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# Bosentan Ameliorates Diabetic Angiopathy and Nephropathy in Streptozotocin-Induced Diabetic Model in Albino Rats

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***Abstract:*** *Angiopathy and nephropathy are serious problems encountered in management of diabetes mellitus. Angiotensin II (AII) and endothelins (ETs) receptors play an important role in the pathogenesis of diabetic complications. The purpose of this study was to investigate the possible renoprotective and antiangiopathic effects of the non-selective endothelin (ET) receptor blocker bosentan in type 1 diabetic model of albino wister rats. These rats were divided into four groups ( each group , N= 12 rats): control group (1), control group (2) treated with bosentan (50 mg/kg/day), untreated diabetic group (3) and diabetic group (4) treated with bosentan. Induction of type 1 diabetes mellitus in tested rats was performed by a single injection, in the tail vein, of 35 mg/kg streptozotozin after overnight fast. Treatment with bosentan was continued for 12 weeks during which the 24h urine volume, urinary albumin content, urine and plasma levels of creatinine as well as mean non-invasive blood pressure (mean BP) were assessed at the end of each 4 weeks. At the end of the 12$^{th}$ week rats were sacrificed then the thoracic aortae were dissected for assessment of the vasorelaxant effect of acetylcholine. Diabetic rats showed hyperglycemia, polyuria, albuminuria, elevated mean BP, reduced response to vasorelaxant effect of ACh. Bosentan significantly reduced albuminuria and lowered elevated mean BP. In addition the drug restored the normal values of creatinine clearance and improved vascular reactivity to ACh. The present study suggested a possible renoprotective and aortic vasorelaxant effects by bosentan without a significant effect on the control of blood glucose. The results of the present study was directed towards a possible role of bosentan, as a drug acting on Endothelin receptors, in the improvement of diabetic angiopathy and nephropathy.*

***Keywords:*** *Bosentan, diabetic angiopathy, diabetic nephropathy, type 1 diabetes mellitus, albino rats.*

## I. Introduction

The effects of bosentan on diabetic complications is clinically highly recommended to get more information about its possible beneficial effects when administered to diabetic patients.

Interestingly, it was demonstrated that there is a hypertrophic and mitogenic effects mediated by endothelin-1 (ET-1) **(Gilbert et al, 2000).**

ETs play an important role in the genesis of progressive renal damage especially diabetes-induced leisons **(Hocher et al., 1996-A; and Nakamura et al., 1996).** Additionally, in rats with renal ablation, $ET_A$ receptors are overexpressed in smooth muscle cells of intrarenal arteries **(Hocher et al., 1996-B)**. More interestingly, increased tubular ET-1 synthesis may induce fibroblast proliferation, interstitial matrix deposition, and infiltration of inflammatory cells, features typical of progressive tubulointerstitial fibrosis **(Orth et al, 1998)**.

Therefore, it seems that blocking of ET receptors by a drug like bosentan can significantly retard the progression of diabetic renal and vascular complications. In the present study, the influence of the mixed $ET_{A\&B}$ receptor antagonist bosentan was tested in a streptozotocin-induced model of diabetes type I in albino rats to prove any possible prevention or minimization of the development of either aortic angiopathy (in the form of endothelial dysfunction) and nephropathy.

## II. Materials And Methods

**Materials:**

Bosentan (Actelion Pharm. Ltd.; Canada), streptozotocin (Sigma, St Louis, MO, USA), Nephrat II enzyme-linked immunosorbent assay (ELISA) kit (Exocell INC, PA, USA), glucocard$^{TM}$ test strips (Embee Diagnostics, UK), blood glucose measurement kits (Boerhinger Mannheim, Mannheim, Germany), Ultralente insulin (Mixtard HM, Novapharma, UK), norepinephrine HCl (Sigma) and acetylcholine chloride (Sigma). All drug solutions were freshly prepared. NE was dissolved in 0.03 % HCl solution to avoid its oxidation. Acetylchole (ACh) was dissolved in bidistilled water, while bosentan was dissolved in saline (0.9% NaCl). PowerLab instrument for measurement of non-invasive mean blood pressure (BP) (www.ADinstument.com).

