Original Article

Polycythemia, Thrombocythemia, and Hyperfibrinogenemia are Associated With Streptozotocin-induced Diabetes and Salt-induced Hypertension in Male Wistar Rats

David Ehikhuemen Okonofua[1], Jerome Ndudi Asiwe[1,2*], Emuesiri Goodies Moke[3], Nosakhare Famous Igie[4], Kamaldeen Olalekan Sanusi[1], Jolaoluwa Oluwatosin Yesufu[1], Adesoji Adedipe Fasanmade[1]

1. Department of Physiology, Faculty of Basic Medical Sciences, University of Ibadan, Ibadan, Nigeria.
2. Department of Physiology, Faculty of Basic Medical Sciences, Delta State University, Abraka, Nigeria.
3. Department of Pharmacology, Faculty of Basic Medical Sciences, Delta State University, Abraka, Nigeria.
4. Department of Chemistry, School of Natural Sciences and Mathematics, University of Texas at Dallas, USA.

*** Corresponding Author:**
Jerome Ndudi Asiwe, PhD.
Address: Department of Physiology, Faculty of Basic Medical Sciences, University of Ibadan, Ibadan, Nigeria.
Phone: +98 (81) 63727468
E-mail: asiwejerome@yahoo.com

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ABSTRACT

Background: Diabetes mellitus and anaemia are frequently reported to be associated with polycythemia in several studies. Furthermore, some studies also have linked polycythemia with hypertension. However, whether hypertension and diabetes comorbidity had polycythemia/erythrocytosis, thrombocythemia, or hyperfibrinogenemia is unknown.

Objectives: This study investigated the incidence of polycythemia, thrombocythemia, and hyperfibrinogenemia in diabetic and hypertensive male Wistar rats.

Methods: Thirty male Wistar rats were categorized into five groups, each with six animals: negative control (zero-salt diet), positive control (standard salt diet – 0.3% salt), high salt diet – 8% salt (HSD only), Streptozotocin (STZ)-induced diabetes fed with normal salt diet (STZ only), and high salt diet with STZ-induced diabetes (HSD+STZ). Hematological variables and fibrinogen concentration were measured after a 4-week experimental period. One-way ANOVA was used for statistical analysis and a $P<0.05$ was considered significant.

Results: The heart rate and mean arterial pressure increased significantly in the HSD, STZ, and HSD+STZ groups, suggesting salt-induced hypertension. Compared to the controls, the STZ and HSD +STZ groups had significantly higher hematocrit, platelet estimate, and fibrinogen concentration. The STZ and HSD+STZ groups had a shorter clotting period, which correlated with higher platelet counts and fibrinogen levels. Compared to the controls, the HSD group had a lower platelet count and fibrinogen concentration, as well as a longer clotting time.

Conclusion: This study suggests that polycythemia, thrombocythemia, and hyperfibrinogenemia are potential risk factors for hypertension in people with diabetes mellitus.

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Introduction

Polycythemia/erythrocytosis has been identified in Diabetes Mellitus (DM) in some studies, and several researchers have linked it to the development of DM with anemia [1]. However, because blood viscosity is dependent on hematocrit, plasma proteins (fibrinogen and globulin), and plasma water, the documented increase in blood viscosity in DM patients is a cause for concern [2]. To assess the activity of erythrocytes in Type 2 DM, the red blood cells (RBC) biorheology and biomechanics in type 2 DM were modeled [3]. Extrinsic erythrocyte deformability is reduced under high-shear stress, while intrinsic erythrocyte aggregation is increased under low-shear stress [4, 5]. This may explain why DM showed an increase in hematocrit as well as a decrease in plasma water. Furthermore, experimental studies have identified a connection between polycythemia and hypertension [4]. The role of platelets in maintaining normal homeostasis cannot be over-emphasized. For example, in living systems, platelets are also linked to cardiovascular and metabolic events [6, 7]. There have been several studies on the function of platelets in cardiovascular events, with an emphasis on thrombotic complications. Increased platelet reactivity with the prothrombotic condition has been linked with DM [8]. Also, changes in the morphology of platelet and increased activity (thrombocythemia) have been identified in DM patients [9, 10]. Fibrinogen, a large glycoprotein molecule secreted by liver cells that serves as a natural thrombin substrate, plays a role in the inflammatory process, hemostatic equilibrium, tissue regeneration, and angiogenesis [11]. By converting to fibrin through the action of thrombin and modulating neutrophil, platelet, and endothelial cell adhesion to fibrinogen at the site of injury, fibrinogen mediates the coagulation cascade and inflammation [12, 13]. Hyperfibrinogenemia has also been identified in patients with type 2 diabetic patients as well as insulin resistance [14, 15]. However, this present study investigated the incidence of polycythemia/erythrocytosis, thrombocythemia, or hyperfibrinogenemia in diabetic male Wistar rats with hypertension.

