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Mitigation of Endothelial Dysfunction by Saxagliptin in High-Fat Diet/Streptozotocin-Induced Diabetic Rats

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Abstract **Background and Aim** Saxagliptin is a dipeptidyl peptidase-4 (DPP-4) inhibitor that reduces postprandial plasma glucose associated with type 2 diabetes mellitus. It is a novel class of drugs for the treatment of diabetes mellitus. Diabetes mellitus is associated with an induction of vascular endothelial dysfunction (VED), an initial event that could lead to the pathogenesis of atherosclerosis and hypertension. Saxagliptin effects on endothelial dysfunction function have not been fully elucidated. This study was designed to assess the possible effect of saxagliptin on impaired endothelial function, oxidative stress, and inflammation beyond blood glucose control in type 2 diabetic rats. **Material and Methods** adult male Wistar rats were randomly divided into 3 equal groups. Group 1, Normal control group; rats were fed normal diet for 16 weeks; group 2 (diabetic group), rats were fed with a high fat diet for 8 weeks followed by administration of streptozotocin (100mg/kg body weight) by intraperitoneal injection and group 3, diabetic rats received saxagliptin (10mg/kg/day, orally) for 8 weeks. Fasting blood glucose and glucose tolerance test, blood pressure, nitric oxide (NO) bioavailability in the aortic tissue and soluble intracellular adhesion molecule -1 (sICAM-1) were determined in serum of the different groups. **Results:** high fat fed diabetic rats showed increased fasting glucose levels, systolic and diastolic blood pressure, and vascular oxidative stress. Saxagliptin treatment significantly decreased fasting blood glucose level, improved glucose tolerance test and decreased systolic blood and diastolic blood pressure. Saxagliptin also decreased serum sICAM-1 levels by 37% ($p \leq 0.05$), aortic NO was increased by 22% ($p \leq 0.05$) in diabetic treated rats compared with diabetic non treated group. **Conclusion:** The results of the present study support the concept of anti-inflammatory properties of saxagliptin through inhibition of the pro-inflammatory mediator, including sICAM-1, which are independent of its glucose-lowering properties. In addition, it reduces the vascular oxidative stress by increasing the NO vascular levels, which resulted in an improvement of endothelial function in a diabetic rat model. These beneficial effects of saxagliptin on vascular function with its hypotensive effects might serve it as a potential therapy for type 2 diabetes with endothelial dysfunction.

Keywords Saxagliptin, DPP-4 inhibitor, Endothelial cell (EC), Type 2 diabetes, sICAM-1molecule, High-fat diet

1. Introduction

The worldwide prevalence of diabetes mellitus, particularly type 2 has increased significantly in recent years. Type 2 diabetes alters the vascular responsiveness to several vasoconstrictors and vasodilators and is a major factor underlying development of cardiovascular disease**[6]**. Many of the complications of diabetes are related to increased serum glucose and increased generation of reactive oxygen species, which lead to endothelial dysfunction. Impaired endothelium-dependent relaxation to vasodilators such as acetylcholine is a common feature in both conduit and resistance arteries from experimental models of type 1 and type 2 diabetes**[5; 7; 8; 34; 39]**.

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Endothelial cell (EC) dysfunction is associated with a loss of normal vasodilation, a characteristic feature of hypertension and a major contributor to atherothrombotic disease**[17; 23]**. EC dysfunction, along with reduced nitric oxide (NO) bioavailability, has been observed in patients and animal models of diabetes**[16; 43]**. Diabetes and hypertension are associated with the expression of intercellular adhesion molecule-1 (ICAM-1), which mediates adhesion and transmigration of leukocytes to the vascular endothelial wall where they promote plaque growth and instability**[25]**.

saxagliptin is a dipeptidyl peptidase-4 (DPP-4) inhibitor that reduces postprandial plasma and glycated hemoglobin A_{1c}**[14; 44]**. Inhibition of DPP4 increase the glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide half-life, which improve postprandial glucose clearance**[4]**. They do so by stimulating pancreatic insulin release and inhibiting hepatic glucose release**[4; 48]**. Chronic inhibition of DPP4 leads to elevated GLP-1 levels

and improved metabolic control, resulting in enhanced insulin sensitivity and protection against β cell apoptosis and hyperglycemia in experimental models**[4]**. These agents reduce postprandial plasma glucose even in lean animals with normal insulin sensitivity**[20]**.

