contrast, treated groups show significant improvement in lipid profile. Table (3) show that no significance difference between groups in urea and creatinine levels but control and Diabetic groups Show significant increase in SGPT levels if compared with treated groups.

Table (4) showed significant increase in insulin, Glucagon and TSH levels in Diabetic groups if compared with results of control and treated groups

Fig. (1, 2) show that no significant difference in lipase levels between all groups with significant decrease in Amylase levels in Diabetic groups and in contrast, treated groups show significant improvement in Amylase levels.

Discussion
Type 2 Diabetes mellitus is a metabolic syndrome which is associated with defect in insulin action or decreased insulin secretion or both with decline in insulin sensitivity (Polonsky et al., 1998; Taylor et al., 1994). Many previous studies have reported that rats which fed with HFD are already show mild hyperglycemia, and more susceptible to develop significant hyperglycemia and hyperlipidemia with development of insulin resistance with injection of low dose of STZ which mimics the human type 2 Diabetes (Bansal et al., 2011). In the present study, combination of various antidiabetic drugs was investigated on high fat diet and low dose of STZ induced diabetic rat model.

The HFD was formulated to causes insulin resistance over a short period of time. Thus, the feeding of HFD for a period of 6 weeks produced rats with insulin resistance syndrome as was characterized by the increased bodyweight (obesity) and this which shown in diabetic group (group B). The significant increase in body weight which found in diabetic rats might be due to consumption of a diet rich in saturated fats (lard) and its deposition in various body fat pads with decrease in energy expenditure as compared to control animals(Srinivasan et al., 2004).

Briefly, the presence of high level of triglycerides due to high fat content of diet could consider a source of increased fatty acid available for oxidation. The increased fatty acids for oxidation blunts the insulin ability for reduction of hepatic glucose output and reduces the glucose utilization in skeletal muscle leading to compensatory hyperinsulinemia, the common feature of insulin resistance which indicated by significant increase in HOMA-IR (Belfiore and Iannello , 1998).

As The conversion from prediabetes to permanent hyperglycemia in type 2 diabetes is associated with decline in secretory capacity of beta cells of pancreas to compensate insulin resistance. There is a relative insulin deficiency as the circulating insulin concentrations in patients with type 2 diabetes are comparable in absolute terms or slightly elevated to the values seen in non-diabetic individuals and this shown in insulin levels in diabetic groups after 8 weeks

Diabetic group show significant increase in HbA1c in relation to hyperglycemia as the erythrocyte is freely permeable to glucose and within each erythrocyte, glycosylated hemoglobin is formed continuously from hemoglobin A at a rate dependent on the blood glucose concentration. HbA1c reflects the average plasma glucose over the previous eight to 12 weeks (Nathan et al.,2007). HbA1c became a diagnostic test for DM and screening test for persons at high risk of diabetes (IEC, 2009).It can be performed at any time of the day and does not require any special preparation such as fasting, can avoid the problem of variability of glucose values from day to day. Also recent report from Australia has shown that HbA1c most favorable for predicting incident of retinopathy than one including fasting plasma glucose (Tapp et al.,2008).

C-peptide measurements have also been done as Palmer et al., 2004 reported that type 2 diabetes mellitus is associated with insulin resistance and typically initially has normal or elevated levels of C-peptide, which can decrease over the course of the disease and this in agreement with my result which shown in Diabetic group , C-peptide measurements now used to classify diabetes mellitus and as a marker of pancreatic β-cell function.

Apart from glucose Hemostasis, diabetic rats also showed abnormalities in lipid metabolism as evidenced from increased TG ,TC and LDL-C levels with significant decreased HDL-C levels which might contribute to cardiovascular complications. The hypertriglyceridemia observed in these rats may be due to increased absorption and formation of triglycerides in the form of chylomicrons following consumption of diet rich in fat or through increased production of TG-enriched hepatic very low density lipoprotein (VLDL) and decreased TG uptake in peripheral tissues (Srinivasan et al., 2004) .Hypercholesterolemia may be attributed to high level of cholesterol absorped from the small intestine following the intake of HFD in a diabetic rats (Shafrir,2003).

