# COMPARATIVE GLUCOSE-LOWERING AND RENOPROTECTIVE EFFECTS OF SITAGLIPTIN AND INSULIN AND THEIR COMBINED THERAPY ON TYPE-2 DIABETES MELLITUS WITH NEPHROPATHY IN RATS

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## ABSTRACT

**Aims:** The study compared the glucose-lowering and renoprotective effects of sitagliptin, insulin and their combination on high-fat, high-sugar diet, streptozotocin (STZ)-induced type-2 diabetes mellitus (T2DM) with nephropathy in rats. **Methods:** In addition to the normal control group (n= 8), diabetes was induced in adult male Spurge-Dawley rats by 6-week high-fat, high-sugar diet followed by a single intraperitoneal injection of streptozotocin 30 mg/kg BW. For four weeks thereafter, diabetic rats (n= 32) were divided into 4 equal groups (n = 8) and received daily: vehicle (untreated diabetic group), insulin 10 IU/kg SC, sitagliptin 30 mg/kg PO, or combined sitagliptin-insulin, and continued on the same diet. We assessed a group of blood/serum measures of glucose metabolism and kidney functions and histopathology. **Results:** Compared to control group, the untreated diabetic rats developed significant decreases in body weight (BW) and serum insulin, increases in kidney weight (KW), KW/BW ratio, blood glucose and AGEs levels, increases in blood urea, creatinine, urine output, albuminuria and renal tissue TGF-β1 levels, and decrease in the creatinine clearance and showed variable glomerular, tubule-interstitial and vascular kidney lesions. Sitagliptin monotherapy stabilized the BW, KW and KW/BW ratio, reduced the blood glucose, AGEs, urea, creatinine, urine output, albuminuria and renal tissue TGF-β1 levels and increased the serum insulin and creatinine clearance, with greater improving the biochemical parameters (and not blood glucose), and ameliorating kidney histopathological lesions more than insulin alone. In contrast, insulin produced better effects on BW and KW/BW ratio. Importantly, sitagliptin-insulin co-treatment highly and greatly improved measures of glucose metabolism and kidney functions, and highly ameliorated kidney lesions when compared to treatment with insulin or sitagliptin alone. **Conclusion:** Combined sitagliptin-insulin therapy, in rats with T2DM and nephropathy, produced greater glycemic control and renoprotective effects more than treatment with sitagliptin or insulin alone. This combined therapy could have clinical unique application in the management of T2DM with nephropathy.

**Keywords:** Sitagliptin; insulin; diabetes mellitus; nephropathy; rats

**Abbreviations:** DM: Diabetes mellitus, T1DM: Type-1 diabetes mellitus, T2DM: Type-2 diabetes mellitus, IP: Intraperitoneal, STZ: Streptozotocin, FBG: Fasting blood glucose, 2hBG: 2-hour blood glucose postload, AGEs: Advanced glycation end-products, GIP: Glucose-dependent insulinotropic peptide, DPP-IV: Dipeptidyl peptidase-IV, GLP-1: Glucagon-like peptide-1, GLP-1R: GLP-1 receptor, TGF-$\beta$1: Transforming growth factor-$\beta$1, TNF-$\alpha$: Tumor necrosis factor-$\alpha$, IL-1β: Interleukin-1β

## INTRODUCTION

Type-2 diabetes mellitus (T2DM) is a complex disease that involves a variety of pathophysiologic abnormalities, including insulin resistance, increased hepatic glucose production, and abnormalities in the secretion of hormones, such as insulin, glucagon, amylin, and incretins [(1)]. Glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) are the two primary incretin hormones secreted from the intestine in response to food digestion in humans and rodents, to stimulate glucose-stimulated insulin secretion from pancreatic β-cells [(1, 2)]. GLP-1 also suppresses glucagon secretion from pancreatic α-cells. Incretin dysfunction in diabetes can be treated with GLP-1 receptor agonists e.g. exenatide or inhibitors of dipeptidyl peptidase-IV (DPP-IV), the enzyme that degrades GLP-1 and GIP, e.g. sitagliptin [(1, 2, 3)]. DPP-IV inhibitors are a new class of oral hypoglycemic agents approved for the treatment of T2DM, with low risk of hypoglycemia and no weight gain, that suppress DPP-IV-dependent inactivation of GIP and GLP-1, thereby enhancing their biological activities. Sitagliptin is the first approved, oral, once-daily, highly selective DPP-IV inhibitor

that enhances postprandial levels of active GLP-1, leading to a rise in insulin release and decrease in glucagon secretion [4, 5, 6]. Diabetic nephropathy (DN) is the leading cause of end-stage renal disease in patients with diabetes mellitus [7, 8]. The risk of DN in patients with type-1 (T1DM) and T2DM is strongly linked to poor glucose control [9, 10]. As T2DM progresses, most patients with uncontrolled diabetes will require a combination of various glucose-lowering agents including insulin replacement therapy. However, the evidence for rationale to combine oral antidiabetic drugs, except metformin, with insulin is less clear [11]. While the glucose-lowering and renoprotective effects of sitagliptin monotherapy have been previously studied [12], the magnitude of its effect when combined with insulin remains to be elucidated. To our knowledge, the expected benefit effects of combined sitagliptin and insulin in T2DM with diabetic nephropathy have not been investigated. The present study aimed to compare the effects of sitagliptin, insulin and their combination therapy on the high-fat and high-sugar diet streptozotocin-induced T2DM with nephropathy in rats.