**Animal grouping:**

Forty-eight albino wister rats weighing 200- 250 g. Model of type 1 Diabetes was induced by a single injection, in the tail vein, of 35 mg/kg STZ diluted in 0.1 M citrate buffer (pH 4.5) after an overnight fast (**Mifsud et al, 2002)**.

Rats were divided into four groups ( each, N= 12 rats):

**1)** Control group : received saline alone.
**2)** Control bosentan-treated group : received bosentan 50 mg/kg dissolved in saline. **(Muller et al; 2000)**
**3)** Type 1 diabetic non-treated group: received saline alone ip.
**4)** Type 1 diabetic Bosentan-treated group: received an ip daily dose of 50 mg/kg bosentan **(Muller et al; 2000)**

Administration of bosentan was done at 8.00 a.m. daily starting one week after diabetes mellitus induction (diabetic group) or saline administration (non-diabetic group) and continued for 12 weeks. All rats were given free access to water and standard rats' chow. In diabetic groups, rats showed a blood glucose level of > 300 mg/dl 24 h after STZ injection. Diabetic animals received daily intraperitoneal injections of ultralente insulin (3U/rat) to avoid ketonemia and promote the well-being of animals. Prior to insulin administration, blood glucose level was assessed using super-glucocard$^{TM}$ glucose test meter GT-1640 and glucocard$^{TM}$ test strips. At the ends of weeks 4, 8 and 12 blood samples were collected from the tail vein for assessment of glucose levels by kits using standard spectrophotometric methods. At the end of the 12$^{th}$, week after STZ or saline administration. All tested rats were anaesthetized with urethane ( 1 g/kg ip) and the thoracic aortae were removed for ACh- vasorelaxant reactivity study.

- Measurement of non-invasive mean blood pressure (mean BP) in all rats of tested groups as indicated by manual of the AD instrument (PowerLab) as follows:

The Blood Pressure Analysis View displays the average cycles and the mean BP was calculated by the module. In the Analysis pane of the Blood Pressure Settings dialog you set the number of Cycles to average. The values of mean non-invasive blood Pressure of all tested rats were recored on a window of the monitor of PowerLab AD Instrument.

**Renalfunction**

Rats were individually housed in metabolic cages for 24 h, at the ends of the 4$^{th}$, 8$^{th}$ and 12$^{th}$ week after STZ or saline administration. During the 24-h period, animals continued to have free access to tap water and standard rats' chow. Urine volume/24h was calculated for each animal and aliquots of 5 ml urine were centrifuged for 10 minutes and stored at -20ºC for subsequent determination of albumin content using Nephrat II ELISA kits as previously described **(Isshiki et al, 2000).** Plasma and urine creatinine concentrations were measured using a Beckman analyzer (Beckman Instruments, USA) according to the picric acid colorimetric method **(Wallach J, 1978)** and creatinine clearance in different groups was calculated, after STZ or saline administration then at the end of the 12$^{th}$ week, using the following formula: {[(Urine creatinine$_{(mg/dL)}$ x Urine volume per day$_{(mL)}$/1440$_{(min)}$]/Serum creatinine$_{(mg/dL)}$}/Body weight$_{(g)}$ x 100 **(Lu et al., 2003).**

**Preparation of thoracic aortae for ACh-vasorelaxant reactivity study:**

All tested rats were dissected, thoracic wall was opened and the thoracic aorta was then clearly dissected, removed and placed in a dish containing Krebs's solution. Under the dissecting microscope, the aorta was thoroughly cleaned from any adhering fat and connective tissue then cut into rings (about 4 mm long, each). The rings were then carefully suspended at 37°C under 2g tension in 40 ml organ baths filled with Krebs' solution of the following composition (mM/L); NaCl 118.4, KCl 4.7, $KH_2PO_4$ 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, D-glucose monohydrate 11.1, ascorbic acid 0.022 & EDTA 0.026. The tissue preparations were gassed continuously with a mixture of 95% $O_2$ and 5% $CO_2$ and left to equilibrate for a period of 90 minutes during which they were repeatedly washed with Krebs' solution. Responses to vasoactive drugs were measured isometrically using a Grass FT O3 force-displacement transducer, and recorded on a polygraph. At the end of the equilibration period, the tissues were contracted by norepinephrine (NE) ($10^{-6}$ M), a concentration known to produce a submaximal contractile response. On top of the plateau response to NE, cumulative concentration-response curves were constructed in response to the relaxant effect of acetylcholine (ACh) in preparations with either intact or denuded endothelium. In denuded preparations, the intimal surface of the rings was finely mechanically rubbed with a wooden applicator before the vascular reactivity study. Relaxation was expressed as a percentage decrease in NE-induced constrictory tone.