DPP-4 is widely expressed in the cardiovascular system, especially in endothelial cells**[28]**. DPP4 inhibition with saxagliptin was recently shown to reduce blood pressure and increase sodium excretion in young, hypertensive rats, but not in adult animals**[33]**. A small but statistically significant reduction in blood pressure was observed in non diabetic subjects with hypertension treated with a DPP inhibitor, but the mechanisms are not understood**[29]**. Saxagliptin was shown to improve endothelial NO release and reduce CD40 and peroxynitrite levels in Zucker obese rats, independently of changes in the fasting glucose**[26]**.

This study was designed to assess the effect of saxagliptin on the blood pressure, to study the antioxidant and anti-inflammatory effects of saxagliptin on the vascular endothelium in type 2 diabetes mellitus beyond blood glucose control.

2. Experimental Protocol

The current study was carried on 36 male adult albino rats at 4 weeks old (body weight 130- 160 gm), rats were housed under hygienic conditions at 20°C–25°C in a 12 hr/12 hr light/dark cycle for 16 weeks**[22]**. They had free access to standard laboratory chow (El-Nasr Company, Abou-Zaabal, and Cairo, Egypt) and water. They have acclimatized for one week and were caged (6/cage) in fully ventilated room (at room temperature) in pharmacology department, Benha Faculty of Medicine. All experimental protocols were approved by the committee of Benha and Zagazig University.

3. Drugs and Chemicals

- Urethane (Ethyl Carbamate); (Prolabo, Paris) white crystals.
- Saxagliptin (onglyza, Astra-Zennica).
- Streptozotocin (STZ) powder creamy white: (Sigma Chemicals Co., U.S.A).
- Carboxy-methyle cellulose (powder) (El Nasr Pharmaceutical Chemicals Co.)

4. Study Design

After acclimatization for 1 week, the rats were assigned into 3 experimental groups (12 rats each) and treated for 8 weeks as follow:

Group (1):normal control group: Rats fed normal diet (the composition of the standard chow was 20% protein, 40% carbohydrates, 5% fat, 3% fiber and other constituents composed 32%., received physiological saline and served as normal control group.

Group (2): diabetic non-treated group: rats that were given high fat diet (HFD) with 60% kcal% fat that consists of 16.45 proteins, 25.6% carbohydrate and 55.0% fat (total 23.4kJ/g) in the form of cotton seed oil added to the laboratory chow for 8 weeks then injected with single dose of streptozotocin (100mg/kg, i.p).

Group (3): saxagliptin treated group: Rats had taken a HFD+STZ injection and received saxagliptin (10 mg/kg/day) by gavage. Administration of saxagliptin was started after the 8 weeks of high fat feeding and continued for 8 weeks. The doses and schedules of the study were chosen according to our preliminary study and in consistence with previous reports **(26; 21; Preston et al., 2012)**. All experimental protocols were approved by the ethical committee of the Faculty of Medicine, Benha and Zagazig University.

All drugs were dissolved in saline except streptozotocin (STZ 100mg/kg) dissolved in cold 0.1 mole citrate buffer (pH 4.5). 72 hours after STZ injection, diabetes was confirmed in rats by showing blood glucose levels increased to > 140 mg/100 ml (***Trinder, 1969***). The blood glucose concentration was measured using a glucometer from blood samples obtained by tail prick.

5. Biochemical Measurements

5.1. Determination of Serum Fasting Glucose Level

According to**[46]** using glucose enzymatic (GODPAP) - liquizyme Kits (Biotechnology, Egypt).

5.2. Oral Glucose Tolerance Test

Glucose levels were measured using Acc-Check Compact plus glucometer (Sanmina-SCI, San Jose, CA). All glucose measurements were preceded by 6-8 hours fasting periods. Glucose was administered orally at (1.5g/kg b.w) blood samples were taken at 5, 15, 30, 60, 90 and 120 minutes after glucose administration.

