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Possible Protective Effect of Endothelin-1 Receptor Antagonist on Renal Impairment in Type 2 Diabetic Rats

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Abstract

Background: Diabetic nephropathy (DN) is characterized by persistent albuminuria and progressive decline in glomerular filtration rate (GFR)**.** Bosentan is a non-peptide mixed antagonist of ET-1. **Aim**: To investigate the possible protective role of bosentan on renal impairment in T2DM rats and the mechanisms concerned**. Materials:** Fifty rats were randomly divided into five equal groups; Non-Diabetic (ND), Diabetic non-treated (DN), Diabetic Bosentan-treated (D-Bos.), Diabetic Metformin-treated (D-Met.), Diabetic combined Bosentan and Metformin-treated groups (D-Bos& Met.). T2DM was induced by Streptozotocin (STZ) (35mg/kg) and high fat diet (HFD) for 3 weeks. Both Bosentan and metformin were given orally for 4 weeks. At the end of the experiment, 24hrs urine samples were collected to measure urine (volume, albumin, NAG and creatinine), renal blood flow velocity (RBFV) and peripheral renal resistance (RVR) were measured using Doppler technique. Blood was collected directly from abdominal aorta to estimate fasting serum glucose, glycosylated hemoglobin (HBA1c), serum insulin, complete lipid profile, renal function tests and parameters of oxidative stress: malondialdehyde (MDA), total antioxidant capacity (TAC) and tumor necrosis factor-α (TNFα). Insulin resistance, serum LDL-cholesterol, urinary albumin creatinine ratio (UACR) and creatinine clearance (CC) were calculated. **Results:** Serum cysteine c, BUN, urinary albumin and UACR were significantly lowered in both D-Bos. & D-Met when compared to the corresponding values in DN while urinary volume, urinary creatinine, CC and RBFV were significantly elevated but serum creatinine, NAG & RVR were significantly lowered in D-Bos. only**. Conclusion:** Bosentan and metformin treatment both have a renoprotective effect. Combined treatment showed better results.

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INTRODUCTION

Diabetes Mellitus (DM) is a common and serious metabolic condition which imposing itself as one of the largest health threats of the 21st century. In the past three decades the prevalence of type 2 diabetes mellitus (T2DM) has risen dramatically in all countries. These massive numbers led to the prediction that >600 million individuals would develop T2DM worldwide by 2045, with around the same number developing pre-DM (1). It is characterized by chronic hyperglycemia together with disturbances of carbohydrate, fat and protein metabolism resulting from defects of insulin secretion, insulin action or both. T2DM is a heterogeneous disorder caused by a combination of genetic and environmental factors which adversely affect β - cell function and tissue insulin sensitivity (2).

DN is one of the most feared diabetic chronic microvascular complications and the major cause of end-stage renal disease (ESRD). One in 3 diabetic patients will develop DN (3). The classical presentation of DN is characterized by hyperfiltration and albuminuria in the early phases which is then followed by a progressive renal function decline. The multifactorial disease, type 2 DN (T2DN), may be associated with factors such as hyperglycemia, hyperlipidemia, oxidative stress and inflammatory cytokines (4).

Substantial evidence suggests a potential link between Endothelin-1 (ET-1) and the pathogenesis of metabolic and renal impairments associated with T2DM (5). ET-1 directly affects podocytes as revealed that a podocyte-specific double deletion of endothelin A receptor (ETAR) and endothelin B receptor (ETBR) resulted in less proteinuria and protection from podocyte dysfunction and glomerulosclerosis in diabetic mice (6).

Bosentan can inhibit the stimulation of both ETA and ETB receptors. Oral administration of the dual endothelin receptor antagonist was found to improve peripheral endothelial function in patients with T2DM and microalbuminuria (7).

Metformin, which is an oral hypoglycemic biguanide drug, has been widely used in the treatment for T2DM, particularly in patients with obesity. Metformin can decrease blood glucose levels, as well as partially reversing the renal damage caused by diabetic nephropathy and prolonging the survival of diabetic mice (8). SO, the aim of the present investigations was to study the possible protective effect of endothelin 1 receptor antagonist on renal impairment in type 2 diabetic rats and the underlying mechanisms concerned.

Material and Methods

Animals

This study was conducted in accordance with the regulations of Animal Experimentation Ethics Committee of Faculty of Medicine Menoufia University. Fifty adult white male albino rats of local strain, weighing 150 ± 10 grams each, were used in this investigation. All rats were housed in groups of 5 per cage in standard rat cages under controlled temperature (22-24 °C), humidity (30-40%) in a light-controlled room with an alternating 12 h light/12 h dark cycle with free access to food and water ad libitum. Animals were acclimatized to these conditions for 1 week before the experiment. Appropriate care to all rats was in compliance with the Public Health Service Policy

on Use of Laboratory Animals published by the Ethical Committee of the College of Medicine, Menoufia University, Egypt.