## MATERIALS AND METHODS

**1. Drugs and chemicals:** Streptozotocin (STZ) was purchased from Sigma Chemical Co, USA. Sitagliptin was a gift from Merck (Rahway, USA). Insulin NPH (Mixtard 40 IU/ml) was purchased from Novo Nordisk Co., Denmark. The chemicals such as cholesterol, ursodeoxycholic acid (livagoal 450 mg), tried lard, sucrose and 10% formalin solution were procured from the local commercial sources. Citric acid-natrium citricum buffer (pH 4.5), Phosphate buffer (0.1M, Ph7.4), and Tris-hydrochloric buffer were purchased from Biodiagnostic Co., Egypt. All chemicals were of analytical grade.

**2. Animals:** Forty adult male Spurge-Dawley rats, weighed 200-300 grams, were obtained from the laboratory animal house (Zagazig Faculty of Medicine, Egypt). The rats were housed in fully ventilated cages and kept on a 12-hour light–dark cycle, under a temperature-controlled environment at 22 ± 2º C. The rats had free access to water and fed on the respective diets of different rats' groups throughout the experiment period (4 weeks) as described below. The experiments were performed according to the guidelines of the Institutional Animal Care at The Faculty of Medicine, Zagazig University, Egypt.

**3. Establishment of rat model of T2DM [13]:** Thirty-two rats were fed with high-fat and high-sugar 'diabetogenic' diet (67% normal diet, 20% sucrose, 10% tried lard, 2% cholesterol and 1% bile salts) and water ad libitum for six weeks.. The rats were then injected intraperitoneally (IP) with a single dose of streptozotocin (STZ) (30 mg/kg body weight) freshly dissolved in a pH 4.5 citric acid–natrium citricum buffer, after an overnight fasting. A week after STZ injection, diabetes was identified by measuring fasting blood glucose (FBG) and 2-hour blood glucose postload (2hBG) using a MediSense blood glucose meter and strips method. For 2hBG measuring, rats were given 50% glucose solution at a dose of 2 g/kg body weight by oral gavage after an overnight fasting. The rats with FBG ≥ 126 mg/dl (≥ 7.0 mmol/L) and/or 2hBG ≥ 200 mg/dl (≥ 11.1 mmol/L) were considered to be diabetic.

**4. Experimental design and animal groups:** The rats' body weights (BW) and blood glucose levels were measured before, during (weekly) and at the end of the experiment. The rats were classified into five groups (n = 8 rats in each):

**i. Normal control group:** The normal rats received equivalent amounts of vehicles; of a single IP injection of citric acid-natrium buffer and of daily oral distilled water and were fed with the laboratory normal diet for the experimental period (4 weeks).

**ii. Untreated diabetic group:** The diabetic rats continued to feed with the diabetogenic diet and received daily oral distilled water for four weeks.

**iii. Insulin-treated diabetic group:** The diabetic rats continued to feed with the diabetogenic diet and received subcutaneous injection of NPH insulin 10 IU/kg body weight/day for the 4 weeks [14]. The blood

glucose levels were cheeked weekly to avoid hypoglycemia and the last injection was given 24 hrs before sacrificing the rats.

**iv. Sitagliptin-treated diabetic group:** The diabetic rats continued to feed with the diabetogenic diet and received sitagliptin 30 mg/kg BW/day suspended in distilled water by oral gavage for four weeks [15].

**v. Sitagliptin-insulin co-treated diabetic group:** The diabetic rats continued to feed on the diabetogenic diet and received sitagliptin and insulin as described above for 4 weeks.

**5. Experimental procedures:** At the end of the experiment, the rats underwent the following procedures:

**i. Collection of urine samples:** The rats were accommodated in metabolic cages for two days before the end of the experiment. On day one, rats were allowed to explore and become familiar with the environment of the cage. On day two, 24-hours urine was collected to measure urine volume/24 hours and microalbuminuria [16]. Fresh urine sample was taken to measure urine creatinine.

**ii. Collection of blood samples and separation of serum:** After urine collection, the rats were fasted overnight and then held in a glass chamber to be anesthetized with diethyl ether. Venous blood samples were collected by heparinized microcapillary tubes from the retro-orbital plexus (24 hours after the last drugs' administration). The samples were incubated at 37°C until blood clotted and then centrifuged (5000 g, 10 min) for separation of serum which was stored at -20°C till used for biochemical estimations as described below.

**iii. Tissue sampling:** At the end of the experiment, the rats were weighed and then sacrificed. The kidneys were removed and washed carefully. The right kidney was weighed for calculating the kidney weight/ body weight (KW/BW) ratio (g/Kg). Then, kidneys were immediately kept in 10% phosphate buffered formalin and stored frozen in –80°C until required for the rat kidney transforming growth factor-beta1 (TGF-β1) and histopathological examination.

**6. Assessment of diabetic nephropathy:** At the end of the experiment, the following procedures were done for all rats:

**i.** The BW (g) (after 8 hours of fasting, in daytime), KW (g) and KW/BW ratio (g/Kg), as renal trophy indexes [12].

**ii.** Biochemical assays as the followings:

**a)** Blood glucose (mg/dL) was measured using glucose oxidase kit (Sigma, USA).

**b)** Serum insulin level (ng/mL) was measured using rat insulin ELISA kit (DRG International, Inc., Germany).

**c)** Serum advanced glycation end-products (AGEs) level (ng/L) was measured using rat ELISA kits (GSCIENCE, Inc., USA) [17].

**d)** Blood urea level was measured using urea kits (Diamond Diagnostic, Egypt) [18].

**e)** Serum and urine creatinine levels were measured using creatinine kits (Diamond Diagnostic, Egypt) [19].

**f)** Creatinine clearance was estimated using the following equation [20]:

Creatinine clearance (mL/min) = U X V/P, where U: Urine creatinine level (mg/dL), V: Volume of urine per minute (mL/min), P: Plasma creatinine level (mg/dL)

**g)** Urinary albumin (microalbuminuria) (mg/dL) was measured using microalbumin immunoassay kits (i-CHROMA Reader. Boditech Med. Inc., Korea) [21].