5.3. Measurement of Serum sICAM-1 Molecule Levels

Blood was collected from retro-orbital plexus into MiniCollect K_3 EDTA tubes (Greiner BioOne, Monroe, NC). Plasma was obtained by centrifugation (2700g for 10 minutes at 4 C), transferred into cryogenic vials, and stored at -80 C for future analysis. Enzyme-linked assay method was used to measure levels of sICAM-1 (Millipore Core, St.Charles, MO) according to**[37]**.

5.4. Blood Pressure Measurements for Rats

Blood pressure was measured using a BP-98A indirect (tail cuff) blood pressure meter (Softron, Tokyo, Japan) after warming the animals to 37ºC for 15 minutes. All animals were acclimated to this procedure for 3 days before measurement to minimize stress-induced variations in blood pressure. At least 3 measurements were made in every session, and the mean blood pressure was calculated as the

mean of 3 representative measurements differing from each other by no more than 5mm Hg. The pulse sign should be monitored to see when the pulse signal begins to become detectable and reach the maximum pulse height. The start of pulsation is viewed on the tracing and is referenced to the pressure curve signal at that point, this reading is analogous to systolic blood pressure, while, the mean blood pressure measured at stability of pulsation and referenced to the pressure curve**[13]**. Rats of systolic blood pressure of 140 mmHg or more were considered hypertensive**[13]**.

5.5. Aortic Tissue Content of NO

Aortic tissue content of NO was measured as total nitrite (NO_x), the stable degradation products of NO, after reduction of nitrate to nitrite by copper-cadmium alloy and measuring total nitrite (nitrite+nitrate) using Griess Reaget**[38]**.

5.6. Histopathological Aortic Examination

The rats were sacrificed by a blow on the head, the chest was opened, and the thoracic aorta was cut as near the heart as possible and dissected free as far as diaphragm. The aortic arteries were cleaned from surrounding tissues and fat then fixed in 10 % phosphate buffered formalin Fixed specimens were prepared for paraffin sections . Aorta cross sections (4μm) were cut at the aorta and stained with hematoxylin & eosin.

6. Statistical Analysis

Results are presented as the mean±S.E.M. Statistical differences between the means of the various groups were evaluated using one-way analysis of variance (ANOVA) followed by Tukey's test. Data were considered statistically significant at a p value <0.05.

Results:

6.1. Diabetes Induced Changes in Rats

High fat diet for 8 weeks with streptozotocin (100mg/kg, i.p) significantly increased blood glucose level, blood pressure, and serum soluble intercellular adhesion molecule-1. However, it reduced aortic nitric oxide levels. High cholesterol diet produced significant pathological changes in aorta.

6.2. Effects of Saxagliptin on Blood Glucose

It was observed that treatment with saxagliptin (10mg/kg, day) modestly decreased fasting blood glucose level in comparison with diabetic non treated group (table 1).

6.3. Effects of Saxagliptin on Glucose Tolerance Test

Saxagliptin treatment improved the response of diabetic rats to a glucose challenge test over 60 minutes when compared with diabetic non-treated group (figure 1D).

6.4. Effects of Saxagliptin on Nitric Oxide

Treatment with saxagliptin for 8 weeks significantly mitigated the effect of diabetes on aortic nitric oxide (NO) as compared with diabetic non-treated group (figure 1A, B).

6.5. Serum sICAM-1 Levels

Saxagliptin significantly reduced the effects of diabetes on sICAM-1 molecule as compared with diabetic non treated group (figure 2C). Plasma sICAM-1 decreased by 37% with saxagliptin (8.9±2.9 ng/ml) versus (14.0±3.7 ng/ml) ($p<0.05$) (Fig. 1C).

6.6. Effects of Saxagliptin on Blood Pressure

Saxagliptin treatment was associated with reduced systolic blood pressure and diastolic blood pressure by 6%, 9% respectively as compared with diabetic non treated group (figure 2).

