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Risk of Endothelial Dysfunction in Streptozotocin-Induced Diabetic Sprague-Dawley Rats: Nitric Oxide in Focus

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Abstract

Endothelial dysfunction in diabetes manifests in part as reduction of nitric oxide (NO) bioavailability, which leads to inadequate relaxation of the vascular smooth muscle and a disparity between the vasoconstrictive and vasorelaxant intracellular pathways which favours a rise in vasoconstriction. In this current work, we evaluated serum Nitric oxide (NO) concentration and the activity of Nitric oxide synthase (NOS) in streptozotocin-induced diabetic male and female rats' serum. Our results showed initial and final fasting blood glucose concentration of 5.30±0.16mmol/l and 5.24±.015mmol/l versus 5.68±0.18mmol/l and 5.43±0.15mmol/l in male and female controls. Among the diabetic rats, the initial and final fasting blood glucose concentration was 17.50±1.91mmol/l and 15.68±2.84mmol/l versus 20.50±4.76mmol/l and 19.40±4.13mmol/l in male and female rats respectively. NO concentration was 106.12±7.23µmol/l and 131.81±12.54 µmol/l in male and female control rats compared to 52.38±3.01µmol/l and 65.29±16.19 µmol/l in male and female diabetic rats respectively. Serum activity of NOS were 133.72±10.92µIU/l and 156.06±18.22 µIU/l in control male and female rats compared to 98.01±1.48 µIU/l, 78.89±8.39µIU/l in diabetic male and female rats respectively. The difference in both NO concentration and NOS activity was significant between diabetics and non-diabetic rats as well as between male and female diabetics rats ($p< 0.05$). The endothelial dysfunction in diabetic animals regardless of gender may be an initial pointer to upcoming pathogenesis and we recommend routine evaluation of NO concentration and NOS activities among diabetics.

Key words: Diabetes mellitus, endothelial dysfunctions, Nitric Oxide, Nitric Oxide synthase, Streptozotocin

Introduction

Diabetes mellitus (DM) a disorder of carbohydrates metabolism is characterized by under-utilization of glucose, which leads to persistent hyperglycaemia. It occurs due to insulin absence or other factors present that opposes actions of insulin (Bernal-Mizrachi *et al*., 2004). Noticeable symptoms of hyperglycaemia include polyuria, polydipsia, weight loss, sometimes with polyphagia, and blurred vision (Dallatu *et al.,* 2011). Endothelial dysfunction in DM is manifested partly as the decreased bioavailability of Nitric Oxide (NO), resulting in insufficient relaxation of the vascular smooth muscles (American Heart Association, 2014). This causes imbalances between vasoconstrictive and vasorelaxant intracellular pathways favouring increased vasoconstriction (American Heart Association, 2014). Endothelial nitric oxide synthase (eNOS), an enzyme that produces the vasoprotective chemical nitric oxide (NO), is a essential tool in the endothelial cells' arsenal in the fight against vascular disease. Unfortunately, the human endothelium cells are unintentionally exposed to harmful risk factors like cigarette smoke, high blood pressure, high glucose levels, or high lipid levels. Despite all of this, the endothelium puts up with all of this for a while, strives maintaining NO production, and preservation of vascular protection (American Heart Association, 2014). However, the risk factors lead to excess production of superoxide ($O_2^{\cdot -}$ i.e they produce oxidative stress). $O_2^{\cdot -}$ reacts with NO to form peroxynitrite, and vascular protection slowly vanishes. But that is only the beginning of the calamity. eNOS now enters into a vicious biochemical cycle. It changes its enzymology, starts making peroxynitrite ($ONOO^-$) itself, and eventually becomes an enzyme that generates only $O_2^{\cdot -}$ (American Heart Association, 2014). Cardiovascular

risk factors increase the expression and/or activity of NADPH oxidases (NOX) in the vascular wall, thereby enhancing the production of reactive oxygen species (ROS). Animal models of hypertension, including those that receive angiotensin II infusions, spontaneously hypertensive rats (SHRs), and models of diabetes mellitus, have provided evidence for NOX activation. eNOS uncoupling plays a major role in endothelial dysfunction seen in diabetes mellitus. Tetrahydrobiopterin (BH4), a cofactor in NO production is extremely vulnerable to oxidation by ONOO. Reduced amount of BH4 encourages eNOS production of O2- . These transformations of eNOS from a beneficial enzyme to a contributor of oxidative stress have been reported in numerous in vitro models, diabetic animal models, and patients with cardiovascular risk factors (Stuehr *et al*., 2001; Muniyappa *et al.,* 2007; Mohar *et al.,* 2012).