**h)** Rat kidney TGF-β1 (pg/mL): The right kidney was washed in ice cold saline (0.9 %), weighted and homogenized in chilled phosphate buffer (0.1M, Ph7.4) using glass tissue homogenizer. The homogenate was centrifuged for 20 minutes at 3000 rpm; the supernatant was stored at −80°C till used for biochemical assay, using rat TGF-β1 ELISA kits (eBioscience, Inc., USA).

**iii. Light microscopy:** The stored left kidneys were prepared and sectioned for **histopathological examination** (glomeruli, tubules, interstitium and vasculature):

**a)** Haematoxylin and eosin (H&E) stained sections were used for assessment of histopathological parameters [22].

**b)** Masson trichrome technique was used for detection of collagen [23].

**7. Statistical analysis:** The parameters were presented as means and standard error of means (mean ± SEM) for all groups. One-way analysis of variance (one-way ANOVA) and Fisher's least significant difference (LSD) test were applied for comparisons between experimental groups. All statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) v.16.0 (IBM Corp., Armonk, NY, USA).The minimal level of significance was identified at $P < 0.05$.

## RESULTS

**1. Effects of insulin, sitagliptin and combined sitagliptin-insulin treatments on the BW, KW and KW/BW ratio in diabetic rats (Table 1):** Concerning the BW, no significant differences were encountered between the different groups of rats at the beginning of diabetogenic diet feeding, and the results were excluded from tables in order to facilitate data comparison and interpretation. The untreated diabetic group showed significant decrease in the BW and increases in the KW and KW/BW ratio ($P0 < 0.05$) when compared to the normal control group. The insulin-treated diabetic group showed significant increase in the BW and decrease in the KW/BW ratio ($P1 < 0.05$), but insignificant change in the KW ($P1 > 0.05$) compared to the untreated group. The sitagliptin-treatment in diabetic group showed insignificant changes in the BW, KW and KW/BW ratio ($P1 > 0.05$) when compared to the untreated diabetic group. However comparing to sitagliptin, insulin produced greater effects on the BW and KW/BW ratio ($P2 < 0.05$) but insignificant effect on the KW ($P2 > 0.05$). The sitagliptin-insulin- co-treated diabetic group showed significant increase in BW and decreases in the KW and KW/BW ratio when compared with the untreated diabetic group ($P1 < 0.05$). However, the sitagliptin-insulin co-treated group did not differ in these three parameters when compared with the insulin-treated group ($P2 > 0.05$). When compared with the sitagliptin-treated group, this co-treated group showed significant increase in BW and decrease in the KW/BW ratio ($P3 < 0.05$), but insignificant change in the KW ($P3 > 0.05$) (i.e.in this co-treated group, effects greater than insulin or sitagliptin alone).

**Table 1:** Effects of insulin, sitagliptin and combined sitagliptin-insulin treatments (for 4 weeks after induction of DM) on the body weight (BW), kidney weight (KW) and KW/BW ratio in diabetic rats

| Parameter \ Rat group | Normal control group | Untreated diabetic group | Insulin-treated diabetic group (10 IU/Kg) | Sitagliptin-treated diabetic group (30 mg/Kg) | Sitagliptin-insulin- co-treated diabetic group |
|---|---|---|---|---|---|
| BW (g) | 279.48 ± 3.98 | 113.11 ± 4.88<br>P0 < 0.05 | 182.78 ± 3.09<br>P1 < 0.05 | 115.96 ± 3.08<br>P1 > 0.05<br>P2 < 0.05 | 177.18 ± 2.79<br>P1 < 0.05<br>P2 > 0.05<br>P3 < 0.05 |
| KW (g) | 0.61 ± 0.01 | 0.99 ± 0.01<br>P0 < 0.05 | 0.89±0.03<br>P1 > 0.05 | 0.93 ± 0.03<br>P1 > 0.05<br>P2 > 0.05 | 0.83 ± 0.02<br>P1 < 0.05<br>P2 > 0.05<br>P3 > 0.05 |
| KW/BW ratio (g/Kg) | 2.18 ± 0.06 | 8.76 ± 0.20<br>P0 < 0.05 | 4.89 ± 0.15<br>P1 < 0.05 | 8.08 ± 0.19<br>P1 > 0.05<br>P2 < 0.05 | 4.69 ± 0.17<br>P1 < 0.05<br>P2 > 0.05<br>P3 < 0.05 |

- Values are expressed as mean ± SEM. Number of rats in each group = 8.
- P0: Compared with the normal control group. - P1: Compared with the untreated diabetic group.
- P2: Compared with the insulin-treated diabetic group. - P3: Compared with the sitagliptin-treated diabetic group.

**2. Effects of insulin, sitagliptin and combined sitagliptin-insulin treatments on the blood glucose, serum insulin and AGEs in diabetic rats (Table 2):** The untreated diabetic group showed significant increases in blood glucose and serum AGEs and decrease in serum insulin (P0 < 0.05) when compared to the normal control group. The insulin-treated diabetic group showed significant decreases in blood glucose and AGEs (P1 < 0.05) and an insignificant change (P1 > 0.05) in serum insulin when compared with the untreated group. When compared to the untreated group, the sitagliptin-treated diabetic group showed significant decreases in the blood glucose and AGEs and an increase in serum insulin (P1 < 0.05). When compared with the insulin-treated group, the sitagliptin-treated group showed a significant increase in serum insulin and a decrease in AGEs (P2 < 0.05). However, insulin produced greater lowering effect on blood glucose than sitagliptin (P2 < 0.05), while sitagliptin produced rising effect on serum insulin and lowering effect on AGEs greater than insulin treatment (P2 < 0.05). The sitagliptin-insulin co-treated diabetic group showed significant decreases in blood glucose and AGEs and an increase in the serum insulin (P1 < 0.05) when compared with the untreated group. When compared with insulin alone, sitagliptin-insulin co-treatment significantly increased serum insulin and decreased serum AGEs (P2 < 0.05). However, the glucose-lowering effects of sitagliptin-insulin co-treatment and of insulin treatment alone were not different (P2 > 0.05). In contrast, when compared with sitagliptin alone, the co-treatment significantly decreased blood glucose and AGEs (P3 < 0.05), but the effects on serum insulin were not different between the two treatments (P3 > 0.05). Collectively, the effects of co-treatment on blood glucose, insulin and AGEs levels were greater than that of treatment with sitagliptin or insulin alone.