Materials and Methods

Experimental design

Thirty male Wistar rats (120-150 g) from the College of Medicine Central Animal House, University of Ibadan were used for the study. The experimental animals were housed under normal laboratory conditions and fed standard rat pellets and water ad libitum for two weeks prior to the start of the experiment. Animals were handled in compliance with the National Institute of Health's guidelines for the treatment and use of research animals, which were adopted by the University research ethics committee (UI-ACUREC/18). The rats were divided into five groups, each containing six animals (n=6):

Group 1: Negative control (zero-salt diet)

Group 2: Positive control (normal salt diet–0.3% salt)

Group 3: High salt diet–8% salt (HSD only)

Group 4: STZ induced diabetes fed with a normal salt diet (STZ only)

Group 5: High salt diet with STZ-induced diabetes (HSD+STZ)

Preparation of feed rations

For the report, three separate rations were used [16]. Eight grams of table salt was mixed with 92 g of regular rat chow to make the high-salt diet. This ration and unlimited water were given to the HSD only and HSD+STZ groups. The usual salt diet was made by combining 0.3 g of table salt with 99.70 g of regular rat chow and unlimited water for the positive control group. The negative control group was administered a zero-salt diet.

Induction of diabetes mellitus

Diabetes was induced by a single intraperitoneal injection of 60 mg/kg body weight of streptozotocin (STZ) dissolved in freshly prepared 0.1 M cold citrate buffer of pH 4.5 into the animals [16, 17]. Prior to diabetes induction, the experimental animals fasted for 18 hours despite having access to water. Blood was taken from the rats' tail veins 72 hours after the STZ injection to test their blood glucose level. Animals having fasting blood glucose levels ≥200 mg/dL were considered diabetic and used in the study.

Measurement of blood glucose level

In order to ascertain the diabetic state of the animals, the fasting blood sugar level was measured every week after being fasted overnight using the ACCU-CHEK Active glucometer (Model GB06140695). Blood samples were taken from the rats' tail arteries in order to test their blood glucose levels. Animals' body weights were also assessed weekly in grams to see if induced diabetes and high salt diet affected their body composition. A standard weighing scale was used to determine the weight.

Blood pressure and heart rate

Blood pressure and heart rate were measured at the Veterinary Clinic, University of Ibadan's Small Animal Ward in Ibadan, Nigeria. The tail-cuff plethysmography method was used to measure systolic blood pressure (SBP) in a conscious and slightly restrained rat (Kent Scientific, USA). During the BP measurement, heart rate tracings were detected and reported. Rats were conditioned to the restraint (cone) and the warming chamber for around 20 minutes prior to the measurement for these tests. To prevent sound interference from the same investigator, SBP and HR measurements were taken in a very quiet environment. Two sensors were used to test blood pressure and vascular peripheral resistance. Following the chamber's stabilization, an acclimatization run of five cycles was performed, followed by a standard run of ten repetitions of the automatic inflation-deflation cycle.

Blood collection

At the end of the 4-week cycle, blood samples were collected from animals through the retro-orbital sinus and stored in heparinized tubes, which were used to calculate hematological indices and fibrinogen concentration. The rats were euthanized by prolonged chloroform exposure and then buried at the conclusion of the study.

Hematological analysis

"Unico" microhaematocrit reader was used to calculate hematocrit. Cronkite's ammonium oxalate method was used to calculate platelet count. The clot weight method of Ingram was used to calculate plasma fibrinogen concentration [18]. The capillary method was used to test the clotting time [8].

Statistical analysis

Data were analyzed by GraphPad Prism Software, version 7.0 (San Diego, CA) and expressed as Mean ± SEM. One-way ANOVA was used for comparisons, followed by a post-hoc Newman-Keuls multiple comparison test. Results were statistically significant at $P<0.05$.