6.7. Aortic Histopathological Structure

Histological examination of a cut section of the aorta of control group showed that the wall of the aorta consists of tunica intima, tunica media, and tunica adventitia. The tunica intima consists of an endothelial coat of flattened squamous cells resting on a complete basal lamina and is supported by a subendothelial loose connective tissue. The tunica media consists largely of elastic, concentric laminae and variable amounts of smooth muscle cells. The tunica adventitia contains bundles of collagen fibers and a few elastic fibers, both of which have a loose, helical arrangement (Figure 3A). In diabetic none treated group there were ulcerated endothelial cells of the intima with collection of foamy histocytes and fat globules and formation of fatty streaks with degeneration. The media and adventitia showed fibrosis and inflammatory cell infiltration (Figure 3B). saxagliptin treatment markedly decreased the size of fatty streaks, foamy and inflammatory cell infiltration. Regeneration of the endothelial cells of the intima was observed (Figure 3C).

Table (1). Effects of chronic treatment with saxagliptin (10mg/kglday, orally) for eight weeks on fasting glucose levels (FBG), systolic blood pressure (SBP), diastolic blood pressure (DBP) in diabetic rats

Animal groups	FBG (mg/dl)	SBP (mmHg)	DBP (mmHg)
Normal control	76±2.1	102.25±1.31	68.25±2.88
Diabetic non treated	169±3.1*	127.58±1.38*	99.25±2.69*
Saxagliptin treated	138±1.9	111.92±1.30	91.91±2.46