**Table 2:** Effects of insulin, sitagliptin and combined sitagliptin-insulin treatments (for 4 weeks after induction of DM) on the blood glucose, serum insulin and serum AGEs in diabetic rats

| Parameter \ Rat group | Normal control group | Untreated diabetic group | Insulin-treated diabetic group (10 IU/Kg) | Sitagliptin-treated diabetic group (30 mg/Kg) | Sitagliptin-insulin- co-treated diabetic group |
|---|---|---|---|---|---|
| Blood glucose (mg/dL) | 102.12 ± 2.31 | 395.81 ± 18.70<br>P0 < 0.05 | 126.78 ± 9.41<br>P1 < 0.05 | 188.11 ± 16.70<br>P1 < 0.05<br>P2 < 0.05 | 115.41 ± 10.50<br>P1 < 0.05<br>P2 > 0.05<br>P3 < 0.05 |
| Serum insulin (ng/mL) | 3.02 ± 0.09 | 0.11 ± 0.02<br>P0 < 0.05 | 0.13 ± 0.02<br>P1 > 0.05 | 0.92 ± 0.04<br>P1 < 0.05<br>P2 < 0.05 | 1.11 ± 0.06<br>P1 < 0.05<br>P2 < 0.05<br>P3 > 0.05 |
| Serum AGEs (ng/mL) | 82.71 ± 0.42 | 251.26 ± 2.47<br>P0 < 0.05 | 181.401 ± 3.01<br>P1 < 0.05 | 159.47 ± 2.09<br>P1 < 0.05<br>P2 < 0.05 | 101.56 ± 3.12<br>P1 < 0.05<br>P2 < 0.05<br>P3 < 0.05 |

- Values are expressed as mean ± SEM. Number of rats in each group = 8.
- P0: Compared with the normal control group. - P1: Compared with the untreated diabetic group.
- P2: Compared with the insulin-treated diabetic group. - P3: Compared with the sitagliptin-treated diabetic group.

**3. Effects of insulin, sitagliptin and combined sitagliptin-insulin treatments on the blood urea, serum creatinine, urine output, creatinine clearance, albuminuria, and renal tissue TGF-β1 in diabetic rats (Table 3):** The untreated diabetic group showed significant increases in blood urea, serum creatinine, urine output, albuminuria, and renal tissue TGF-β1, and a decrease in creatinine clearance ($P0 < 0.05$) when compared to the normal control group. Treatment with insulin produced significant decreases in blood urea, serum creatinine, urine output, albuminuria, and renal tissue TGF-β1, and an increase in creatinine clearance ($P1 < 0.05$) when compared with the untreated diabetic group. Treatment with sitagliptin produced significant decreases in the blood urea, serum creatinine, urine output, albuminuria and renal tissue TGF-β1 and an increase in creatinine clearance ($P1 < 0.05$) when compared with the untreated group, and also, produced significant decreases in the blood urea, serum creatinine, albuminuria and renal tissue TGF-β1 (even all greater than treatment with insulin), and in the urine output (though lesser than treatment with insulin) and an increase in creatinine clearance (lesser than with insulin) ($P2 < 0.05$) when compared with the insulin-treated diabetic group. The sitagliptin-insulin- co-treated diabetic group showed significant decreases in blood urea, serum creatinine, urine output, albuminuria, and renal tissue TGF-β1, and an increase in the creatinine clearance ($P1 < 0.05$) when compared with the untreated group. These changes in the above measures that were greater in the co-treatment group than those in the insulin-treated ($P2 < 0.05$), and sitagliptin-treated diabetic groups ($P3 < 0.05$) (i.e. generally overall effects were greater than sitagliptin or insulin alone).

**Table 3:** Effects of insulin, sitagliptin and combined sitagliptin-insulin treatments (for 4 weeks after induction of DM) on the blood urea, serum creatinine, creatinine clearance, urine output, albuminuria and renal tissue TGF-β1 in diabetic rats