Results

Red blood cell count and hematocrit level

Figures 1 and 2 show the red blood cell count (10^6/mm^3) and hematocrit (Packed Cell Volume (PCV)) four weeks after the induction of diabetes and receiving a high-salt diet. There was a significant increase in red blood cell count [$F_{(4, 15)}=13.5$, P<0,0001] and hematocrit [$F_{(4, 15)}=54$, $P<0.0001$] when other groups were compared to the controls.

Platelet count

The STZ only and HSD+STZ groups showed a significant ($P<0.05$) increase in platelet count [$F_{(4, 15)}=24.1$, $P<0.0001$] compared to the control groups (Figure 3).

Fibrinogen estimates

This study showed that the fibrinogen levels significantly ($P<0.05$) increased in the STZ only and HSD+STZ groups [$F_{(4, 15)}=8$, $P<0.0011$] compared to the control groups (Figure 4).

Clotting time

A significant ($P<0.05$) decrease was found in the clotting time [$F_{(4, 15)}=32$, $P<0.0001$] of the STZ and HSD+STZ groups compared to the control groups (Figure 5).

Discussion

This study reported a significant increase in red blood cell count and hematocrit when other groups were compared with the controls (Figures 1 and 2). The significant increase in erythrocyte count (Figure1) and PCV (Figure 2) when the experimental groups were compared with the control groups contradicted the findings of Uko et al. [1], Oyedemi et al. [19] and Kothari and Bokariya [20] who reported a decrease in PCV in type 2 DM. This report suggested that the incidence of anemia in DM is linked to the increased RBC membrane protein non-enzymatic glycosylation, which correlates with increased blood glucose [19, 21]. The occurrence of erythrocytosis in type 1 DM subjects has also been recorded [22]. The significant increase in PCV in the HSD in this study is in concordance with other studies reporting that polycythemia could be a risk factor for the etiology of hypertension [23-25]. A possible mechanism to support the increase in PCV could be the behavior of erythrocytes in subjects with DM. The structure of a healthy human red blood cell is a biconcave disc, 8 μm in diameter, 2 μm thick at the rim, and 1 μm thick at its center [26]. Red blood cells are capable of losing their shape as they progressively stretch under shear stress and form ellipsoids with their long axis aligned with the flow [4, 27]. The red blood cell is capable of deformations when passing through narrow capillaries as small as 3 mm in diameter without any damage due to the fluid nature of the lipid bilayer and the elastic nature of the cytoskeleton. In

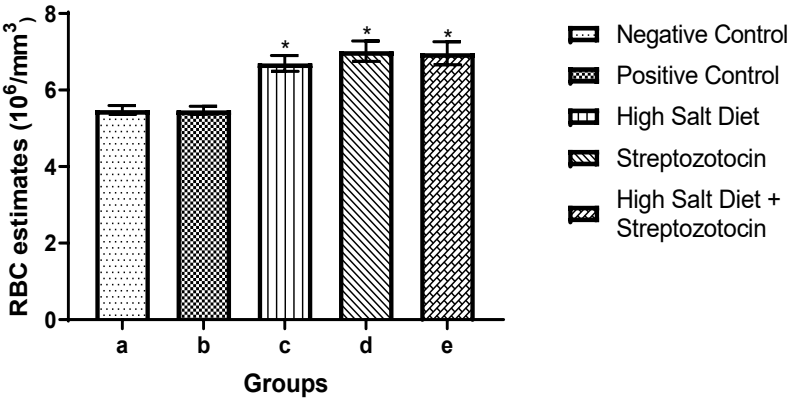

Figure 1. Red blood cell count (10^6/mm^3) four weeks after the induction of diabetes and receiving a high-salt diet