Also LDL-C often is increased in association with insulin resistance with a reduction in HDL-C and this result is in agreement with Reaven , 1995 as Glucose oxidation produces free radicals which damage cellular proteins and mitochondrial DNA. Increased oxidative stress reduces nitric oxide levels, decreased the levels of antioxidants such as GSH, vitamin C, and vitamin E, while the levels of some markers of oxidative stress are increased, e.g. oxidized low-density lipoprotein cholesterol (Garg et al.,1988).

Diabetic group show hypergluconemia associated with hyperinsulinemia and this result in agreement with Björn et al.,2011 as insulin and glucagon participate in glucose homeostasis, insulin secreted in the fed state, promoting glucose uptake by its target organs where glucagon mobilizes hepatic glucose in the fasting state to ensure the maintenance of normoglycemia (Walker et al.,2011)as inhibitory effects of insulin and somastostatin on glucagon secretion are relatively well established (Gromadaet al.,2007). The loss of the inverse relationship between these two hormones in T2D patients might be secondary to the diminished mass of insulin pulses, and suggests that alterations in the cross-talk between beta- and alpha-cells may underlie hyperglucagonemia (Menge et al. ,2011). Also, Lund et al. 2011 evaluated the role of glucagon-like peptide -1(GLP-1) and glucagon-like peptide-2 (GLP-2) in this response due to the fact that the incretin effect might be reduced in T2DM, so improper hyperglucagonemic response occur.

In this study Diabetic groups show elevation in TSH levels while decrease in amylase levels as many theory connecting thyroid dysfunction and T2DM as one of them there is an interaction between thyroid hormones and adipose tissue derived cytokines. Effects of thyroid hormones on production rates and plasma levels of these cytokines could be used to explain mechanisms of insulin resistance in hypothyroidism. Adipose tissue derived cytokines are adiponectin, leptin, interleukin-6 and tumor necrosis factor-α (Mitrou et al., 2010). Laloo and Salam, 2012, reported in their study that prevalence of hypothyroidism is quite high in type 2 DM patients associated with uncontrolled hyperglycemia and obesity.

Adib et al., 2005 illusterated the reduction in serum pancreatic amylase was recorded in both types of diabetes; similarly, the reduction in serum amylase in type 2 diabetes was higher in patients with longer duration of illness (59%) and in patients with low serum insulin value (79%). The finding thought to be due to reduced acinar cell function in insulin-depleted islets. Analysis of pure pancreatic juice aspirated directly from the pancreatic duct showed a significant decrease in amylase activity with little changes in lipase and bicarbonate concentrations in diabetic patients with uncontrolled hyperglycemia (Kawamori et al.,1995).

The effectiveness of combination therapy may be more clinically significant for long term management of diabetes However, treatment protocol are based on initial monotherapy, usually with metformin, and only move to combination or add-on therapy when treatment has failed and disease has progressed. Combination therapy used in this study indicating their potent antihyperglycemic and hypolipidemic activity and this result in agree with Mohamed et al., 2010 . Metformin, a biguanide agent acts as an insulin sensitizer. As it improved hepatic insulin sensitivity and decreased hepatic gluconeogenesis. Metformin also improved peripheral insulin sensitivity, primarily at muscle and adipocyte by phosphorylation and activation of AMP-activated protein kinase (Kirpichnikov et al., 2002). Pioglitazone, one of the thiazolidinedione classes, is a potent peroxisome proliferator activated receptor (PPAR)-gamma receptor agonist. PPAR-gamma is a transcription factor involved in glucose and lipid metabolism. PPAR activation increases insulin sensitivity, intracellular fatty acid oxidation and reducing intracellular lipid levels. This effect may be enhanced by or due to an increase in circulating adiponectin which increases peripheral fat oxidation and decreases vascular inflammatory responses (Phillips et al., 2003). Sitagliptin, this new class of drugs is called DPP4 inhibitors. Which prevent GLP-1 degradation and improve circulation time of the active forms of GLP-1 and GIP thereby increasing the biological activity of this incretin hormones in improving the glycaemic dysmetabolism. Also GLP-1 inhibits intestinal lipoprotein secretion and may lower post-prandial hyperlipidaemia (Scrocchi et al.,1998). As it corrected hypertriglyceridaemia, inflammation and hypertension, A small but significant decrease in plasma cholesterol (total- and non-HDL-cholesterol) and triglycerides was observed with Sitagliptin treatment(Ferreira and Pinto,2010).