| Parameter \ Rat group | Normal control group | Untreated diabetic group | Insulin-treated diabetic group (10 IU/Kg) | Sitagliptin-treated diabetic group (30 mg/Kg) | Sitagliptin-insulin- co-treated diabetic group |
|---|---|---|---|---|---|
| Blood urea (mg/dL) | 26.12 ± 0.51 | 63.41 ± 0.50<br>P0 < 0.05 | 56.72 ± 0.21<br>P1 < 0.05 | 51.31 ± 0.34<br>P1 < 0.05<br>P2 < 0.05 | 42.038 ± 0.48<br>P1 < 0.05<br>P2 < 0.05<br>P3 < 0.05 |
| Serum creatinine (mg/dL) | 0.44 ± 0.01 | 1.48 ± 0.02<br>P0 < 0.05 | 0.99 ± 0.03<br>P1 < 0.05 | 0.84 ± 0.03<br>P1 < 0.05<br>P2 < 0.05 | 0.71 ± 0.01<br>P1 < 0.05<br>P2 < 0.05<br>P3 < 0.05 |
| Creatinine clearance (mL/min) | 0.78 ± 0.02 | 0.25 ± 0.01<br>P0 < 0.05 | 0.51 ± 0.01<br>P1 < 0.05 | 0.44 ± 0.03<br>P1 < 0.05<br>P2 < 0.05 | 0.71 ± 0.03<br>P1 < 0.05<br>P2 < 0.05<br>P3 < 0.05 |
| Urine output (mL/24h) | 9.22 ± 1.09 | 35.14 ± 0.66<br>P0 < 0.05 | 21.21 ± 0.44<br>P1 < 0.05 | 32.1 ± 0.37<br>P1 < 0.05<br>P2 < 0.05 | 17.10 ± 0.66<br>P1 < 0.05<br>P2 < 0.05<br>P3 < 0.05 |
| Albuminuria (mg/dL) | 4.81 ± 0.81 | 9.91 ± 0.18<br>P0 < 0.05 | 7.78 ± 0.28<br>P1 < 0.05 | 6.61 ± 0.26<br>P1 < 0.05<br>P2 < 0.05 | 5.31 ± 0.27<br>P1 < 0.05<br>P2 < 0.05<br>P3 < 0.05 |
| Renal tissue TGF-β1 (pg/mL) | 28.2 ± 0.41 | 118.61 ± 2.09<br>P0 < 0.05 | 102.21 ± 2.11<br>P1 < 0.05 | 82.71 ± 1.51<br>P1 < 0.05<br>P2 < 0.05 | 59.52 ±2.8<br>P1 < 0.05<br>P2 < 0.05<br>P3 < 0.05 |

- Values are expressed as mean ± SEM. Number of rats in each group = 8.
- P0: Compared with the normal control group. - P1: Compared with the untreated diabetic group.
- P2: Compared with the insulin-treated diabetic group. - P3: Compared with the sitagliptin-treated diabetic group.

**4. Effects of insulin, sitagliptin and combined sitagliptin-insulin treatments on the histopathology of kidney in diabetic rats (Figure 1A-E):**

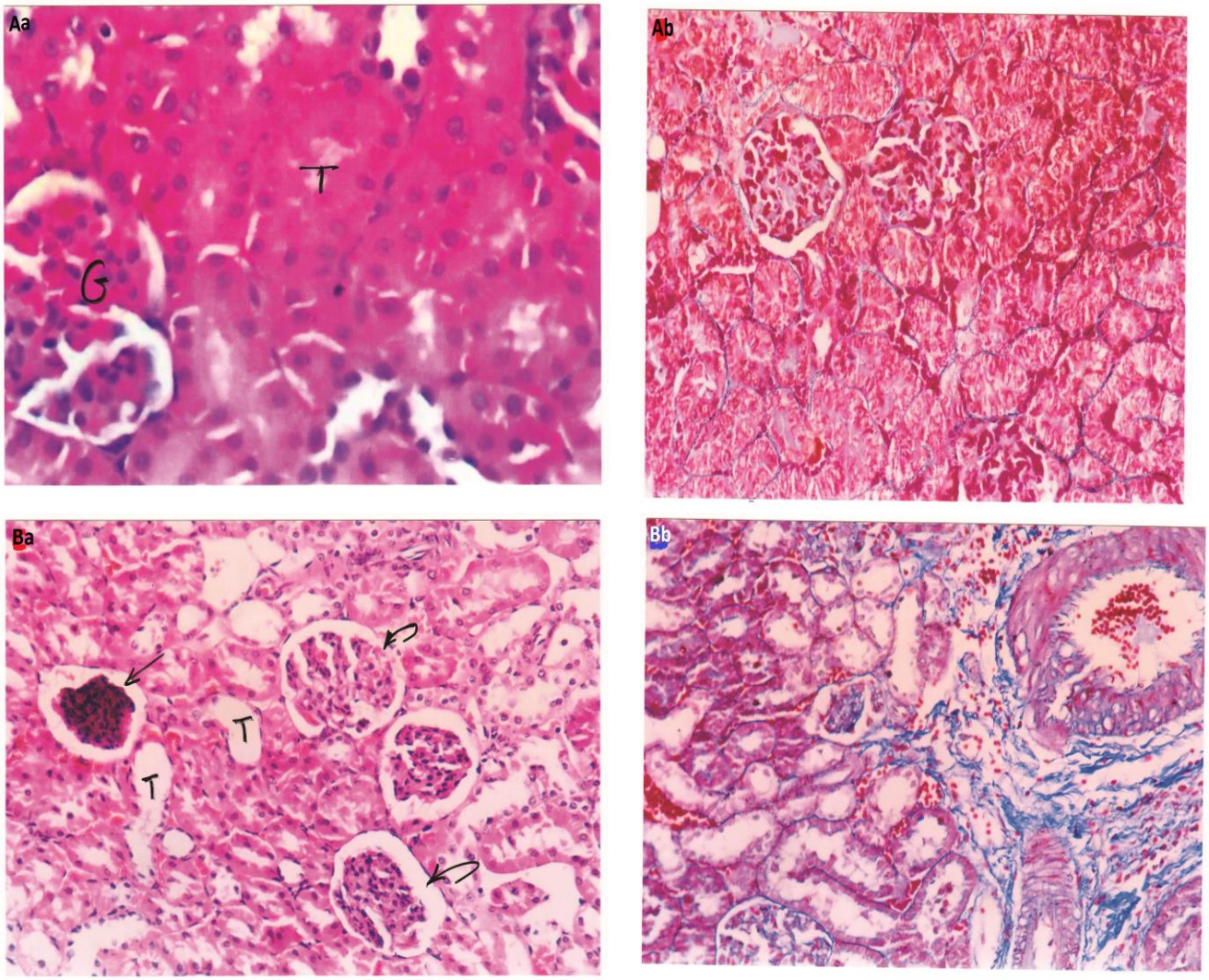
**A.** The control rat's kidney showed normal histological structure of the renal tubules, glomeruli and vasculature (Figure 1Aa). The connective tissue interstitium revealed minimal amount of collagen fibers among renal tubules (Figure 1Ab).