Data are mean ±SEM. (n=12 per group). *p<0.05

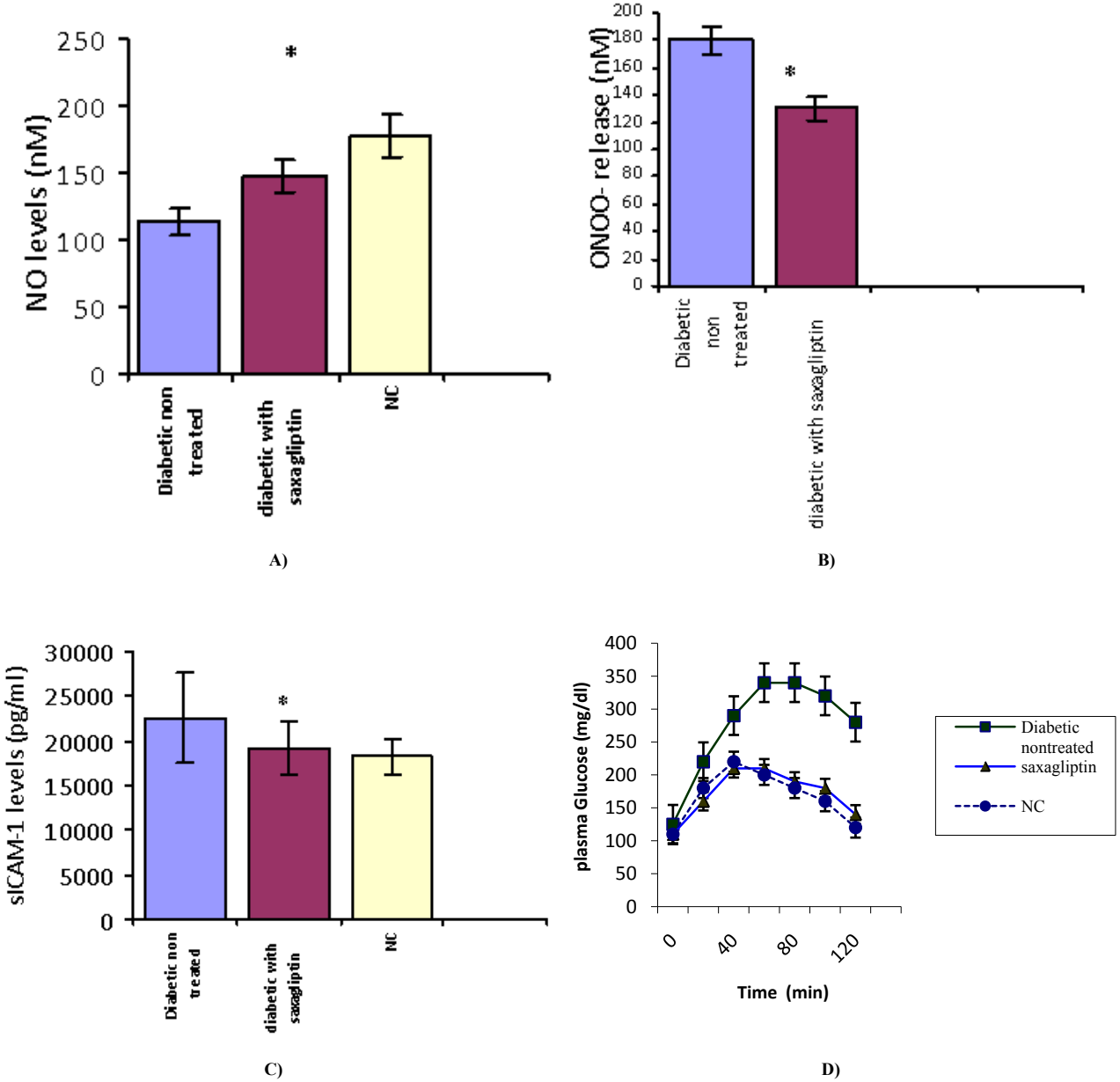
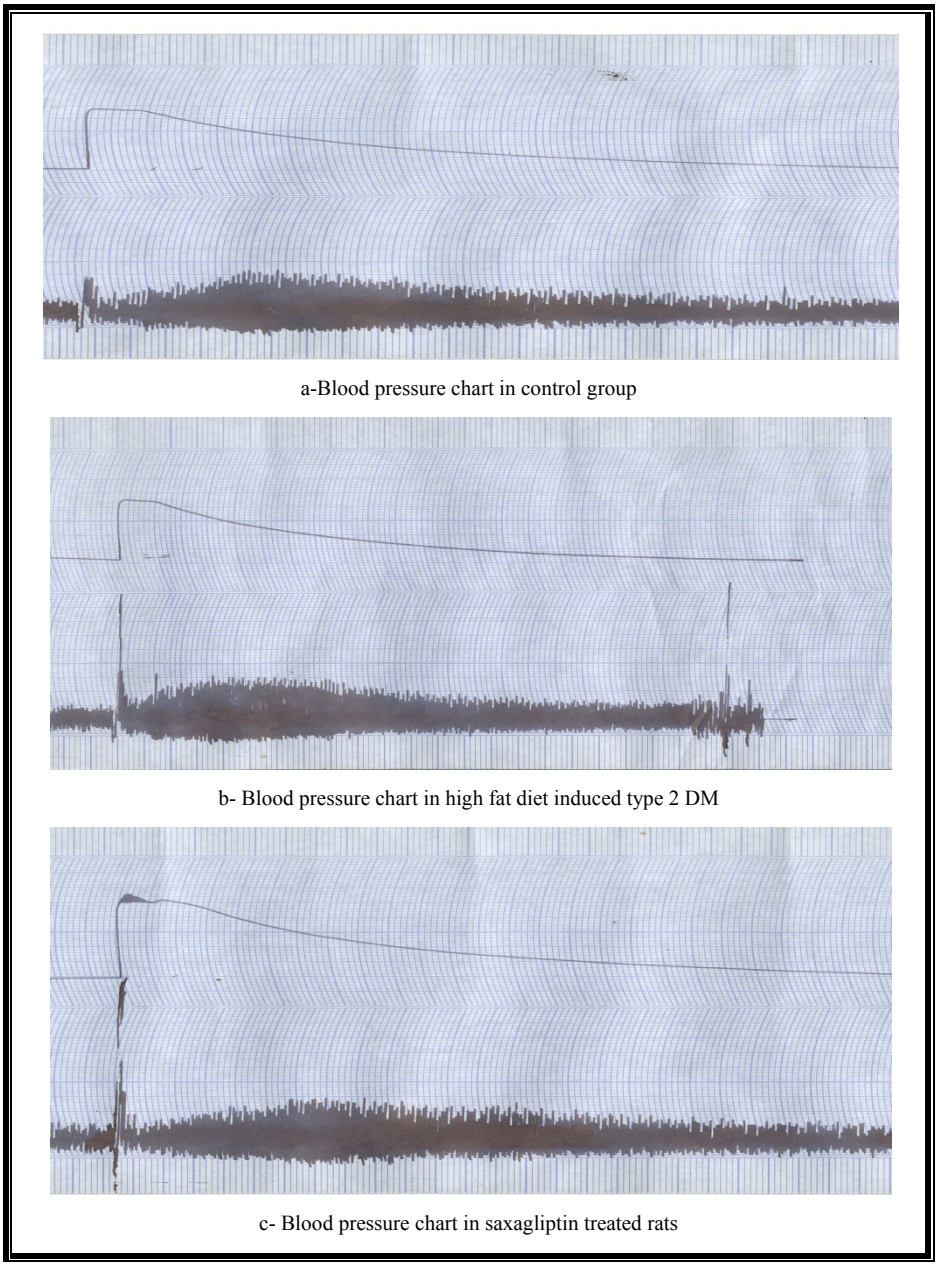