Experimental design

Rats were randomly divided into 5 equal groups: I) Non-Diabetic group (ND):rats with fasting blood glucose less than 110 mg/dl were selected in this group (9). Vehicle (sterile saline solution 0.9 NaCl) was administered via gastric gavage, II) Diabetic non-treated group (DN):T2DM was induced by giving a HFD containing 50% carbohydrate, 13% protein, 30% fat and 7% fiber for 3 weeks (10) followed by a single intraperitoneal (i.p.) injection of STZ (35 mg /kg of body weight (B.W.) of rats) in 0.2 ml of 10 mmol /l citrate buffer (pH 4.5) mimicking the picture of T2DM (11). Vehicle was administered via gastric gavage. Half ml dextrose 5% was given intraperitoneally 30 minutes before STZ injection as a protective dose and rats were supplied with sucrose in the drinking water (10%) to avoid sudden hypoglycemia after injection. DM was confirmed 72hrs after injection by measuring tail vein blood glucose level for two consecutive days using glucometer (ACCU-CHEK) in overnight fasted rats (12). Rats were considered diabetic when fasting blood glucose levels exceeded 150 mg/dl (13) then will be left untreated for 4 weeks (experimental period), III) Diabetic Bosentan - treated group (D-Bos.):After being diabetic as in previous group, treatment was continued with bosentan (100/mg/kg) (14). The drug was administered once daily via gastric gavage for 4 weeks, IV) Diabetic Metformin - treated group (D-Met.): After being diabetic as in previous group, treatment was continued with metformin (250 mg/kg) (15). The drug was administered once daily via gastric gavage for 4 weeks, V) Diabetic combined Bosentan and Metformin -treated group (D-Bos. & Met.)**:** After being diabetic as in previous groups, treatment was continued with both bosentan & metformin by same doses mentioned above for 4 weeks.

The first 2 groups were done before the next 3 groups to be sure that the DN has developed renal impairment after 4 weeks (experimental model).

At the end of the experiment, 24 hours urine samples were collected for rats by using metabolic cages. In the next day, 12hrs fasted rats were anaesthetized using thiopental sodium (50 mg/kg, i.p.), and each rat was placed on a suitable rodent surgical table. The skin on the ventral aspect of abdomen was opened to expose renal artery for measurement of renal blood flow velocity and peripheral renal resistance using Doppler technique. After that in the same day, direct cannulation of abdominal aorta was done for taking blood samples. Four milliliters of blood were collected directly from abdominal aorta. One milliliter of blood was collected in lavender top tube containing EDTA as an anticoagulant for measuring of HBA1c. Another 3 milliliters of blood were collected in a clean graduated centrifuge tube.

Collection of 24hrs urine sample

At the end of the experiment, 24hrs urine samples were collected and registered for each rat by using metabolic cages to measure urine volumes (ml/min). Aliquots of 5 ml urine were centrifuged for 10 minutes and stored at -20°C for subsequent determination of urinary albumin (mg/24h), urinary N-acetyl-β-D-glucosaminidase (NAG) (U/g UCr) and creatinine concentrations

(mg/ml). Urinary albumin creatinine ratio (UACR) was calculated.

Measurement of renal blood flow velocity and resistance:

Each rat was anesthetized with a single dose (50 mg/kg) of thiopental sodium by i.p. injection. Then, each rat was placed on a suitable rodent surgical table. The skin on the ventral aspect of the abdomen was carefully shaved and disinfected. Midline laparotomy was done to expose renal artery. After setting the mode of pulsed blood flow meter (Bi-directional Printing Doppler with LCD, Smartdop®50 Series / 50EX Series, HADECO, Japan), ultrasonic probe was pressed softly on the measured area at an angle of 40º-50º. After hearing optimal sound, the probe was kept still for 5 seconds. The freeze key was pressed allowing the wave form to freeze then the RBFV and peripheral renal resistance were measured in the studied groups **(16).**

Blood sampling and assay

12hrs fasted animals were anaesthetized as mentioned above**.** After that, direct cannulation of abdominal aorta was done. Blood samples were left for 10 minutes, and then centrifuged at 3000 r.p.m for 20 minutes. The supernatant serum was collected in a dry clean tube and kept at -20 °C to estimate serum levels of the following parameters: fasting serum glucose, HBA1c, fasting serum insulin, complete lipid profile: serum TC, serum TGs, HDL-cholesterol, renal function tests: serum creatinine, serum Cystatin C, serum BUN, parameters of oxidative stress: serum MDA, serum TAC and serum TNFα. Insulin resistance, LDL-cholesterol and CC were calculated.

