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# UNCOUPLING OF NITRIC OXIDE SYNTHASE PREDISPOSES DIABETIC RATS TO CARDIOVASCULAR RISK EVENTS

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## ABSTRACT
It has previously been established that diabetes is associated with endothelial dysfunction resulting from decrease in the activities of endothelial nitric oxide synthase (eNOS) leading to reduced nitric oxide (NO) production. In the current work, we hypothesized that acute hyperglycaemia alone may affect the vascular beds differently, leading to endothelial dysfunction and the loss of cardiovascular protection in diabetes. Adult male and female Sprague-Dawley rats, 9–11 weeks of age were divided into three groups: controls (5 males and 5 female), diabetics (5 males and 5 females) and diabetics supplemented with tetrahydrobiopterin, 20mg/kgbw/day for two weeks. Diabetic groups received a single i.v. injection of streptozotocin (STZ, 60 mg/ kg) while the control group were injected with a similar volume of citrate buffer. Results shows total cholesterol was 144.30±3.51 mg/dl, 145.66±3.78 mg/dl in controls, 165.30±3.84 mg/dl and 177.01±2.49 mg/dl in diabetics and 152.57±2.75 mg/dl, 157.70±2.02 mg/dl in diabetics supplemented respectively. HDLC was 34.40±0.42 mg/dl, 39.28±1.45 mg/dl in controls, 31.49±1.11 mg/dl, 26.59±3.12 mg/dl in diabetics and 35.05±0.73 mg/dl, 36.35±1.24 mg/dl in diabetic supplemented respectively. LDL-C was 91.87±3.48 mg/dl, 86.98±3.36 mg/dl in controls, 108.72±2.81, mg/dl 124.40±4.41 mg/dl diabetics, 94.31±3.38 mg/dl, 97.79±2.46 mg/dl diabetics supplemented respectively. VLDL-C was 18.08±1.10 mg/dl, 19.40±0.70 mg/dl in controls, 25.08±0.41 mg/dl, 26.00±0.82 mg/dl in diabetics, 23.04±0.83 mg/dl, 23.50±0.29 mg/dl in diabetics supplemented. TG was 123.50±5.39 mg/dl, 97.00±3.49 mg/dl in controls, 125.00±2.61 mg/dl, 130.00±4.08 mg/dl in diabetics, 115.20±4.13 mg/dl, 117.75±1.60 mg/dl diabetics supplemented. AIX was 4.21±0.14, 3.72±0.15 in controls, 5.26±0.13, 6.98±0.92 in diabetics, 4.36±0.10, 4.37±0.15 in diabetics supplemented respectively. With the exception of TG and VLDL-C, differences observed between controls, diabetics and diabetics supplemented were significant ($P<0.05$). Treatment with tetrahydrobioptherin, a known cofactor of NOS, tend to reversed all the anomalies to near control values, hence we conclude, uncoupling of NOS by diabetes, may predispose these subjects to cardiovascular events that may be reversed by treatment with tetrahydrobiopterin.

**KEYWORDS:** Diabetes mellitus, Lipid Profile, Cardiovascular events, Tetrahydrobiopterin.

## INTRODUCTION
Cardiovascular disease is the largest cause of mortality and morbidity in Western societies and also an emerging health burden in developing countries (Bendall *et al*., 2014). Nitric oxide (NO), produced by endothelial NO synthase (e NOS) in the vascular endothelium, is a critical signaling molecule in vascular homoestasis (Bendall *et al*., 2014). Loss of NO bioavailability is a key feature of endothelial dysfunction in vascular disease states such as hypertension, diabetes and atherosclerosis. Furthermore, impaired NO –mediated endothelial function is an independent risk factor for cardiovascular diseases (Heitzer *et al*., 2001).

Several factors contribute to loss of NO bioavailability. These include reduced NO synthesis from defective eNOS activity, NO scavenging by reactive oxygen species (ROS) and certain disease conditions (Cai and Harriosn, 2000), and these processes are now understood to be not a separate entities, rather they interact in a cascade response to vascular. n the vasculature, e NOS is critical to these signaling pathways (Rebelink and Luscher, 2006).