Figure 1. Effects of diabetes and saxagliptin treatment on nitric oxide **(A)**, peroxynitrite **(B)** release from aorta endothelial cells from diabetic rats. Values are mean ±SEM (n=12), *p<0.05 versus vehicle treated group. **(C)** Effects of diabetes and saxagliptin treatment on soluble intercellular adhesion molecule-1 (sICAM-1) levels. Values are mean ± SEM (n=12). *p<0.05 versus compared with non-treated diabetic group**. (D)** saxagliptin treatment normalized the response of diabetic animals to a glucose challenge test. Diabetic rats (black squares) showed evidence of insulin resistance in response to a glucose challenge test as compared with normal control animals (black circles). Treatment with saxagliptin (10mg/kg/day) for 8 weeks (black triangles) reproduced the normal response. Saxagliptin or salin was administered per orally 4 hr. prior to starting the glucose tolerance test. Glucose was administered at 1.5g/kg/body weight

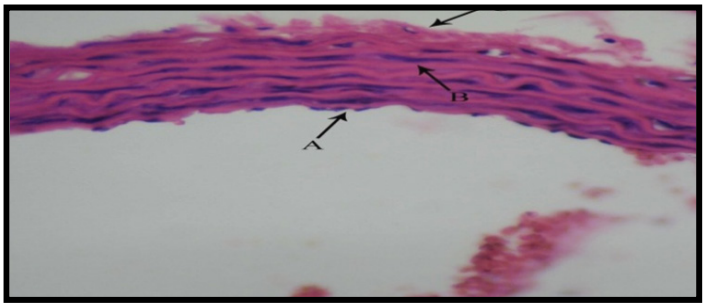


a-Blood pressure chart in control group

b- Blood pressure chart in high fat diet induced type 2 DM

c- Blood pressure chart in saxagliptin treated rats

Figure 2. Effects of saxagliptin (10mg/kg/day, orally) treated diabetic rats on blood pressure showing modest decrease of blood pressure

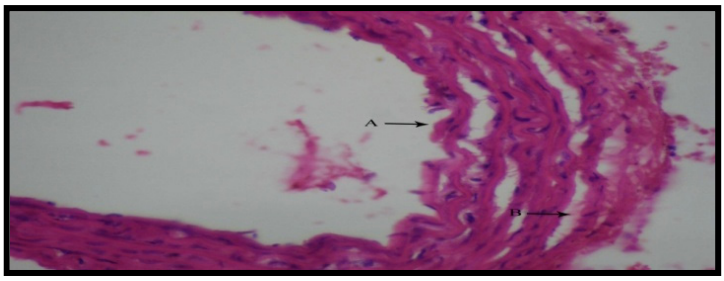


a)