Biochemical Analysis

1. **Estimation of fasting serum glucose (FSG) (17)**

FSG was estimated using a test reagent kit (Bio diagnostic Company, Egypt) according to manufacturer's instructions. The results were expressed as mg/dl.

2. **Estimation of Glycosylated hemoglobin (HbA1c) (18)**

HBA1C was estimated using a test reagent kit (Bio diagnostic Company, Egypt) according to manufacturer's instructions. The results were expressed as % of normal

3. **Estimation of serum insulin (19)**

Insulin was estimated using enzyme-linked immunosorbent assay (ELISA) kit (Sigma Company, USA) according to manufacturer's instructions. The results were expressed as µU/ml.

Calculation of HOMA-IR index (17)

Insulin resistance was calculated by homeostasis model assessment for insulin resistance (HOMA-IR index) using the following formula:

$$\frac{\text{Fasting serum insulin (µu/ml)} \times \text{fasting blood glucose mg/dl}}{405}$$

To define insulin resistance using HOMA-IR, the best cutoff is 3.8.**(20)**

4. **Estimation of serum total cholesterol and HDL-cholesterol (21)**

Serum total cholesterol, HDL-cholesterol and LDL-cholesterol were estimated by enzymatic method using diagnostic kits (Bio diagnostic Company, Egypt) according to manufacturer's instructions. The results were expressed as mg/dl.

Calculation of LDL-cholesterol (22)

C_{LDL} was calculated according to the formula:

$C_{LDL} = C_{plasma} - C_{HDL} - TG/5$

5. Estimation of serum total triglycerides (21)

Total triglycerides were estimated by enzymatic method using diagnostic kits (Bio diagnostic Company, Egypt) according to manufacturer's instructions. The results were expressed as mg/dl.

6. Estimation of serum and urinary creatinine (23)

Serum and urinary creatinine were estimated using a test reagent kit (Bio diagnostic Company, Egypt) according to manufacturer's instructions. The results were expressed as mg/ml.

Calculation of creatinine clearance (24)

Creatinine clearance (ml/min) in each group was calculated by using the formula:

$$\text{Creatinine Clearance} = \frac{U \times V}{P}$$

U: Creatinine concentration in urine (mg/ml).

V: Volume of urine per minute (ml/min).

P: Creatinine concentration in plasma (mg/ml).

7. Estimation of serum Cystatin C (25)

Serum Cystatin C was estimated using ELISA kit (DRG International, Inc., Germany) according to manufacturer's instructions. The results were expressed as mg/L.

8. Estimation of serum BUN (26)

BUN was estimated using a standard diagnostic kit (Bio-diagnostic Company, Egypt) according to manufacturer's instructions. The results were expressed as mg/dl.

9. Determination of urinary albumin (27)

Quantitative measurements of micro albuminuria using ELISA kit (DRG International, Inc., Germany) according to manufacturer's instructions. The results were expressed by mg/24h.

Calculation of urinary albumin-to-creatinine ratio (UACR) (28)

UACR (mg/g creatinine) was calculated as albumin concentration (mg/l) divided by creatinine concentration (g/l).

10. Estimation of urinary NAG (29)

NAG was estimated using a test reagent kit (Bio diagnostic Company, Egypt) according to manufacturer's instructions. The results were expressed as U/g UCr.

11. Estimation of serum Malondialdehyde (MDA) (30)

MDA was estimated using a test reagent kit (Bio diagnostic Company, Egypt) according to manufacturer's instructions. The results were expressed as µmol/l.

12. Estimation of serum Total antioxidant capacity (TAC) (31)

TAC was estimated using a test reagent kit (Bio diagnostic Company, Egypt) according to manufacturer's instructions. The results were expressed as µmol/l.

13.Estimation of serum TNFα (32)

TNF α was estimated using ELISA kit (Assay Pro Company, USA) according to manufacturer's instructions. The results were expressed as pg /ml.