In rodents, pioglitazone has been shown to enhance islet cell mass and restore normal structure and function (Diani et al., 2003). The mechanism of beta-cell modulation is unclear but is likely due to direct PPAR activation at the beta cell (Kawasaki et al., 2005 and Rasouli et al., 2007). Also DPP4 inhibitors exhibit favourable actions on islet and beta cell mass, morphology and survival in animal studies (Drucker, 2007) and this illustrate the improvement in HOMA data in treated groups. Also, there was a trend for

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DPP4 therapy to decrease HBA1c more compared with control as sitagliptin combined treatments lowered HBA1c by 0.89% compared to another combination of anti-diabetic agents (Richter et al.,2008).HgA1c lowered with pioglitazone/metformin was −2.3% when compared with metformin alone (Oerter et al.,2006).

Treated groups also showed improvement in glucagon levels as pioglitazone therapy led to decreased hepatic gluconeogenesis while insulin levels were lowered, it appeared that pioglitazone acted to restore sensitivity to insulin's inhibitory actions on phosphoenolpyruvate carboxykinase transcription (Hofmann et al., 1992). Also, glucagon level are significant decreased in Janumet Groups as GLP-1 inhibits glucagon secretion by the pancreatic α-cells (Weir et al.,1989), resulting in a further decrease of hepatic glucose production in the postprandial state in subjects treated with GLP-1-receptor agonists or DPP-4 inhibitors. GLP also potentiates glucose disposal. Moreover, GLP-1 reduces food intake and gastric emptying through either direct effects on the gastrointestinal tract or indirect actions via the central nervous system (Baggio and Drucker, 2007) promoting satiety and improving weight maintenance and this explain the reduction of glucagon levels in Janumet treated group and improvement in body weight gain

Estimation of serum Urea, Creatinine and SGPT levels is done for detect harmful effect of this drugs on renal and hepatic tissue but our obtained results show no significant difference between treated groups and control group while SGPT level is elevated in control and diabetic groups may be due to associated with increased body weight in control groups and for the hepatic IR, metabolic syndrome in T2D. As ALT, it can be a strong and independent predictor for T2D (Harris, 2005), Hyperlipidemia is another factor directly linked to hepatic IR as high circulating lipid concentrations in the blood with increased levels of LDL, VLDL and total cholesterol in rats are a risk factor for hepatic IR as well as T2D (Benado et al., 2005).