The objective of this study is to assess the association between diabetes mellitus and CVD risk factors notably NOS uncoupling and NO depletion and whether it is affected by gender. In the current work, we evaluated serum concentration of NO and serum Nitric Oxide Synthase (NOS) in streptozotocin-induced diabetic male and female rats.

Materials and Methods

Experimental Animals

Twenty (20) Sprgue-Dawley rats (10 males and 10 females) 100-120g were purchased from the Animal House, National Veterinary Research Institute (NVRI), Vom. The animals were housed in standard cages at room temperature, in the month of July/August in the animal house, Department of Pharmacology, Faculty of Pharmaceutical Sciences, UDU, Sokoto. The twenty (20) Sprague-Dawley rats (10 male and 10 female) were divided into 4 groups:

Group 1: Five (5) male non-diabetic rats as control.
Group 2: Five (5) female non-diabetic rats as controls.
Group 3: Five (5) male diabetic rats.
Group 4: Five (5) female diabetic rats**.**

Induction of Diabetes Mellitus

A single intraperitoneal injection of freshly dissolved Streptozotocin (60 mg/kg), in normal saline maintained at 37 °C, was used to induce diabetes mellitus in diabetic group rats that had been fasting for 12 hours (Dallatu *et al.,* 2009). Tetrahydrobiopterin 20mg/kg bw/day was administered orally to a similar diabetic group for two weeks as previously reported (Kase *et al*., 2005).

Rats in the control group received a similar injection of sterile saline alone. To avoid hypoglycaemia brought on by the overt release of insulin from damaged pancreatic cells, they drank 5% glucose solution during the first 24 hours.

The rats fasted overnight, and their fasting blood glucose was determined 72 hours after receiving a dose of streptozotocin. Only rats with fasting blood glucose levels $\geq$ 7.1 mmol/L ($\geq$ 126 mg/dl) were used in the study.

From induction of DM to sample collection, the study lasted for 4 weeks. On the 28th day the overnight fasting mice were anesthetized by dropping them into clear plastic jars filled with chloroform vapour. Blood was drawn from the patient by an abdominal incision and separated into fluoride oxalate and lithium heparin anti-coagulated containers for analysis. This humane technique previously reported by Dallatu *et al.* (2009) was used.

Biochemical Analysis.

Plasma glucose estimation—GOD-POD method Trinder (1969) Randox, Switzerland. Plasma Nitric oxide assay—Griess method Schmidt (Schmidt, 1995) Cayman Chemicals, USA. Plasma Nitric oxide synthase activity—Griess method Schmidt (Schmidt, 1995) Cayman Chemicals, USA.

With the use of statistical software called "SPSS version 20," the data obtained were processed, and the results were displayed as mean Standard Error (SE) of the concentration. Using the one way ANOVA and student's t-test, the differences between the variable means were compared. A significant P-value was one below 0.05.

Results

Table 1 shows the ± Standard error of mean of fasting blood glucose (FBG) of Streptozotocin induced diabetic rats. There was substantial rise ($p<0.05$) in the fasting blood glucose mean concentration of the diabetic group injected with Streptozotocin when compared with controls ($p<0.05$). There is no gender disparity among the groups.

Table 1: Mean and Standard error of mean of fasting blood glucose (FBG) of Streptozotocin induced diabetic rats

Group	Gender (n)	Initial FBG (mmol/l)	Final FBG (mmol/l)
1(Control)	Males (5)	5.30±0.16	5.24±.015
2 (Controls)	Females (5)	5.68±0.18	5.43±0.15
3 (Diabetic)	Males (5)	17.50±1.91*	15.68±2.84*
4 (Diabetic)	Females (5)	20.50±4.76*	19.40±4.13*

±±*Values are Mean ± Standard Error of the Mean of Streptozotocin-Induced diabetic rats. Values differ significantly (P<0.05) from non-diabetic controls. Group 1controls were not diabetic! Group 2 rats not were not diabetic! Group 3 rats were diabetic! Group 4 rats were diabetic!*

Table 2 shows the mean ± Standard error of mean of plasma Nitric oxide (NO) concentration among the groups. There was statistically significant difference ($p<0.05$) between mean NO concentration of controls and diabetic groups ($p<0.05$). Gender disparity in mean NO concentration exist ($p<0.05$) among between the groups.