**Ethics**

All procedures were in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals, as well as the guidelines of the Animal Welfare Act.

**Statistical analysis**

Results are expressed as mean±SD [Standard Deviation]. Statistical analysis was performed by analysis of variance followed by Tukey's post hoc using GraphPad Prism version 3.00 for Windows 97 (Graph Pad Software, San Diego, CA, U.S.A.). Differences with $p< 0.05$ were considered to be statistically significant.

## III. Results

**Blood glucose and Urine volume**

Bosentan treatment did not produce any significant changes in neither urine volume nor blood glucose level compared to diabetic non-treated group **(Table 1)**.

**Albuminuria**

Treatment with bosentan produced significant ($p< 0.05$) reduction in albumin content of urine in mg/24 h. compared to diabetic non-treated group. **(Table 1)**

**Mean non-invasive blood pressure ( mean BP)**

Bosentan treatment for 12 weeks significantly ($P< 0.05$) lowered mean BP compared to diabetic non-treated group. **(Table 1)**.

**Creatinine clearance**

Treatment with bosentan significantly ($p< 0.05$) increased creatinine clearance values in comparison to diabetic non-treated group at the end of the 12$^{th}$ week of treatment.

**(Table 1)**.

| Parameter | Control | Control Bosentan-treated | Diabetic non-treated | Diabetic bosentan-treated |
|---|---|---|---|---|
| **Urine Volume (mL/24h/kg)** | 15.3 ± 1.1 | 17.6 ± 1.7 | 355.4 ± 1.2 | 360 ± 8 |
| **Urine Albumin (mg/24h)** | 4.2 ± 0.5 | 4.4 ± 0.6 | 20.5 ± 0.4 | 4.1 ± 1.2 |
| **Blood Glucose (Mmol/L)** | 8.6 ± 0.9 | 8.3 ± 0.7 | 49.6 ± 0.5 | 48.8 ± 3.9 |
| **Mean BP (MmHg)** | 103 ± 2.3 | 104 ± 3.4 | 135 ± 3.5 | 100 ± 2.7 |
| **Creatinine Clearance (mL/100 g.bwt/min)** | 2.3 ± 0.04 | 2.7 ± 0.03 | 0.16 ± 0.05 | 2.9 ± 0.06 |

**Table (1):** Effect of treatment with bosentan (50 mg/kg/day) on urine volume, urinary albumin content, , blood glucose level, mean non-invasive BP and creatinine clearance in both non-diabetic and diabetic albino rats compared to control groups. All results recorded are those obtained at the end of 12$^{th}$ week which is the duration of the present study. Treatment of diabetic rats with bosentan significantly ($p< 0.05$) reduced albuminuria, improved creatinine clearance and lowered elevated mean BP compared to diabetic non-treated group.

**Response to relaxant effect of acetylcholine**

Induction of diabetes significantly decreased relaxant effect of acetylcholine compared to control non-diabetic group. Bosentan treatment significantly ($p< 0.05$) augmented relaxation responses to ACh compared to diabetic non-treated group **(Figure 1)**.