At present, there are no adequate and well controlled studies are available that illustrates the safety use of janumet (Sitagliptin /metformin).In this study Creatinine and BUN levels is estimated for effect of this drugs on renal tissue and our result show that this drugs made no change in their levels and this in agreement with Chan et al., 2008 as they have looked at the safety of sitagliptin in patients with moderate renal insufficiency, when a dose of 50 mgs daily was used, and in severe renal insufficiency when a dose of 25 mgs daily was used. Many studies reported that Sitagliptin decreased liver lipid accumulation while increasing pancreatic insulin content and enhancing glucose-stimulated insulin secretion (Mu et al.,2006) also prevent hepatic steatosis after high-fat feeding (Drucker,2007) and this explained normal level of sGPT enzyme in Sitagliptin treated group if compared with Diabetic and Control groups. Although there are benefits to incretin-based treatments of T2DM, questions have been raised regarding to the possibility that long-term treatment might lead to pancreatitis and potential neoplasia (Cervera et al.,2008).As anticipated, in our study levels of amylase and lipase are estimated for this questions and the results show that no combination therapy used in study affect on this enzyme levels and this agree with Thomas et al.2014 which illustrate that Sitagliptin had no adverse effect on the pancreas of the Zucker diabetic fatty (ZDF) rat model of T2DM. There was no evidence of increased risk of pancreatitis, and no difference in exocrine duct and ductular PI between treated and untreated rats. As Sitagliptin had no effect on total GLP-1 levels, but increased active GLP-1 levels approximately 3-fold. There was no evidence of pancreatitis and no evidence of an increase in PI. The absence of an increase in PI in the Sitagliptin treatment groups in this study is also evidence against the hypothesis that Sitagliptin increases the risk of exocrine pancreatic neoplasia by increasing PI. Notably, the mouse and rat bioassays conducted for Sitagliptin did not suggest a risk for pancreatic cancer (Engel et al., 2010)

Also, thyroid tumors were reported to be more common in rodent toxicology studies with the GLP-1 agonist although the relevance of this in humans has been questioned (Victoza,2009).our study showed that no effect of DPP-4 inhibitors on levels of TSH and this in agreement with Elashoff et al.,2011 which explained The reported event rate for thyroid cancer in patients treated with GLP-1 mimetic therapy was increased and reached statistical significance in the exenatide group but not in the sitagliptin group. The new drugs are being used as early monotherapies. Since metformin likely suppresses the putative actions of GLP- based drugs to promote pancreatitis and pancreatic cancer. Other theory was prompted by the reported actions of DPP-4 inhibition on the immune system and concerns raised that these might promote cancer through decreased immunosurveillance (Havre et al., 2008; Matteucci and Giampietro 2009). The most obvious conclusion from these studies is that careful long term monitoring of patients treated with GLP-1 mimetics or DPP-4 inhibitors is required.

Table (1): Body weight (gm), Fasting glucose (mg/dl), HbA1c (%), C-Peptide (ng/ml) levels and HOMA-IR in different treated groups after 4 and 8 weeks from drug administration:

Duration	Body weight (gm)		Fasting glucose level (mg/dl)		HbA1c (%)		C-Peptide level (ng/ml)		**HOMA-IR**	
Groups	4 Weeks	8 Weeks	4 Weeks	8 Weeks	4 Weeks	8 Weeks	4 Weeks	8 Weeks	4 Weeks	8 Weeks
A (control)	$121.67^{b} \pm 7.26$	$281.67^{a} \pm 1.60$	$86.33^{d} \pm 2.33$	$91.00^{c} \pm 2.64$	$3.63^{c} \pm 0.08$	$4.10^{b} \pm 0.11$	$0.60^{b} \pm 0.10$	$0.86^{b} \pm 0.01$	$7.06^{c} \pm 1.33$	$11.30^{b} \pm 0.97$
B (Diabetic)	$193.33^{a} \pm 10.40$	$155.00^{c} \pm 11.90$	$208.67^{a} \pm 4.66$	$351.75^{a} \pm 13.23$	$6.97^{a} \pm 0.44$	$9.97^{a} \pm 0.15$	$0.95^{a} \pm 0.07$	$1.15^{a} \pm 0.07$	$28.60^{a} \pm 1.06$	$19.42^{a} \pm 2.69$
C (Janumet)	$111.67^{b} \pm 2.88$	$172.00^{bc} \pm 6.29$	$154.00^{b} \pm 5.00$	$127.75^{b} \pm 2.01$	$5.65^{b} \pm 0.41$	$3.66^{b} \pm 0.10$	$0.50^{b} \pm 0.01$	$0.85^{b} \pm 0.02$	$14.97^{b} \pm 2.83$	$14.48^{b} \pm 1.07$
D (Jan + Pio)	$121.67^{b} \pm 10.90$	$180.00^{b} \pm 5.00$	$128.33^{c} \pm 8.25$	$113.00^{b} \pm 4.60$	$4.30^{c} \pm 0.11$	$3.71^{b} \pm 0.08$	$0.53^{b} \pm 0.05$	$0.87^{b} \pm 0.004$	$12.74^{bc} \pm 1.41$	$13.23^{b} \pm 1.88$
E (Met +Pio)	$120.00^{b} \pm 0.00$	$170.00^{bc} \pm 2.88$	$141.33^{bc} \pm 1.85$	$132.75^{b} \pm 1.25$	$4.40^{c} \pm 0.25$	$3.90^{b} \pm 0.23$	$0.63^{b} \pm 0.07$	$0.84^{b} \pm 0.02$	$12.89^{bc} \pm 1.80$	$13.50^{b} \pm 1.09$