Statistical Analysis

Results were presented as mean ± standard error of mean (S.E.M) and $p \leq 0.05$ was considered significant. Results were analyzed using the Statistical Package of Social Sciences (SPSS) program version 20, (Chicago, IL, USA). Shapiro-Wilk test was done to check if the data was parametric or non-parametric. One-way analysis of variance (ANOVA) was used for statistical descriptive analysis of the results **(33)**. A Tukey's adjustment for multiple comparisons was used for

all post-hoc mean comparisons for significant effects from all analyses **(34).**

Results

Serum biochemical analysis:

Fasting serum glucose, HBA1c, serum insulin and HOMA-IR in DN, D-Bos., D-Met. and D-Bos. & Met. groups were significantly elevated when compared to corresponding values in ND group, while fasting serum glucose, HBA1c, serum insulin and HOMA-IR in D-Met. and D-Bos. & Met. groups were significantly lowered when compared to corresponding values in DN group but in D-Bos only HOMA-IR was significantly lowered while others were insignificantly changed, while fasting serum glucose, HBA1c, serum insulin and HOMA-IR in D-Met. and D-Bos. & Met. groups were significantly lowered when compared to corresponding values in D-Bos. group while serum insulin and HOMA-IR in D-Bos. & Met. group were significantly lowered when compared to corresponding values in D-Met. group but fasting serum glucose, HBA1c in D-Bos. & Met. group were insignificantly changed.

Table (1): Renal functions (urinary and serum parameters): urinary volume, urinary creatinine, urinary albumin, urinary NAG and UACR serum creatinine, serum Cystatin C, BUN & creatinine clearance in ND, DN, D-Bos., D-Met. and D-Bos. & Met. #

	Serum creatinine (mg/dl)	Serum Cystatin C (mg/l)	BUN (mg/dl)	CC (ml/min)	Urinary Volume (ml/min)	Urinary Creatinine (mg/ml)	Urinary albumin (mg/24 h)	Urinary NAG (U/g Cr)	UACR (mg/g Cr)
ND	0.37±0.01	0.48±0.02	23.43±0.48	2.54±0.09	0.004±0.00006	2.35±0.01	4.29±0.28	5.15±0.24	316.52±1.14
DN	1.96±0.12*	1.12±0.03*	56.8±1.06*	0.69±0.02*	0.018±0.0002*	0.75±0.008*	15.24±0.85*	9.74±0.37*	783.2±3.78*
D-Bos	1.54±0.09 *$	0.69±0.06 *$	27.14±0.79 *$	1.98±0.09 *$	0.015±0.0001 *$	2.04±0.007 *$	6.17±0.48 $	6.08±0.39 $	139.68±1.02 *$
D-Met	1.67±0.1 *	0.83±0.04 *$	34.61±1.19 *$	1.73±0.08 *$	0.013±0.0002 *$Ω	2.22±0.008 *$Ω	8.35±0.44 *$Ω	8.69±0.6 *Ω	198.68±1.86 *$Ω
D-Bos & Met.	0.85±0.07 *$Ω%	0.53±0.03 $Ω%	27.45±0.89 *$%	2.63±0.12 $Ω%	0.01±0.0002 *$Ω%	2.23±0.007 *$Ω	6.34±0.39 $	6.05±0.33 $%	197.6±0.97 *$Ω

the number of rats in each group was 10,* Significant when compared to the corresponding value in non-diabetic group, $ Significant when compared to the corresponding value in diabetic group, Ω Significant when compared to the corresponding value in diabetic bosentan treated group, % Significant when compared to the corresponding value in diabetic metformin treated group, P-Value ≤ 0.05 is significant; P-Value >0.05 is insignificant. The results were expressed as mean ± SEM.

ND: non-diabetic, DN: diabetic non treated, D-Bos.: diabetic bosentan treated, D-Met.: diabetic metformin treated, D-Bos.&Met.: diabetic combined bosentan and metformin, BUN: blood urea nitrogen, CC: creatinine clearance, NAG: urinary N-acetyl-β-D-glucosaminidase, UACR: urinary albumin creatinine ratio.

Table (2): Renal blood flow velocity (RBFV) (cm/sec) and renal vascular resistance (RVR) in the studied groups. #

	RBFV (cm/sec)	RVR
ND	7.22±0.54	0.76±0.0697
DN	2.99±0.19 *	2.34±0.22 *
D-Bos.	6.38±0.49 $	1.34±0.1 *$
D-Met.	5.73±0.25 $	1.87±0.13*
D-Bos&Met.	6.45±0.29 $	1.12±0.1 $%

the number of rats in each group was 10, * Significant when compared to the corresponding value in non-diabetic group, $ Significant when compared to the corresponding value in diabetic group, % Significant when compared to the corresponding value in diabetic metformin treated group, P-Value ≤ 0.05 is significant; P-Value >0.05 is insignificant. The results were expressed as mean ± SEM.