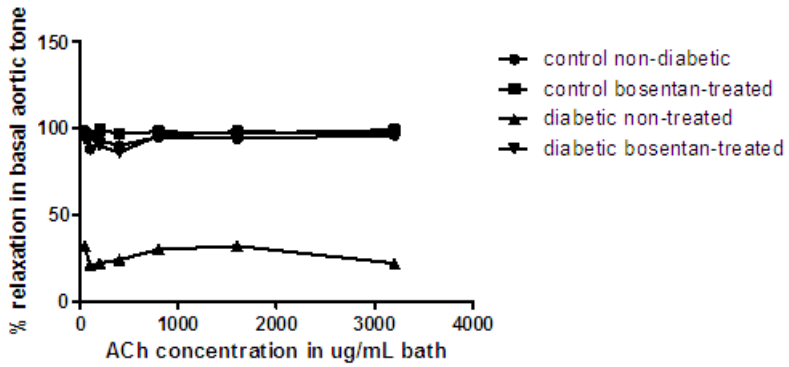

Figure (1): Isolated aortic rings from diabetic bosentan-treated group of albino rats showed a significant ($p<0.05$) increase in the relaxant effect of acetylcholine (ACh) compared to the diabetic non-treated group. Bosentan-treated group significantly ($*p<0.05$) augmented relaxation responses to ACh to a percentage comparable to control groups (1 & 2)

## IV. Discussion

Diabetic angiopathy and nephropathy are considered among the most serious problems encountered in antidiabetic therapy. Diabetic nephropathy is a major cause of end-stage renal disease, and there has been a continuous increase in its incidence worldwide in the past two decades **(Ritz and Orth, 1999).** It is characterized by microalbuminuria, renal and glomerular hypertrophy, mesangial expansion with glomerular basement membrane thickening, arteriolar hyalinosis, and global glomerular sclerosis, which ultimately cause the progression of proteinuria and renal failure **(Parving et al., 2000).** Hyperglycemia is a necessary precondition for the development of the lesions **(Fioretto et al., 1998)**, whereas systemic hypertension is an equally important aggravating factor of the disease **(Ritz and Orth, 1999).** On the other hand, diabetic angiopathy is associated with endothelial dysfunction leading to impaired nitric oxide (NO) release and, thus, to altered regulation of vascular tone **(Olbrich et al., 1996 & 1999).**

In addition to involvement of AII in diabetic leisons, enhanced ET plasma concentrations have been suggested to participate in the pathophysiology of diabetic angiopathy **(Nakamura et al., 1995 and Neri et al., 1998).** ET can be released by many factors, including angiotensin II **(Masaki and Yanagisawa, 1992),** and acts through stimulation of $ET_A$ or $ET_B$ receptors. Endothelial $ET_B$ receptor activation mediates NO release, while $ET_A$ receptor stimulation is involved in vasoconstrictive and proliferative effects of ET **(Ohlstein et al., 1992; and Simonson, 1994).** Synergistic interaction between ET and the RAS has been postulated.Thus, antagonization of either the angiotensin or ET pathway may exert renoprotective and antiangiopathic effects in diabetes mellitus. **(Cameron and Cotter, 1996).**

The present study investigated the possible beneficial therapeutic effects of the non-selective endothelin receptor antagonist bosentan as a protective drug against angiopathy and nephropathy in a diabetic model of albino rats. The probable modifying effects of this studied drug on different parameters involving urine volume, urinary albumin content, creatinine clearance, blood glucose level, mean non-invasive BP and vasorelaxant reactivity to ACh were studied. Bosentan-treated group revealed significant changes in all measured parameters compared to non-diabetic bosentan-treated group. The results were comparable with control groups either with or without bosentan treatment.

The pathogenesis of albuminuria and diabetic nephropathy is still a matter of debate in type 1 diabetes mellitus. However, previous reports have shown that development of diabetes leads to accumulation of advanced glycosylation end products, osmotic diuresis and widening of the glomeruli with consequent activation of the RAS **(Shikata et al., 1995).** This, in turn, increases plasma ET levels **(Neri et al., 1998)** and upregulates ET-1 gene **(Benigni et al., 1998)** with consequent stimulation of extracellular matrix protein production. This results in elevation of the levels of certain extracellular matrix components, such as alpha collagen chains; laminin B1 and B2 chains; and certain growth factors e.g., platelet-derived growth factor **(Ruiz-Ortega et al., 1994)** with subsequent development of nephropathic changes. According to this sequence of events it seems that the key for regression of diabetic nephropathy resides in direct inhibition of the RAS with the resultant decrease in the glomerular capillary pressure **(Imanishi et al., 1997; and Dhein et al., 2000)** and reduced albuminuria.