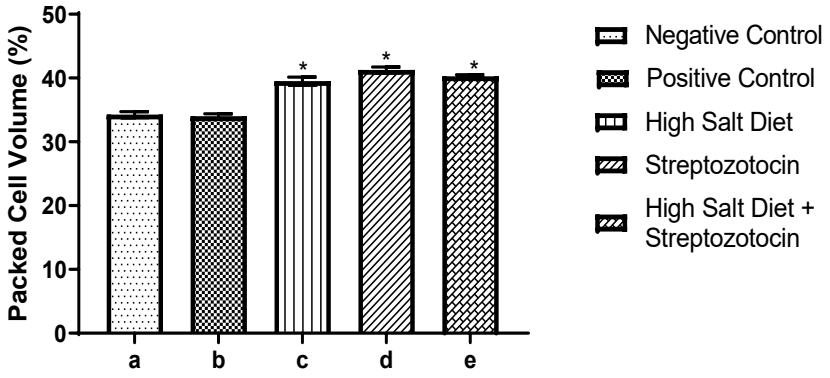

Figure 2. Hematocrit (%) four weeks after the induction of diabetes and receiving a high-salt diet

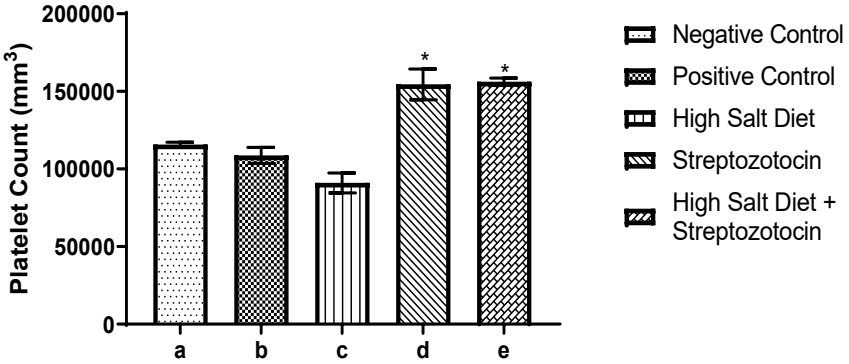

Figure 3. Platelet count (mm^3) four weeks after the induction of diabetes and receiving a high-salt diet

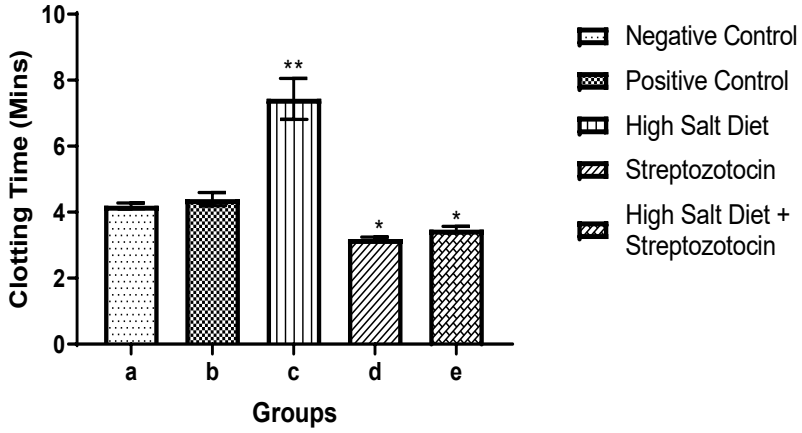

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Figure 4. Fibrinogen estimates (mm^3) four weeks after the induction of diabetes and receiving a high-salt diet

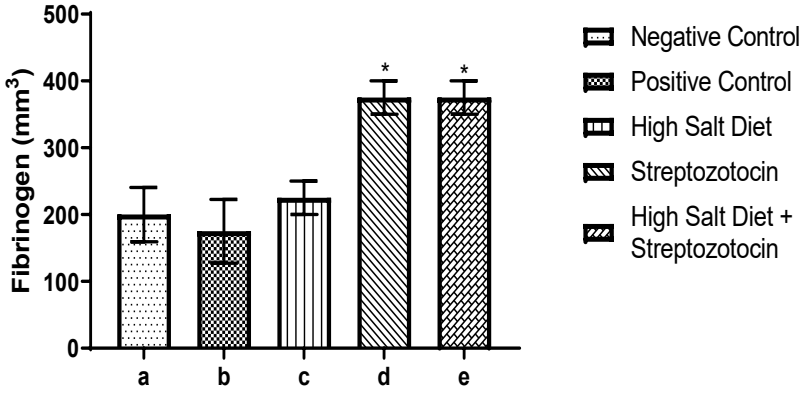

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Figure 5. Clotting time (minute) four weeks after the induction of diabetes and receiving a high-salt diet

disease conditions, the change in cell geometry and the membrane properties of the affected red blood cells can lead to less effective functionality, which includes loss of deformability [28]. Reports have shown that red blood cells are less deformable and become larger (erythrocytosis) in DM [29]. Metabolic disturbances result in the development of irregularities in the contours of red blood cells in DM, which can cause a significant reduction in its deformability [30]. Also, the intrinsic behavior of erythrocytes in the formation of rouleaux is increased in subjects with DM due to increased aggregating factors, like platelets, fibrinogen, and globulins [31, 32]. Although the mechanism is yet to be fully understood.