ND: non-diabetic, DN: diabetic non treated, D-Bos.: diabetic bosentan treated, D-Met.: diabetic metformin treated, D-Bos.&Met.: diabetic combined bosentan and metformin.

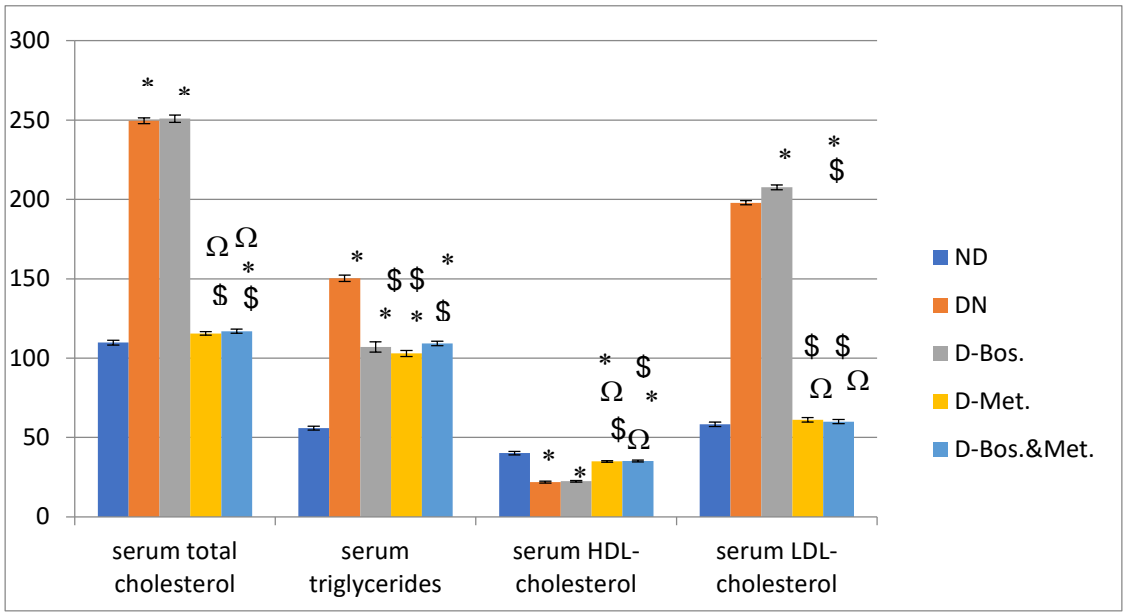

Figure (1): Serum total cholesterol (mg/dl), serum total triglycerides (mg/dl) , serum HDL-cholesterol (mg/dl) and serum LDL-cholesterol (mg/dl) in the studied groups.#, # the number of rats in each group was 10,* Significant when compared to the corresponding value in non-diabetic group, $ Significant when compared to the corresponding value in diabetic non-treated group, Ω Significant when compared to the corresponding value in diabetic Bosentan-treated group.Error bars represent ± SEM.

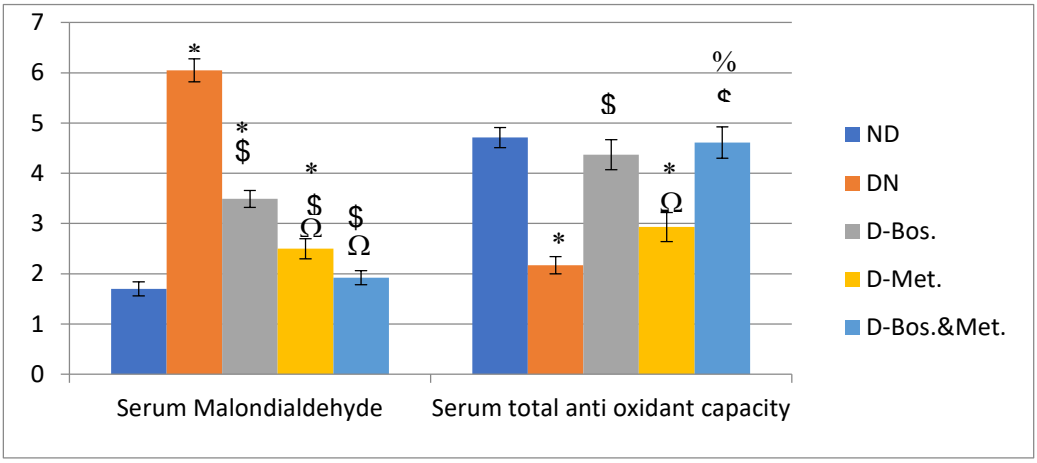

Figure (2): Serum Malondialdehyde (µmol/l), Serum total anti-oxidant capacity (µmol/l) in the studied groups.#, # the number of rats in each group was 10, * Significant when compared to the corresponding value in ND, $ Significant when compared to the corresponding value in DN, Ω Significant when compared to the corresponding value in D-Bos., % Significant when compared to the corresponding value in D-Met..Error bars represent ± SEM.

