

Haematologic Response in Hyperglycaemic Rats Experimentally Induced with Alloxan

Fakoya Olugbenga Olatunde* and Oseni Bashiru Anthony

Department of Biomedical Sciences, Faculty of Basic Medical Sciences, College Of Health Sciences, Ladoke Akintola University of Technology, Ogbomoso, Oyo State, Nigeria.

*Correspondence author: Fakoya O Olatunde, Department of Biomedical Sciences, Faculty of Basic Medical Sciences, College Of Health Sciences, Ladoke Akintola University of Technology, Ogbomoso, Oyo State, Nigeria, Tel: +2348132997431; E-mail: dantuned85@yahoo.com

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Abstract

Background: Severe hyperglycaemia has been associated with metabolic and organ dysfunctions with insufficient information on haematologic abnormalities. This study investigates the effect of induced hyperglycaemia on some peripheral blood and bone marrow indices with a view to mimic haematologic picture of poorly and untreated diabetes mellitus patients.

Methods: Sixty male albino rats weighing 175-250 g used for this study were divided equally into control and test groups. Hyperglycaemia was induced with 170 kg bwt⁻¹ alloxan intraperitoneally in the test group while control groups received sterile normal saline. At day 12 post induction haematological parameters were investigated as packed cell volume, white blood cell count (total and differential), haemoglobin concentration, red cell count, red cell indices of mean cell volume, mean cell haemoglobin and mean cell haemoglobin concentration, platelet count, reticulocytes count, bone marrow myeloid-erythroid ratio and cytochemical-hemosiderin demonstration. Data obtained were analyzed using statistical package for social sciences and expressed as mean \pm 1 standard deviation. Test of significance for control and test was done using student T-Test and expressed as $p < 0.05$.

Results: Results of test compared to control showed significant reduction ($p < 0.05$) in body-weight (188 ± 15 vs. 224 ± 30)g, packed cell volume (24.40 ± 3.87 vs. 40.45 ± 3.93)%, haemoglobin Concentration (7.81 ± 1.45 vs. 13.39 ± 0.40) g/dl, neutrophil count (12.50 ± 2.72 vs. 24.90 ± 5.34)% and red blood cell count (3.47 ± 0.29 vs. 7.16 ± 0.25) $\times 10^{12}/l$, while reticulocyte count (12.4 ± 1.87 vs. 3.69 ± 0.47)%, mean cell volume (70.55 ± 11.16 vs. 56.35 ± 5.18)fl, mean cell haemoglobin (22.44 ± 3.97 vs. 18.71 ± 0.96)pg, eosinophil (13.35 ± 2.32 vs. 2.45 ± 1.53)%, basophil (2.45 ± 1.88 vs. 0.65 ± 0.67)% were significantly ($p < 0.05$) increased. Total leucocytes (5.50 ± 1.62 vs. 5.36 ± 1.67) $\times 10^9/l$, lymphocytes (69.30 ± 3.28 vs. 70.15 ± 4.17)%, monocytes (2.60 ± 0.8 vs. 3.80 ± 1.5)% and platelet count (216.05 ± 26.37 vs. 203.95 ± 23.05) showed no significant difference in both groups. The bone marrow smear revealed marked increase in myeloid-erythroid ratio (6:1 vs. 2.7:1) with increased hemosiderin pigments.

Conclusion: Our data describes body weight loss with regenerative anemia evidenced by peripheral reticulocytosis and neutropenia. This alongside medullary leukaemoid reaction and an undefined dyserythropoiesis arising from megaloblastic maturation characterizes the haematologic response. Conclusively, a complex haematologic abnormality is envisaged to accompany untreated and poorly controlled diabetes mellitus patient.

Keywords: Haematologic; Hyperglycaemic; Alloxan

Abbreviations: ROS: Reactive Oxygen Species; MNPS: Myelomonophagocytic System; EDTA: Ethylene Diamine Tetraacetic Acid; PCV: Packed Cell Volume; RBC: Red Blood Cell; MCV: Mean Cell Volume; MCH: Mean Cell Haemoglobin; MCHC: Mean cell Haemoglobin Concentration; SPSS: Statistical Package for Social Sciences.