One of the typical complications of diabetes mellitus is angiopathy. In the present study, the endothelial dysfunction is reflected by a decreased dilatory response to ACh, which releases endogenous NO from the endothelium, suggesting that endothelial release and/or production of NO is impaired in the diabetic animals. This is in good accordance to previous studies reporting reduced NO release in diabetic rats **(Taylor et al., 1995).** The molecular basis of reduced NO liberation in diabetic animal models is still a matter of debate. Suggested mechanisms for diabetes-induced endothelial impairment include the production of free radicals **(Tesfamariam, 1994),** activation of protein kinase C **(DeRubertis and Craven, 1994),** and enhanced production of advanced glycosylation end products **(Nakamura et al., 1996).** Apart from the role of disturbed NO function in development of diabetic angiopathy, enhanced endothelial production of vasoconstrictive prostaglandins was reported recently to take a part in pathogenesis of diabetes-induced vascular dysfunction **(Okon et al., 2003).**

The present work showed that treatment with bosentan restored a normal response to vasorelaxant effect of ACh. However, the exact mechanisms by which bosentan interferes with diabetic endothelial dysfunction is still unclear. This restoration of vascular reactivity with bosentan treatment might be accounted for by improvement of neurovascular blood flow **( Cameron & Cotter, 1996)** as well as normalization of $ET_B$ receptor density, which is known to be reduced in diabeteic angiopathy and decrease NO liberation **(Kakoki et al., 1999).**

The suggestive vasoprotective effect of bosentan coincides with the findings reporting that bosentan treatment completely abolishes increased vascular tone occurring in diabetic angiopathy **(Dumont et al., 2003, Sahar et al, 2004).** It might be probable that increased release of ET in diabetic angiopathy antagonizes the vasorelxant effect of ACh, thus blocking ET receptors by bosentan effectively abolishes the probable vasoconstrictive effects of enhanced ET release.

Development of hypertension is one of the known complications of diabetes mellitus. Type 1 diabetes mellitus was found to induce hypertension as a primary consequence of poor glycemic control in this type of insulin-dependent diabetes and may be a factor contributing to the initiation of end-organ injury especially renal injury that augments the risk of occurance of hypertension **(Brands and Hopkins, 1996).**

The above mentioned study would support the significant blood pressure lowering effects induced by bosentan in diabetic animals that showed an elevation of mean BP in these rats with poor glycemic control. The lowering of mean blood pressure by bosentan seems to be independent from changes in blood glucose level as this parameter was not affected by treatment with the drug. This effect on elevated mean BP might provide an additional explanation of improved endothelial dysfunction since elevated blood pressure could be a complication to uncontrolled diabetes that is also associated with angiopathy.

Interestingly, an experimental study was designed to identify the roles of the endothelin (ET)-1 in brain damage induced in a streptozocin (STZ)-induced diabetes model in rats as well as the effect of bosentan, as a non-specific ET1 receptor blocker, in the prevention of the diabetes-induced brain damage. The rats were divided into four groups: the sham group (n = 10), the diabetic control group (n = 10), the group of diabetic rats given bosentan 50 mg/kg (n = 10) and the group of diabetic rats given bosentan 100 mg/kg (n = 10). Brain tissues of these rats were measured by molecular, biochemical and histopathological methods. It was found that the brain eNOS levels in the diabetic groups decreased, the ET1 and iNOS levels were found to be increased. While marked brain damage especially in hippocampus and cerebellum with the appearance of pericellular oedema in this diabetes group, but fortunately bosentan-treated groups showed a marked decrease in the incidence of these dangerous pathological changes. Based on all of these results, ET1 is strongly considered in diabetes-induced cerebral complications and bosentan, as ET1 receptor blocker would possess a protective and therapeutic effects against these complications in diabetes mellitus **(Demir et al, 2014).**

In conclusion, chronic treatment with the mixed ET receptor blocker bosentan significantly decreases proteinuria, retards diabetic nephropathic changes, improves vasorelaxant responses and lowers elevated mean non-invasive blood pressure. This would provide a strong suggestion of the beneficial therapeutic use of bosentan in management of nephropathic as well as angiopathic complications of diabetes mellitus.

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