Table 2: Mean ± Standard error of mean of plasma Nitric oxide (NO) concentration among the groups

Group	Gender (n)	NO (µmol/l)
1(Control)	Males (5)	106.12±7.23
2(Control)	Females (5)	131.81±12.54**
3 (Diabetic)	Males (5)	52.38±3.01*
4 (Diabetic)	Females (5)	65.29±16.19*

±±*Values are Mean ± Standard Error of the Mean of Streptozotocin-Induced diabetic rats. Values differ significantly (P<0.05) from non-diabetic controls. Values differ significantly (P<0.05) by gender. Group 1controls were not diabetic! Group 2 rats not were not diabetic! Group 3 rats were diabetic! Group 4 rats were diabetic!*

Table 3 shows mean ± Standard error of mean of plasma Nitric oxide synthase (NOS) activity across the groups. There was statistically substantial difference ($p<0.05$) between mean NOS activity of controls and diabetic groups ($p<0.05$). Gender disparity in NOS activity was significant ($p<0.05$) between the groups.

Group	Gender (n)	NOS (µIU/l)
1(Control)	Males (5)	133.72±10.92
2(Control)	Females (5)	156.06±18.22**
3 (Diabetic)	Males (5)	98.01±1.48*
4 (Diabetic)	Females (5)	78.89±8.39***

±±*Values are Mean ± Standard Error of the Mean of Streptozotocin-Induced diabetic rats. Values differ significantly (P<0.05) from non-diabetic controls. Values differ significantly (P<0.05) by gender. Group 1controls were not diabetic! Group 2 rats were not diabetics! Group 3 rats were diabetic! Group 4 rats were diabetic!*

Discussion

In this study we observed the mean Nitric Oxide concentration in control rats to be significantly higher ($p<0.05$) than the concentration in diabetic rats. Gender disparity exists among the groups of the experiments; with female rats having higher concentrations than male rats of non-diabetic control. In diabetic group, however, the difference between males and females was not statistically significant ($p>0.05$). Diabetic rats showed a decrease in plasma Nitric Oxide concentration. This agrees with the findings of Muniyappa (2007) which showed a reduction in Nitric Oxide level in animal models of diabetes mellitus. American Heart Association (2014) also reported severe depletion in Nitric Oxide bioavailability as a crucial risk factor of cardiovascular disease in diabetes mellitus. This observed decrease in Nitric Oxide bioavailability in diabetes mellitus may be due to rises in glycosylation of products in blood and these products act as quenching agents for Nitric Oxide, further exacerbating endothelium dependent functionality (Mohar *et al.,* 2012). Another reason for Nitric Oxide depletion in diabetes mellitus was reported by Stuehr *et al. (2001)* in which superoxide generation has been implicated, which reacts with Nitric Oxide to form peroxinitrate. Eventually endothelial Nitric Oxide enters into a vicious biochemical cycle and starts generating peroxinitrate. This further depletes Nitric Oxide. Present study agrees with that of Mohar (2012) which reported decreased plasma Nitric Oxide level and oxidative stress in prolonged hyperglycaemia and implication on the major pathways that leads to diabetic complications such as polyol pathway, hexosamine pathway, increased Advanced Glycation End-product and increased protein kinase- pathway. These pathways are implicated in the development of diabetic complications especially cardiovascular disease.

In the current study, gender dimorphism and effect of diabetes mellitus on Nitric Oxide Synthase activity in rats were assessed. Mean Nitric Oxide Synthase activity in control rats were significantly higher ($p<0.05$) than the activity in the diabetic rats. Gender disparity existed across the experimental groups of the rats. Females express greater ($p<0.05$) Nitric Oxide Synthase activity than males in non-diabetic controls rats. However, the activity in diabetic males rats was higher than activity in females rats. In the current study, diabetic rats showed a decrease in Nitric Oxide Synthase activity which is similar to the reported by other researchers (Bauersachs and Widder, 2009). In the current study, the activity of Nitric Oxide Synthase exhibited gender dimorphism. Healthy non-diabetic female rats had higher activity of Nitric Oxide Synthase than male of the same group. This shed more light on why healthy females are naturally more protected against cardiovascular disease than males since Nitric Oxide Synthase produces Nitric Oxide which is a very important vasoprotective agent. This could explain the report of previous study that subjects without diabetes mellitus or baseline cardiovascular disease experiences ischaemic heart disease twice as high in males than in females (Ane *et al*., 2007). Higher Nitric Oxide Synthase activity in females than in males, as demonstrated by the current finding, may therefore be one of the reasons for such gender disparity in cardiovascular disease risk. The current study demonstrated that, both males and females exhibited decreased Nitric Oxide Synthase activity, but the effect is far more severe in females than in males. This could offer an explanation to the notion that, females with diabetes are at heightened risk for coronary heart diseases than males as reported in a similar study (Kanaya *et al*., 2002).

Conclusion

We can therefore conclude that the nitric oxide synthase activity and nitric oxide bioavailability are significantly reduced in diabetic subjects and may be a strong risk factor for endothelial dysfunction seen in diabetic subjects.

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