**B.** The untreated diabetic rat's kidney showed variable histopathological changes in the form of deformed glomeruli with clumped capillary tuft, and hyperplasia of mesangial cells and accumulation of mesangial matrix. Renal tubules showed necrotic epithelial lining and cystic dilatation, with the inflammatory cells reaction (Figure 1Ba). The renal stroma revealed massive perivascular and peritubular accumulation of collagen fibers (Figure 1Bb).

**C.** The insulin-treated diabetic rat's kidney showed mild improvement. Some renal tubules appeared nearly normal, however others renal tubules revealed cystic dilatation and necrotic epithelial cell lining. Some renal glomeruli showed glomerulosclerosis, however others appeared nearly normal (Figure 1Ca). Renal interstitium showed moderate amount of collagen fibers mainly around renal blood vessels which still showed congestion (Figure 1Cb).

**D.** The sitagliptin-treated diabetic rat's kidney showed moderate improvement and better histological appearance than previous diabetic rat's kidney group. Most of renal tubules and glomeruli appeared nearly like control group (Figure 1Da), with nearly normal amount of interstitium collagen fibers (Figure 1Db).

**E.** The sitagliptin-insulin- co-treated diabetic rat's kidney showed good improvement of renal histological appearance, with nearly normal renal glomeruli and tubules like control group (Figure 1Ea), and normal interstitium collagen fibers (Figure 1Eb).



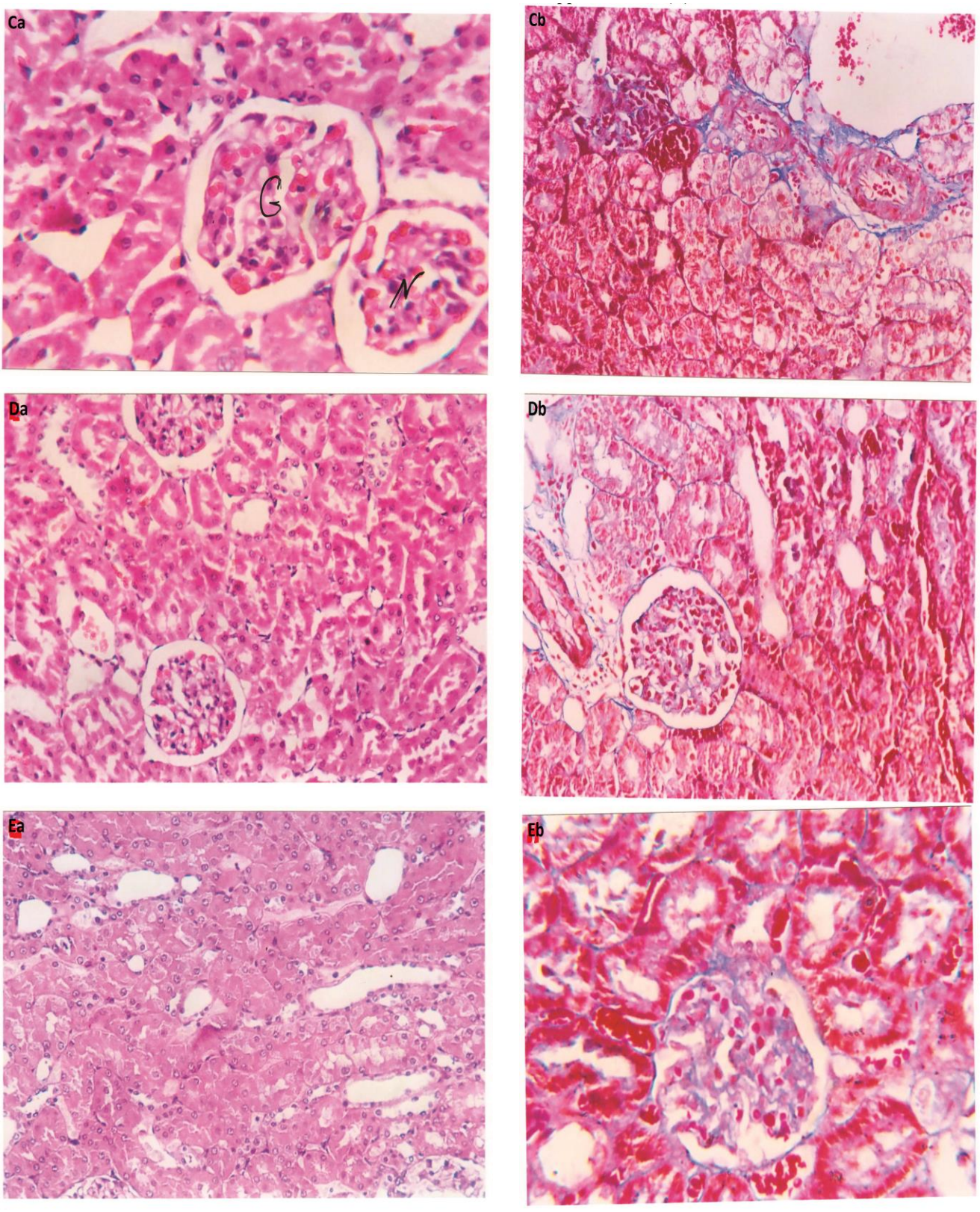



**Figure 1A-E:**

**A.** Section of **control rat's kidney** showing: **(Aa):** normal histological structure of renal tubules (T) and glomeruli (G) (H&E x 400); **(Ab):** Traces of collagen fibers (blue color) among the renal interstitium (MT x 200).