A photomicrograph of a cut section in the aorta of a control rat showing

A) Intact flat endothelial cell lining B) elastic media C) well fitted adventitia. (H & E x 400)

b)



A photomicrograph of a cut section in the aorta of a hypercholesterolemic rat showing A) ulcerated endothelial cells of the intima B) formation of (spindle shaped) cholesterol clefts with degeneration. (H & E x 400)

c)

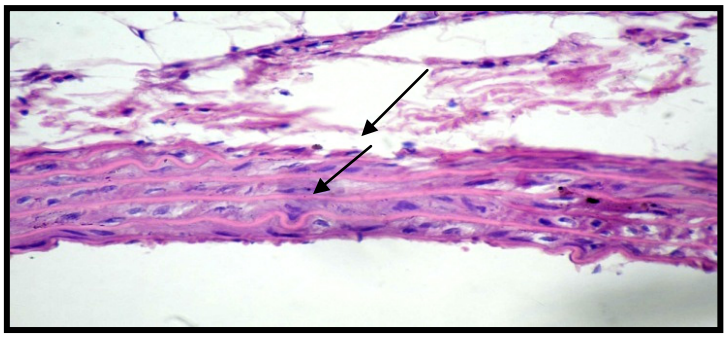


Figure 3. A photomicrograph of a cut section in the aorta of saxagliptin treated rat showing marked decrease of the size of fatty streaks, foamy and inflammatory cell infiltration. Regeneration of the endothelial cells of the intima was observed. (H & E x 400)

7. Discussion

Type 2 diabetes is associated with elevated levels of oxidative stress, glycation and endothelial dysfunction and these are further aggravated by a high-fat diet. DPP-4 inhibitors are novel therapeutic agents for the treatment of diabetes mellitus. These drugs are primarily designed to improve glycemic control. Interestingly, evidence is accumulating that GLP-1 and its analogues can also protect cardiovascular damage via ancillary pathways **(Ban et al., 2008; Sonne et al., 2008)**. In particular, GLP-1 has been shown to induce vasodilatation and improve endothelial function **(Nystrom et al., 2004; Golpon et al., 2001)**. So, this study was designed to assess the effect of saxagliptin on the blood pressure, to study the antioxidant and anti-inflammatory effects of saxagliptin on the vascular endothelium in type 2 diabetes mellitus beyond blood glucose control.

In diabetic animals, oxidative stress is elevated in proportion to the accumulation of AGEs (**Forbes *et al.*, 2002**). AGEs impair endothelial-dependent vascular relaxation mainly by reducing the bioavailability and activity of NO. In the present study, we show that chronic administration of high-fat diet to Wistar rats exhibited endothelial dysfunction, as evidenced by increased blood pressure and aortic histopathological changes. These functional changes were associated with elevation of blood glucose level, impaired glucose tolerance test and inflammatory injury markers such as sICAM-1, in addition to decrease in the NO in tissue homogenate; a marker of oxidative stress injury.

The results from this study revealed that enhanced postprandial glycemic control with a DPP4 inhibitor (saxagliptin) and improved NO release from aortic endothelium. The increase in NO release with treatment was associated with a significant decrease in nitroxidative stress in the aorta vascular tissues. Our results were in line with previous studies which showed that inhibitors of DPP4 such as Saxagliptin, improved postprandial glucose changes even in lean animals**[20]**. Saxagliptin may also enhance eNOS function by reducing postprandial glucose and increasing levels of GLP-1, that increases endothelial-dependent NO release, independent of changes in glucose levels**[2; 31]**. Saxagliptin treatment has also been shown to increase levels of GLP-1 in obese Zucker rats**[45]**. Other important substrates for DPP4 may be involved in this process, including brain natriuretic peptide (BNP), a 32-amino acid hormone that has vasodilating and naturetic properties**[47]**. Pharmacological inhibition of DPP4 enzyme prevents the metabolic degradation of BNP, thereby potentiating its vascular benefits**[3]**. The production of stromal derived factor-1 (SDF-1) is also enhanced with DPP4 inhibition, leading to increased production of endothelial progenitor cells essential for normal vascular function and repair**[11]**.