Endothelial NOS must be in an active dimer state to produce NO. Regulation of the dimeric e NOS complex is important for proper functioning of e NOS.L-arginine and tetrahydrobiopterin (BH4) are two critical factors

that maintain the dimeric state of e NOS allowing electron flow across the homodimer to generate NO from the ferrous-dioxygen complex (Hingorani *et al*., 2000). BH4 deficiency renders e NOS in an uncoupled state, generating more singlet oxygen implicated in a variety of experimental and clinical vascular disease states including diabetes, hypertension and atherosclerosis (Festa *et al*., 2000)**.** Hyperglycaemia also results in BH4 deficiency and hence e NOS dysfunction characterized by a decrease in NO (Guzik *et al.*, 2000).

Physiologically, the liver responds to free fatty acid flux by increasing very-low-density lipoprotein production and cholesteryl ester synthesis (Sniderman *et al*., 2002). This increased production of triglyceride-rich proteins and the diminished clearance by lipoprotein lipase results in hypertriglyceridemia, which is typically observed in diabetes (Cummings *et al*., 1995). Elevated triglyceride concentrations lower HDL by promoting cholesterol transport from HDL to very-lowdensity lipoprotein (Sniderman *et al*., 2002). These abnormalities change LDL morphology, increasing the amount of the more atherogenic, small, dense LDL (Dimitriadis *et al.*, 1995)**.** Both hypertriglyceridemia and low HDL have been associated with endothelial dysfunction (deMan *et al*., 2000).

Studies have shown that, exogenous addition of BH4 increase both e NOS activity and dimerization (Read *et al*., 1994).

Although studies indicate that BH4 facilitates electron transfer and maintain the dimerized state of the enzyme, the complete role of BH4 in e NOS regulation is still unknown. We hypothesized that increasing endothelial BH4 bioavailability would have a salutary effects on vascular inflammation and remodeling after vascular injury secondary to diabetic induction. The current work was designed to evaluate the effect of BH4 supplementation on some cardiovascular risk events in streptozotocin induced diabetic rats.

## MATERIALS AND METHODS

Thirty (30) Albino Wistar rats (15 males and 15 females) 100-120 g divided in to control group (5 males and 5 females), diabetic group (5 males, 5 females) and diabetic group supplemented (5 males, 5 females) were used in the study.

Diabetes mellitus was induced by a single intraperitoneal injection of freshly dissolved Streptozotocin (60mg/kg) (Dallatu *et al*., 2009), in normal saline maintained at 37$^{o}$C, to diabetic group rats, fasted for 12 hours. Similar diabetic group received Tetrahydrobiopterin 20mg/kg b.w./day orally for two weeks as reported (Kase *et al*., 2005).

Control rats received a similar injection of normal saline alone. Glucose solution 5% was used as their drinking water for the first 24 hours to prevent hypoglycaemia due to overt release of insulin from disrupted pancreatic cells.

Seventy two (72) hours after Streptozotocin injection, the rats were fasted overnight and their fasting blood glucose was estimated. Only rats from groups 2 and 3 that have fasting blood glucose level of ≥ 7.1mmol/L (≥126mg/dl) were included in the study.

Fourteen days after diabetes induction, rats were fasted overnight and anaesthetized by dropping each in a transparent plastic jar saturated with chloroform vapour. Incision was made on the abdomen, and blood sample was obtained through cardiac puncture and divided into fluoride oxalate and lithium heparin anti-coagulated containers until analysed. Humane procedure was used as adopted by Dallatu *et al.*, (2009).

### Biochemical Analysis

Total cholesterol (TC) was estimated by Enzymatic (colorimetric) method of Trinder, *(*1969), High Density Lipoprotein-Cholesterol (HDL-C), Trinder, *(*1969), Low Density Lipoprotein-Cholesterol (LDL-C) and Very Low Density Lipoprotein Cholesterol (VLDL-C) were calculated by the formula of Friedewald, *et al; (*1974) and atherogenic Index as described by Tiez, (1994).

The data generated were analyzed using 'SPSS version 20' statistical software and presented as mean ± Standard Error (SE) of the concentration. Differences between means of the variables were compared using one way ANOVA. P-value <0.05 was considered significant.

## RESULTS

Results of the current study, showing lipid profile of male and female controls, streptozotocin-Induced diabetic rats untreated and those treated with tetrahydrobiopterin was shown on table 1.