Values are means ± SE
Means carrying different superscripts considered significant (P < 0.05).

Table (2): Serum levels of Urea (mg/dl), Creatinine (mg/dl) and SGPT (mg/dl) in different treated groups after 4 and 8 weeks from drug administration:

Groups \ Duration	Urea (mg/dl)		Creatinine (mg/dl)		SGPT (mg/dl)	
	4 Weeks	8 Weeks	4 Weeks	8 Weeks	4 Weeks	8 Weeks
A (control)	23.67^{a} ± 0.66	21.67^{a} ± 0.33	0.30^{a} ± 0.00	0.36^{a} ± 0.03	64.67^{a} ± 11.39	84.00^{a} ± 5.68
B (Diabetic)	25.00^{a} ± 5.13	24.50^{a} ± 2.39	0.37^{a} ± 0.03	0.35^{a} ± 0.06	56.00^{a} ± 10.50	80.00^{a} ± 14.00
C (Janumet)	22.00^{a}± 5.85	21.50^{a} ± 4.21	0.33^{a}± 0.03	0.32^{a} ± 0.07	73.33 ^{a} ± 4.70	65.75^{ab} ± 14.90
D (Jan + Pio)	23.67^{a} ± 2.40	18.75^{a} ± 2.01	0.37^{a} ± 0.03	0.35^{a} ± 0.05	61.67^{a} ± 8.64	38.75^{b} ± 5.40
E (Met +Pio)	23.00^{a}± 6.83	24.00^{a} ± 1.15	0.33^{a}± 0.03	0.37^{a} ± 0.02	56.33^{a}± 3.80	42.50^{b} ± 4.50

Values are means ± SE
Means carrying different superscripts considered significant (P < 0.05).

Table (3): Lipid profile in different treated groups after 4 and 8 weeks from drug administration:

Groups \ Duration	TG (mg/dl)		TC (mg/dl)		HDL-C (mg/dl)		LDL-C (mg/dl)	
	4 Weeks	8 Weeks	4 Weeks	8 Weeks	4 Weeks	8 Weeks	4 Weeks	8 Weeks
A (control)	41.33^{b} ± 0.88	57.30^{b} ± 3.28	68.00^{b} ± 3.21	66.30^{b} ± 4.84	31.40^{b} ± 0.88	32.40^{b} ± 3.28	31.34^{bc} ± 0.59	36.43^{b} ± 3.28
B (Diabetic)	94.00^{a} ± 3.46	146.00^{a} ± 4.26	111.60^{a} ± 6.06	136.25^{a} ± 2.95	21.00^{c} ± 3.46	19.80^{c} ± 4.26	60.00^{a} ± 3.46	72.80^{a} ± 4.26
C (Janumet)	71.00^{b}± 4.90	44.66^{bc} ± 1.5	86.30^{b}± 7.35	51.50^{c} ± 5.90	31.00^{b} ± 1.52	33.50^{b} ± 4.90	41.22^{b}± 4.90	35.00^{b} ± 1.50
D (Jan + Pio)	44.00^{bc} ± 1.52	41.50^{c} ± 4.90	69.30^{b} ± 9.13	47.00^{c} ± 6.12	36.00^{a}± 4.90	38.00^{a} ± 3.50	32.30^{bc} ± 1.52	30.50^{bc} ± 4.90
E (Met +Pio)	59.70^{bc}± 1.52	46.00^{bc} ± 3.40	80.30^{b}± 6.83	39.55^{c} ± 2.95	37.70^{a}± 1.52	39.00 ^{a} ± 3.40	35.50^{b}± 1.52	33.00^{bc} ± 3.40

Values are means ± SE
Means carrying different superscripts considered significant (P < 0.05).