**B.** Section of **untreated diabetic rat's kidney** showing: **(Ba):** deformed renal glomeruli with clumped capillary tuft (arrow) and other glomeruli with hyperplasia of mesangial cells and accumulation of mesangial matrix (arrow), and also, cystic dilatation and necrotic epithelium lining of renal tubules (T) (H&E x 200); **(Bb):** perivascular accumulation of collagen fibers (blue color) (MT x 200).

**C.** Section of **insulin-treated diabetic rat's kidney** showing: **(Ca):** mild improvement; some renal tubules with cystic dilatation and atrophy of cell lining. Some glomeruli appear with glomerulosclerosis (G), while others appear normal (N). (H, E x 400); **(Cb):** moderate amount of collagen fibers around renal vessels. (MT x 200).

**D.** Section of **sitagliptin-treated diabetic rat's kidney** showing: **(Da):** moderate improvement, most of renal tubules and glomeruli appearing nearly normal; however few tubules appear necrotic (H&E x 200); **(Db):** nearly normal amount of collagen in the C.T. stroma (MT x 200).

**E.** Section of **sitagliptin-insulin- co-treated diabetic rat's kidney** showing: **(Ea):** good improvement of renal tissues, appearing nearly the same as control rat (H&E x 200); **(Eb):** normal collagen fibers in the interstitium (blue color) (MT x 400).

## DISCUSSION

DM causes vascular complications which are the leading cause of morbidity and mortality in diabetic patients. Diabetic nephropathy (DN) is a common microvascular complication of diabetes mellitus and the leading cause of end-stage renal disease [9]. There is a strong association between DN and poor glucose control in patients with type-2 diabetes mellitus (T2DM), which mostly requires a combination of various glucose-lowering agents. The advent of new oral antidiabetic agents, such as the incretin enhancers (including sitagliptin), has expanded the therapeutic armamentarium of diabetes and its complication. The ability of antidiabetic drugs to ameliorate DN might be as important as their capability to control blood glucose level [7, 9, 12]. The DPP-IV inhibitors may be used as monotherapy or in combination with other antidiabetic compounds, metformin, thiazolidinediones or even sulfonylureas [1, 3, 24].

The present study assessed and compared the glucose-lowering and renoprotective effects of four-week treatment with insulin or sitagliptin alone and sitagliptin-insulin combined therapy in rats with T2DM and nephropathy. In the present study, we established the rat model of T2DM with nephropathy using high-fat and high-sugar diet for six weeks and a single dose of streptozotocin 30 mg/kg as described in Ren et al. [13]. This animal model of T2DM is useful for testing the effects of the antidiabetic agents in controlling diabetes and diabetic nephropathy [25]. In our study, the untreated diabetic rats, four-weeks after induction of diabetes, developed weight loss, hyperglycemia, insulinopenia, and increases in serum AGEs, and also experienced both renal hypertrophy (increased KW and KW/BW ratio) and dysfunctions (increased blood urea, serum creatinine, urine output, albuminuria and renal tissue TGF-β1, and decreased creatinine clearance). These changes were associated with variable glomerular, tubulo-interstitial and vascular kidney pathological lesions. Four-week sitagliptin treatment (30 mg/kg/day) led to reductions in blood glucose, serum AGEs, renal tissue TGF-β1, blood urea, serum creatinine and albuminuria, and elevations in the serum insulin and creatinine clearance. In addition, sitagliptin moderately ameliorated kidney lesions but produced no effect on, though stabilized, the BW, KW and KW/BW ratio. However, while insulin produced greater effects on BW, KW/BW, blood glucose level and urine output than sitagliptin, the latter was superior in elevating serum insulin, lowering AGEs, improving kidney functions and in ameliorating these pathological kidney lesions.

Our findings are consistent with those reported by other authors who found that six-week treatment with low-dose sitagliptin (10

mg/kg BW), in diabetic ZDF (fa/fa) rats, stabilized the loss in BW, improved the hyperglycemia and partially prevented insulinopenia, but did not change kidney trophism viewed by the increased KW and KW/BW ratio. Also, sitagliptin improved the kidney functions and ameliorated the kidney lesions [12, 26].

Other study also found that six-week low-dose sitagliptin (10 mg/kg/day) ameliorated kidney lesions and promoted partial improvements in metabolic and renal profiles, with exception of serum creatinine [27]. Liu et al. [2] induced diabetes in normally-fed rats by a single intraperitoneal injection of STZ 60 mg/kg BW and reported similar findings to our study (i.e. hyperglycemia and kidney dysfunction) in the untreated diabetic rats. Also, DPP-IV inhibitors decreased proteinuria, urinary albumin and serum creatinine, improved creatinine clearance and delayed kidney injuries in diabetic rats [2]. However, some differences, either in the effects or magnitudes, between the present and previous studies could be explained on the basis of the differences in the doses of drugs used, periods of treatments, initial weights of the animals, feeding diets, humans or animal models of DM, and types of DM.

To our knowledge, this is the first experimental study to examine the effects of the sitagliptin-insulin combined therapy on T2DM with diabetic nephropathy in rats. The combination of the incretin-based therapies e.g. DPP-4 inhibitors with insulin has, in theory, logical appeal. While basal insulin primarily improves fasting plasma glucose control, the glucose-dependent effect of incretins will additionally benefit postprandial plasma glucose control leading to reduced HbA1c without weight gain or increase in hypoglycemia [28]. However, the magnitude of the effect of the drug combination on diabetic nephropathy is still unknown. In the present study, four-week co-treatment with sitagliptin (30 mg/kg/day) and insulin (10 IU/kg/day) corrected the loss in BW and the increases in KW and KW/BW ratio. Also, this combined therapy produced greater glycemic control, more reductions in the high serum AGEs and renal tissue TGF-β1 and more elevations in the lowered serum insulin. This combination also produced greater improvements in the above kidney functions parameters and more ameliorations in kidney lesions compared to those associated with sitagliptin or insulin monotherapy. The superiority of sitagliptin-insulin combined therapy in improving kidney functions and morphology when compared to treatment with sitagliptin or insulin alone is likely owing to the additional glucose-lowering effect of insulin. This would suggest that targeting hyperglycemia by insulin, while increasing the active levels of GLP-1 and GIP by sitagliptin, might become a proper therapeutic approach to reverse the pathogenesis of kidney injuries in T2DM.