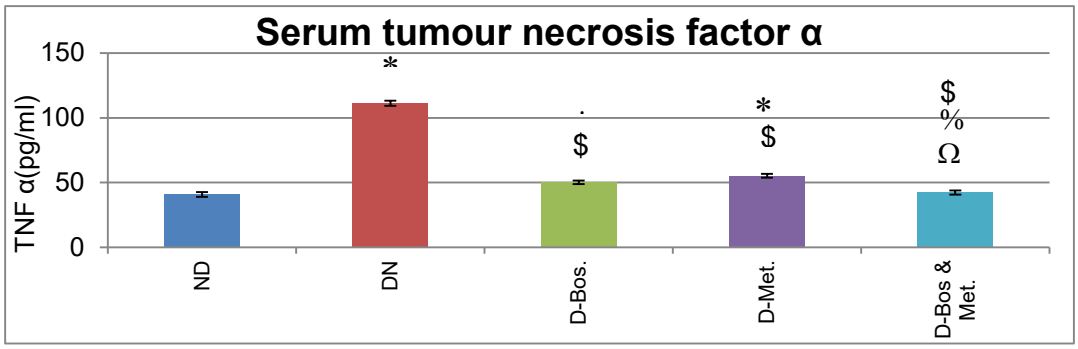

Figure (3): Serum tumor necrosis factor α (pg/ml) in the studied groups.#, # the number of rats in each group was 10, * Significant when compared to the corresponding value in ND group, $ Significant when compared to the corresponding value in DN, Ω Significant when compared to the corresponding value in D-Bos., % Significant when compared to the corresponding value in D-Met.**.** Error bars represent ± SEM.

Discussion

In the present investigation, diabetic rat model was successfully established by HFD and low dose of STZ injection as evidenced by the significant elevation of blood glucose, glycated hemoglobin with insulin resistance which was evidenced by elevated fasting serum insulin level and HOMA-IR. Insulin resistance induced by HFD can be explained by a reduction in the total number of insulin receptors without modification of receptor affinity **(35).**

In addition, HFD decreases insulin receptors autophosphorylation and IRS-1 Phosphorylation **(36).** Injection of low dose of STZ that lead to partial damage of the β-cells, triggering an inflammatory process and further loss of β-cell activity, a process that closely resembles the pathogenesis of T2DM**.** Also hyperglycemia can be explained by reduced entry of glucose to the peripheral tissues; skeletal muscle and adipose tissue due to insulin resistance **(37)**.

The results of the present investigation concerning glucose homeostasis were in agreement with the results of ***Oza and Kulkarni*****, (38)** and ***Guo et al.,*** **(39)** who reported that there was significant elevation in fasting serum glucose, HBA1c, fasting serum insulin and HOMA-IR values indicating development of insulin resistance in type 2 diabetic rats.

In addition, there were significant changes in renal haemodynamics including lowered RBFV and elevated RVR and this resulted in significant reduction in renal functions. This can be explained by elevated intraglomerular pressure and glomerular hyperfiltration, during the initial stages of DN, due to reduced tubuloglomerular feedback (TGF) and dilatation of afferent arterioles due to increased reabsorption of sodium along with glucose via sodium glucose transporter-2 **(40)**. Also, changes in renal haemodynamics can be explained by hyperglycemia that may cause decreased NO production, increased oxidation of NO, endothelial dysfunction of renal vessels and alterations in vascular resistance and ultimately reduced RBF **(41).** These results were in agreement with that of ***Freitas et al.,*** **(42)** mentioned that STZ-induced diabetic rats showed significant lowering in RBFV and significant elevation of RVR.