**Table 1: Shows lipid profile of controls, diabetic and diabetic rats treated with tetrabioptherin.**

| Group | Gender (n) | TC mg/dl | HDL-C mg/dl | LDL-C mg/dl | VLDL-C mg/dl | TG mg/dl | AIX |
|---|---|---|---|---|---|---|---|
| 1(Control) | Males (5) | 144.30±3.51 | 34.40±0.42 | 91.87±3.48 | 18.08±1.10 | 23.50±5.39 | 4.21±0.14 |
| | Females (5) | 145.66±3.78 | 39.28±1.45 | 86.98±3.36 | 19.40±0.70 | 97.00±3.49 | 3.72±0.15 |
| 2(Untreated) | Males (5) | 165.30±3.84 | 31.49±1.11 | 108.72±2.81 | 25.08±0.41 | 125.00±2.61 | 5.26±0.13 |
| | Females (5) | 177.01±2.49 | 26.59±3.12 | 124.40±4.41 | 26.00±0.82 | 130.00±4.08 | 6.98±0.92 |
| 3 (Treated) | Males (5) | 152.57±2.75 | 35.05±0.73 | 94.31±3.38 | 23.04±0.83 | 115.20±4.13 | 4.36±0.10 |
| | Females (5) | 157.70±2.02 | 36.35±1.24 | 97.79±2.46 | 23.50±0.29 | 117.75±1.60 | 4.37±0.15 |

±± Values are Mean ± Standard Error of the Mean of Streptozotocin-Induced diabetic rats treated with tetrahydrobiopterin for 14 days.

∗ Values differ significantly ($p<0.05$) from non-diabetic controls.

∗∗ Values differ significantly ($p<0.05$) from untreated.

∗∗∗ Values differ significantly ($p<0.05$) by gender.

## DISCUSSION

Tetrahydrobiopterin(BH4) is reported to act as a redox regulator of e NOS by promoting and stabilizing e NOS protein monomers in to the active homodimeric form, which in turn maintains the healthy state of the endothelium.[7] It plays important role in maintainance of cardiac and vascular homeostasis and its bioavailability modulate the development of cardiovascular diseases such as atherosclerosis, heart failure diabetes and hypertension (Bendall *et al.,* 2014).

In the current work, we were able to report that, supplementation with BH4 in streptozotocin induced diabetic rats, improves the lipid profile of the rats to near normal, especially the HDL-C and atherogenic index.

Circulating levels of free fatty acids are elevated in diabetes because of their excess liberation from adipose tissue and diminished uptake by skeletal muscle (Fujimoto, 2000). Free fatty acids may impair endothelial function through several mechanisms, including increased production of oxygen-derived free radicals, activation of PKC, and exacerbation of dyslipidaemia (Inoguchi *et al*., 2000)**,** as can be evidenced in our findings.

Another important mechanism of endothelial dysfunction in diabetes mellitus is alteration of the signaling pathways that lead to eNOS inactivation in the endothelium. NO production in endothelial cells depends on the enzymatic conversion of Larginine to NO and citrulline by eNOS, which also requires availability of BH4.The enzyme is constitutively expressed in endothelial cells and is localized to caveolae, which are specialized invaginations of the plasma membrane that are rich in specific lipids and proteins, including caveolin-1 (Shaul *et al*., 1996)**.** eNOS has a low level of basal activity because of its association with caveolin-1, but widely reported to be activated by numerous cofactors such as BH4.Once produced, eNOS-derived NO diffuses locally in the arterial wall and activates guanylyl cyclase in vascular smooth muscle cells, platelets, and endothelial cells to induce its biological effects as observed here (Krumenacker *et al*., 2004).

Abnormalities in endothelial and vascular smooth muscle cell function, as well as a propensity to thrombosis, as observed in the current work, contribute to atherosclerosis and its complications. Endothelial cells, because of their strategic anatomic position between the circulating blood and the vessel wall, regulate vascular function and structure. In normal endothelial cells, biologically active substances are synthesized and released to maintain vascular homeostasis, ensuring adequate blood flow and nutrient delivery while preventing thrombosis and leukocyte diapedesis. NO bioavailability, protects the blood vessels from atherosclerosis by mediating molecular signals that prevent platelet and leukocyte interaction with vascular beds (Mark *et al*., 2003).

BH4 has proven to be an established therapeutic agent in restoring e NOS-mediated NO formation and endothelial function in hypertension, hypercholesterolemia, and diabetes (Vasquez-vivar *et al*., 2002).Together, these findings suggest that supplementation of BH4 may be useful to alleviate vascular complications, reduced CVD risk probably through restoration of endothelial function/e NOS activity in streptozotocin induced diabetic rats.

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