Our results of these beneficial effects of sitagliptin add-on therapy to insulin in controlling T2DM with diabetic nephropathy are supported by several studies which reported that most patients with T2DM will need incrementally more complex therapeutic regimens to control hyperglycemia and will require insulin therapy as the disease progresses [3, 11, 29]. Insulin is very effective in reducing hyperglycemia and may improve β-cell function in patients with T2DM. Thus, adding oral DPP-IV inhibitors to insulin can improve glycemic control and lower the required insulin dose, resulting in less weight gain and lower risk for hypoglycemia [11, 29]. DPP-4 inhibitors are thought to exert their blood glucose-lowering effects *via* mechanisms other than increasing peripheral insulin levels; the possible mechanism is suppressing glucagon secretion. Thus, sitagliptin may contribute to improving blood glucose control in T2DM patients inadequately controlled with insulin monotherapy [3].

Based on the literature, the other potential antidiabetic actions of DPP-IV inhibitors also include prevention of β-cell failure, stimulation of insulin release, improvement of glycemic and hemoglobin A1c (HbA1c) control, and reduction of triglyceride and free fatty acid

levels, and also have vasculo-protective actions [1, 2, 30]. Glucose-mediated cellular damage and dysfunction are tightly linked to poor glucose control and mediated through different molecular mechanisms. The mechanisms include increased polyol pathway flux, increased intracellular formation of AGEs, activation of protein kinase C (PKC) and hexosamine pathways and increased oxidative stress. Each of these mechanisms reflects a hyperglycemia-induced process: overproduction of superoxide by the mitochondrial electron-transport chain [31]. Some of these mechanisms are potentially modifiable by DPP-4 inhibition [1, 32]. However, it has been suggested that hyperglycemia-induced damage to kidney cells enhances biosynthesis of DPP-IV and decreases levels of GLP-1 and expression of GLP-1 receptor (GLP-1R) [33]. DPP-IV is widely distributed on the surface of the kidney's proximal tubular cells and endothelial cells [34, 35]. GLP-1, in addition to its anti-inflammatory action, has the ability to reduce AGEs production by activation of protein kinase A [36, 37]. Hocher et al. [1] described that DPP-IV inhibitors have both GLP-1 dependent (increase GLP-1 levels in kidney) and GLP-1 independent effects since DPP-IV cleaves a wide range of other substrates (e.g. neuropeptides, hormones, cytokines, and chemokines) [1]. Other studies have shown that treatment with sitagliptin led to a rise in levels of GLP-1 in diabetic kidney [26, 38]. This finding suggests that the renoprotective effects of sitagliptin might derive, at least in part, from GLP-1/GLP-1R activation other than glycemic/insulinemic control. In addition, sitagliptin showed cytoprotective effects on other tissues and cells including heart, kidney, pancreas and retina where DPP-IV is also distributed widely [2, 12, 26].

Also, our study is in consistent with Liu et al. and others who showed that hyperglycemia produced an increase in the levels of TGF-β1 in kidney cortex of rats possibly linked to renal cell hypertrophy, interstitial fibrosis and renal dysfunction. The study demonstrated that sitagliptin was able to ameliorate the increase in renal tissue TGF-β1 levels. Other DPP-IV inhibitors, e.g. vildagliptin, were also found to ameliorate the increase in TGF-β1 expression in kidney. These findings suggested that overproduced TGF-β1 is one of the factors involved in the pathogenesis of diabetic nephropathy, and that down-regulation of the TGF-β1 system is a possible mechanism in the renoprotective effects of DPP-IV inhibitors dysfunction [2, 7, 39, 40]. Also, sitagliptin was also able to prevent the increase in expression of both TNF-α mRNA and IL-1β mRNA in diabetic kidney [26]. Reduction of oxidative stress and inflammation and improvement of endothelial dysfunction are other possible mechanisms underlying the renoprotective effects of DPP-IV inhibitors [12]. Furthermore, our study is in agreement with many authors who stated that GLP-1 receptor agonists, DPP-4 inhibitors, and SGLT2 inhibitors improve glycemic control when added to insulin and have a low propensity for hypoglycemia and weight gain (either no change in BW with DPP-4 inhibitors, or a reduction in BW with GLP-1 receptor agonists and SGLT2 inhibitors), and so may be preferred treatment options for insulin combination when compared with traditional therapies. In T2DM patients managed with diet or oral hypoglycemic agents, DPP-IV inhibitors improved blood glucose control by increasing β-cell responsiveness and ameliorated poor blood glucose control in insulin-treated T2DM patients [3, 11, 29]. However, more research is needed to explore the mechanisms implicated in the cyto- and vasculo-protective properties of DPP-IV inhibitors.

**Conclusion:** This study demonstrated that four-week treatment with sitagliptin, in streptozotocin-induced T2DM with nephropathy in rats, was associated with significant glucose-lowering and renoprotective effects. Co-treatment with sitagliptin and insulin produced greater effects than treatment with sitagliptin or insulin alone. Sitagliptin-insulin co-treatment might have unique clinical application for strict control of blood glucose and preventing the development and

progression of diabetic nephropathy in patients with T2DM.

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