In the present investigation there were stastically insignificant changes of fasting serum glucose, HBA1c and serum insulin when compared to the corresponding values in DN group. These results can be clarified by that ET-1is not a major determinant of hepatic glucose output **(5)**. Also expression of ET-1 receptors was not detected in rat hepatocytes **(43).** In accordance of the present results, ***ShamsEldin*** **(44)** and ***Cosenzi et al.*** **(45)**, mentioned that fasting serum glucose insignificantly changed with bosentan treatment. On the other hand, ***Said et al.,*****(46)** revealed that oral administration of bosentan resulted in decreasing fasting serum glucose level in mild diabetic rats. ***Said's*** results was explained by that Bosentan enhances insulin stimulated glucose uptake although bosentan had no direct effect on secretion of insulin**.**

The improved renal functions seen in the present investigations of D-Bos. group can be explained by bosentan antioxidative and anti-inflammatory effect proved by the elevation of serum TAC, lowering of serum MDA and serum TNF-α as seen in the present investigation. Oral

administration of bosentan was proven to decrease type 1 collagen, cellular fibronectin, the activity of smooth muscle α-actin and extra cellular matrix mRNA in rats **(47).** Also bosentan might be able to regulate the expression of stress-responsive proteins such as TNF-α and TGF-β and prevent the expression of pro-fibrotic genes in response to TGF-β **(48).**

Indeed, ETB receptor activation increased intracellular calcium and triggered the NF-κB and b-catenin signaling pathways, analogous to activation of the ETA receptor. The quantitative contribution of the ETB receptor may be substantial, as suggested by the fact that it is up regulated to a larger extent than the ETA receptors in the podocytes of diabetic mice. This study implies that dual blockade of the ETA and ETB receptors may be necessary to achieve maximal benefit in diabetic nephropathy. These findings are novel and very significant because they question the strategy of the clinical trials targeting the ETA receptor to avoid the adverse effects attributed to ETB receptor blockade **(49)**.

In the present investigation serum creatinine, serum Cystatin C, BUN, urinary volume, urinary albumin, urinary NAG, UACR and RVR were significantly lower; while urinary creatinine, creatinine clearance and RBFV were significantly higher in D-Bos. group when compared to the corresponding values of DN group. Oral administration of the dual endothelin receptor antagonist, bosentan, was found to improve peripheral endothelial function in patients with T2DM and microalbuminuria **(7)**. Researchers have found that ET-1 signals increased heparanase in podocytes, an enzyme that degrades heparan sulfate glycosaminoglycans: the major constituent of glycocalyx **(3)**. However, the exact mechanisms by which bosentan interferes with diabetic endothelial dysfunction is still unclear.

The improved renal functions seen in the present investigations of D-Bos. group can be explained by the improved RBFV associated with lowering RVR when compared to the corresponding values of DN group. This can be explained by vascular remodeling which prevents angiotensin II and aldosterone induced end-organ damage **(50)**. Bosentan treatment has an antiproteinuric effect as evidenced by significant reduction in urinary albumin in D-Bos. group when compared to the corresponding value in DN group. The present results were in agreement with **ShamsEldin (44)**, who revealed, significant ($p< 0.05$) reduction in albumin content of urine in mg/24 h. and significant elevation in creatinine clearance when compared to DN group. Also, in accordance with the present results ***Cosenzi et al.*****(45)** and ***Ding et al.*****(51)** revealed that bosentan treatment resulted in significant reduction in urinary protein excretion. These results can be explained by that the endothelins production in kidney tissue was increased in diabetes. ***Hargrove et al,*****(52)** have observed that the activity of the ET-1 promoter in mesangial cells was stimulated by glucose. As an alternative mechanism, ***Zoja et al,*****(53)** have demonstrated that overloading proximal tubular cells with albumin resulted in a dose-dependent increase in ET-1 synthesis, suggesting a link between proteinuria and ET-1 synthesis. So, proteinuria in animal models of diabetic nephropathy, membranous nephropathy and proliferative nephritis was significantly reduced by unselective blocking of ETRs. In

contrast ***Hocher et al.,*****(54)** have demonstrated that the selective ETA blockade resulted in non-significant decrease in proteinuria in diabetic rats.

In the present investigation there was stastically significant improvement of glycemic state manifested by lowering of fasting serum glucose level, HBA1c, serum insulin and HOMA-IR levels of D-Met group when compared to the corresponding values of DN group. This can be explained by the reduction in hepatic glucose production and enhancing insulin suppression of endogenous glucose production and possibly by improving glucose uptake and utilization by peripheral tissue **(55).** Also, metformin increases glucose utilization by the gut and GLP-1 secretion **(56)** and antagonizes the action of the counter regulatory hormone glucagon **(57).**