DM is associated with increased platelet levels [33]. We observed a significant increase in platelet count in the STZ only and HSD+STZ groups compared to the control groups (Figure 3). Some of the possible factors that play a direct role in increased platelet reactivity are metabolic abnormalities, such as increased blood glucose as well as increased blood lipids through non-enzymatic glycation of platelet protein [33, 34], both insulin resistance and absolute insulin deficiency [35], together with its associated conditions, such as oxidative stress, inflammation, and endothelial dysfunction [36, 37]. A possible mechanism for the increase in platelet levels is oxidative stress. Oxidative stress alters endothelial function and causes a decrease in nitric oxide release [38, 39]. Endothelial dysfunction also results in decreased prostacyclin release [40]. Hence, oxidative stress that follows DM incites greater platelet reactivity through direct effects on platelets and by causing endothelial dysfunction. The decreased production and reduced effect of nitric oxide as well as decreased production of prostacyclin have been linked to the endothelial dysfunction-induced increase in platelet reactivity. However, we

observed no significant decrease in the platelet levels in the HSD group. Conversely, hypertension is closely correlated with an increased platelet count [41]. We showed that the fibrinogen levels significantly increased in the STZ only and HSD+STZ groups compared to the control groups (Figure 4). Studies have reported an associated increase in fibrinogen levels in diabetes and hypertensive subjects [42, 43]. There are several possible mechanisms to explain the increased fibrinogen levels in DM. Most of these mechanisms follow a similar pattern to the mechanism for increased platelet reactivity in DM. DM is associated with mild inflammation, leading to an increase in interleukin-6, which stimulates liver cells to synthesize fibrinogen, which forms the major connection between inflammation and hypercoagulation [42]. Another possible mechanism is insulin resistance and insulin deficiency, which are linked to increased liver fibrinogen production in response to insulin [42, 44]. A reported increase in fibrinogen production has also been demonstrated postprandially in type 2 DM but not in healthy controls, which suggests a possible abnormal regulation of fibrinogen production in the liver [45]. However, an association between oxidative stress and increased plasma fibrinogen level in DM has also been reported [37, 46].

The clotting time results showed that there was a significant decrease in the clotting time of the STZ and HSD+STZ groups compared to the control groups (Figure 5). This could be a result of the increased platelet and fibrinogen levels. Several clotting factors in the plasma, including fibrinogen, factor VII, factor VIII, factor XI, factor XII, and kallikrein, are elevated in DM [47, 48]. Conversely, in diabetic conditions, the level of anticoagulant protein C is decreased [48, 49]. High levels of plasma fibrinogen with low plasma protein C activity could lead to a prothrombotic tendency in both insulin-dependent and insulin-deficient diabetic patients [49]. Thrombosis is also associated with increased blood viscosity and microvascular complications [50]. These complications are possible risk factors for the development of associated hypertension [50, 51]. There was a significant increase in the clotting time of the HSD group. This increase could be a result of the decreased platelet and fibrinogen levels.

Conclusion

This study suggests that polycythemia, thrombocythemia, and hyperfibrinogenemia are potential risk factors for people with hypertension and DM comorbidity. Proper management of hemodynamic characteristics is important to avoid exacerbating the comorbidity of DM and hypertension.

Ethical Considerations

Compliance with ethical guidelines

The institution, University of Ibadan Animal Research Ethics Committee agreed with the “Guide to the care and use of laboratory animals in research and teaching” as prescribed in NIH publications volume 25 No.28 revised in 1996, approved the use of animal for this study with approval number UI-ACUREC/18.

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Authors' contributions

All authors equally contributed to preparing this article.

Conflict of interest

The authors declared no conflicts of interest.

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