Table (4): Serum levels of insulin (ng/ml), glucagon (pg/ml) and TSH (ng/ml) hormones in different treated groups after 4 and 8 weeks from drug administration:

Groups \ Duration	Insulin (ng/ml)		Glucagon (pg/ml)		TSH (ng/ml)	
	4 Weeks	8 Weeks	4 Weeks	8 Weeks	4 Weeks	8 Weeks
A (control)	1.33^{b} ± 0.26	2.01 ^{b} ± 0.11	49.73^{c} ± 1.75	85.50^{b} ± 1.76	0.019^{b} ± 0.002	0.006^{b} ± 0.002
B (Diabetic)	2.30^{a} ± 0.11	2.15^{a} ± 0.07	240.12^{a} ± 18.6	149.80^{a} ± 1.71	0.030^{a} ± 0.001	0.026^{a}± 0.002
C (Janumet)	1.61^{ab}± 0.14	1.85 ^{b} ± 0.08	68.40^{bc} ± 6.72	58.20^{c}± 3.90	0.016^{b} ± 0.002	0.010^{b}± 0.001
D (Jan + Pio)	1.35 ^{b} ± 0.07	1.82^{b} ± 0.11	54.62^{bc} ± 1.56	67.30^{bc} ± 5.67	0.025^{b} ± 0.004	0.007^{b} ± 0.005
E (Met +Pio)	1.44^{b}± 0.34	1.85 ^{b} ± 0.24	85.27^{b} ± 1.69	86.60^{b}± 4.78	0.014^{b}± 0.002	0.009^{b}± 0.002

Values are means ± SE
Means carrying different superscripts considered significant (P < 0.05).

Fig (1): Levels of serum lipase (U/l) in different treated groups after 4 and 8 weeks from drugs administration:

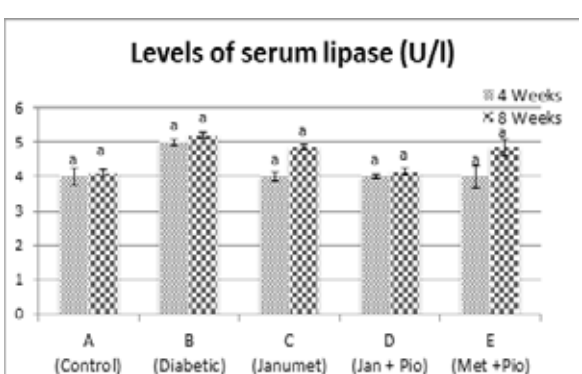
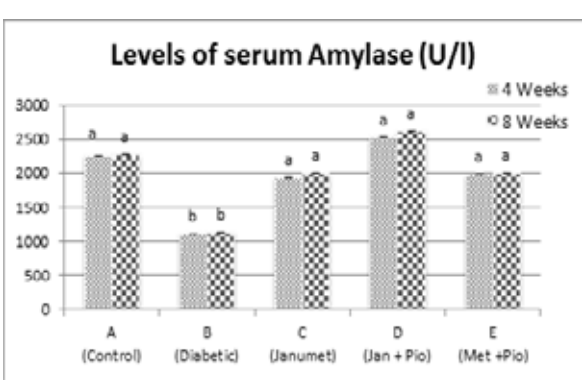


Fig (2): Levels of Amylase (U/l) in different treated groups after 4 and 8 weeks from drugs administration:



Values are means ± SE

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