The improved renal functions seen in the present investigations of D-Met. group can be explained by the beneficial effect on lipid profile as metformin treatment of diabetic rats caused significant lowering of serum TC, serum TGs and serum LDL-cholesterol, but significant elevation of serum HDL-cholesterol when compared to the corresponding values of DN group. This can be explained by the fact that metformin increases insulin sensitivity, reduces the rate of lipolysis and thereby slowing the conversion of free fatty acids to lipoprotein precursors in the liver **(58).** Also, Metformin could reduce ROS by suppressing mitochondrial respiration and repressing AGEs indirectly through reduction of hyperglycemia and directly through an insulin dependent mechanism **(59).** In accordance of the present results**, *Nna et al.*****(60)** mentioned that metformin treatment significantly elevates antioxidant enzymes and reduces lipid peroxidation in STZ-diabetic rats; proved by the significant reduction of serum MDA and TNF-α when compared to the corresponding values in DN group in the present investigation. Moreover the results of the present investigation were in agreement with the results previously reported by ***Abdulmalek and Balbaa,*****(61)** who showed the protective effects of metformin administration in rats with T2DN**.**

In the present investigation serum Cystatin C, serum BUN, urinary volume, urinary albumin, UACR were significantly lower; while urinary creatinine, creatinine clearance and RBFV were significantly higher when compared to the corresponding values of DN group. However the serum creatinine, urinary NAG and RVR was stastically insignificant. Metformin could act as an inhibitor of pro-inflammatory responses through direct inhibition of NF-κB **(62)**. Metformin dose-dependently diminished the production of NO and PGE2 and suppressed the mRNA and protein levels of inducible NOS and COX-2 in lipopolysaccharides (LPS)-activated macrophages. A study of ***Kim et al.*****(63)** demonstrated that metformin inhibits LPS induced production of TNFα and IL-6 in parallel to induction of activating transcription factor-3, a transcription factor and member of the c AMP responsive element binding protein family.

Upon comparing the results of D-Met. group to that of D-Bos. group, it was found that fasting serum glucose, HBA1c, serum insulin, HOMA-IR, serum total cholesterol, serum TGs, urinary volume , serum MDA and serum TAC were significantly lowered; while serum HDL-cholesterol, serum LDL-cholesterol, urinary creatinine, urinary albumin, urinary NAG and UACR were significantly elevated when

compared to the corresponding values of D-Bos.group. In accordance with the present investigation; ***Cayir et al.,*****(64)** illustrated that bosentan treatment, particularly at a dose of 100 mg/kg, prevented the impairment in endothelial dysfunction, the cytokine levels increase and the oxidant/ antioxidant balance progressed in favor of antioxidants**.** Elevated ET-1 levels were claimed to induce the inflammation mechanism in rat kidneys and increase cytokine, growth factors and macrophage chemoattraction **(65).** So ET receptors may be responsible for diabetes-related organ damage, and ET-antagonists may become a promising therapy in preventing diabetic complications **(47).**

In the present investigation there was stastically significant lowering of fasting serum glucose level of D-Bos. & Met. group when compared to the corresponding value of diabetic non-treated group. This can be explained by action of metformin on glycemic state as previously illustrated in D-Met. group.

The improved renal functions in the present investigations of D-Bos. & Met. group when compared to the corresponding values of DN. This can be also explained by renoprotective effect of both bosentan and metformin on kidney functions.

Upon comparing the results of D-Bos. & Met. group to that of D-Bos. group, it was found that fasting serum glucose, HBA1c, serum insulin, HOMA-IR, serum total cholesterol, serum TGs, serum creatinine, serum Cystatin C, urinary volume , serum MDA and serum TNFα were significantly lowered; while serum HDL-cholesterol, urinary creatinine and creatinine clearance were significantly elevated when compared to the corresponding values of D-Bos. group.

Both drugs bosentan and metformin have a renoprotective effect but in two different mechanisms. Metformin depends mainly on hypoglycemic and hypolipidemic effect. Bosentan depends mainly on vascular remodeling **(44),** antioxidative and anti-inflammatory effect **(45).**

There are two major limitations in this study that could be addressed in future research. First, the study focused on renal function urinary and serum parameters including early and specific biomarkers and indicators for diagnosis of diabetic kidney disease not histopathological studies as this research did not receive any specific grant from funding agencies so from authors' point of view upon prioritizing aims of the work extensive renal functions tests coming first of all Second blood pressure wasn't measured in this study. From previous studies bosentan was found to lower blood pressure but it is not authors' scope in this study which mainly focuses on bosentan effect on renal blood flow and renal vascular resistance but for sure these two limitations will be included in authors' further work.

Conclusion

From the results of the present investigation it can be concluded that: bosentan treatment improves the renal functions in diabetic rats. Combined treatment resulted in significant improvement of the glycemic state, renal functions and hemodynamic. This improvement may indicate that bosentan had a complementary effect to metformin in T2DM. So, bosentan is a promising adjuvant therapy for T2DM. Based on all of these results, we hope that ET-1 receptor

blockers will not be ignored in diabetes induced renal complications.

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