Moreover, **Drucker,[9]** reported that enhanced glycemic control in obese Zucker rats was associated with improved NO release and diminished production of superoxide (O_2^-), as evidenced by marked reductions in $ONOO^-$ production. Membrane –bound eNOS, as well as NAD (P) H oxidase, may contribute to the overall generation of $ONOO^-$. These pro-oxidant pathways may be inhibited through increased insulin-coupling efficiency, improved glycemic control, and

enhanced incretin activity.

Insulin resistance is considered a core metabolic dysfunction of type 2 diabetes and is associated with abnormalities in glucose and lipid levels. The insulin-mediated delivery of glucose to skeletal muscle can be reversibly blocked by specific eNO inhibitors**[40]**. Transgenic mice that are eNOS-deficient have vascular abnormalities typically associated with insulin resistance, including hyperinsulinemia**[10]**.

A link between reduced NO bioavailability and insulin resistance has been well characterized in various animal models of diabetes. The specific inducible NOS inhibitor, N^6-(1-iminoethyl)-^{L}-lysine (^{L}-NIL), has been shown to reverse fasting hyperglycemia, improve insulin sensitivity, and ameliorate hyperinsulinemia in genetically obese diabetic (*ob/ob*) mice[**12]**. However, insulin-induced uptake of glucose into skeletal muscle was reversibly blocked by the nonselective NOS inhibitor, N^G-methyl-^{L}-arginine (^{L}-NMMA)**[1; 40]**. In addition, the endogenous eNOS inhibitor, asymmetrical dimethylarginine, has been shown to reduce insulin sensitivity in a transgenic model**[43]**.

This consistent with the observation that patient with type 2 diabetes and insulin resistance have elevated plasma asymmetrical dimethylarginine levels**[42]**. In patients with type 2 diabetes, extended treatment with insulin caused a significant improvement in endothelial-dependent vasodilation**[35; 49]**.

The relationship between NO bioavailability and blood pressure reduction with DPP4 inhibitors is an area of interest. The results of this study showed that saxagliptin significantly decrease elevated systolic and diastolic blood pressure in type 2 diabetic rats by 6% and 9% respectively in comparison with none-treated diabetic group. This result was in consistence with **Pacheo et al.,** who found that, sitagliptin reduced blood pressure in hypertensive rats, but this was only seen in immature rats**[33]**. A small but statistically significant reduction in blood pressure was observed in non diabetic subjects with hypertension treated with sitagliptin, but the mechanism was not elucidated[**30]**. Saxagliptin was shown to increase GLP-1 receptor expression in spontaneously hypertensive rat renal arteries and the upregulation was associated with improvement of endothelial function via restoration of NO bioavailability **(Liu et al., 2012)**.

Another important finding from this study was the significant reduction in levels of the inflammatory marker sICAM-1 levels with saxagliptin treatment. Patients with diabetes are at higher risk of atherosclerosis, a chronic disease of the arterial wall causally associated with endothelial dysfunction, inflammation, and the development of acute or chronic tissue ischemia**[24; 36]**. sICAM-1 is a reliable predictor of cardiovascular events in those without any prior history of coronary artery or other vascular disease**[15; 18]**. Additionally, data from the Framingham Offspring study supports the correlation between sICAM-1 and risk factors such as smoking, high cholesterol levels, high glucose levels and obesity[**19]**.

8. Conclusions

The results of the present study support the concept of anti-inflammatory properties of saxagliptin through inhibition of the pro-inflammatory mediator, including sICAM-1, which are independent of its glucose-lowering properties. In addition, it reduces the vascular oxidative stress by increasing the NO vascular levels, which resulted in an improvement of endothelial function. These beneficial effects of saxagliptin on vascular function might serve it as a potential therapy for type 2 diabetes with endothelial dysfunction.

9. Recommendations

Further studies have to demonstrate whether these antioxidant and anti-inflammatory properties of saxagliptin may translate into improved clinical outcomes in diabetic patients.

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