Background

One of the most notable clinically important human diseases with global prevalence is diabetes mellitus. It is a disorder of impaired carbohydrate metabolism usually secondary to a relative or an absolute deficiency of insulin [1]. Irrespective of classification, diabetes mellitus is usually characterized by an excessively high glucose concentration in the blood a condition known as hyperglycaemia. Uncontrolled

hyperglycaemia appears to be the principal biochemical abnormality that underlies the increased oxidative load contributing to the pathogenesis of the diabetic complications in diabetes mellitus [2].

Deficiencies resulting from hyperglycaemia, causes an accelerated development of diabetic complications such as retinopathy, nephropathy neuropathy and cardiovascular diseases [3].

An effective method of establishing experimental diabetes is chemical induction by Alloxan (2,4,5,6-tetraoxypyrimidine; 2,4,5,6-pyrimidineterone) [4].

It is a well-known diabetogenic agent, found to produce stable hyperglycaemia at 160-180 kgbw⁻¹ for prolong period [5]. The mechanism of alloxan in hyperglycaemia induction has been reported to be cytotoxic sometimes capable of causing wide range damage across the entire body system and predisposing the experimental animals to premature death [6].

Other studies however posit that glucose given at suitable dosage may partly ameliorate the unwanted deleterious effect observed from alloxan induction [2].

Recent research works on diabetic animal have established that chronic hyperglycemia generates Reactive Oxygen Species in the vascular environment (ROS) [7,8]. These reactive substances are able to attack unsaturated fatty acids of membrane phospholipids and cause lipid peroxidation damaging cellular membrane structures most importantly the erythrocytes and other clinically important haematologic cells [9-11].

Haematological parameters are important, powerful and rapid diagnostic tools as well as component of minimum data base which can be used to assess the severity of an illness [12], this can be of high importance in low socioeconomic countries where financial sources to more elaborate investigation can be a challenge.

The erythrocytes, leucocytes and platelets are products of differentiation and maturation of the bone marrow pluripotential stem cell [13]. Induction of cellular injuries by hyperglycaemia has been observed in various tissues in diabetes [14,15] with scarce information on haematologic tissues (blood) and organs (bone marrow). The aim of this study therefore is to evaluate the effect of the hyperglycaemic environment on the peripheral and bone marrow cells.

Materials and Methods

The haematologic response of hyperglycaemic rat was an experimental, case-control study conducted at the mercyland campus of ladoke akintola university of technology, Osogbo. Sixty (60) white male albino rats weighing 175-250 gm were acclimated for 14 days to the animal house of the mercyland campus of ladoke akintola university, college of health sciences, osogbo.

The selected animals were housed in wire mesh well aerated cages at normal atmospheric temperature ($25 \pm 5^\circ\text{C}$) and normal 12 hour light/dark cycle. They had free access to water and were supplied daily with standard diet of known composition ad libitum. All animal procedures were in accordance with the standard recommendations for care and use of laboratory animals.

Chemicals and reagent

Alloxan was purchased from Sigma chemicals Co., St. Louis, MO, USA, protected from light exposure and stored at $2-4^\circ\text{C}$. All other chemicals were of analytical grade and were obtained from licensed laboratory reagent suppliers.

Induction of diabetes

Rats were weighed and blood samples collected from the tail vein for baseline plasma glucose estimation using randox glucose kit, Glucose Oxidase method. Subsequently, the animals were divided equally into two (2) groups.

Group 1: Control group were injected with freshly prepared sterile saline [5].

Group 2: Test group received 170 kgbwt^{-1} alloxan preparation (2,4,5,6-tetraoxypyrimidine; 2,4,5,6-pyrimidineterone) induction. All injections were done through single intraperitoneal administration using a total volume of 0.5 ml, after estimating the effective dose and administered volume with respect to their weights [5].

Experimental design

At days 3,6,9 post injection, rats were re-weighed and glucose estimation was done in all the two (2) groups described above. At day 10 post induction, twenty four (24) rats had very high plasma glucose level greater than 250 mg/dl and were thus included for group 2 while four (4) lower responsive rats were excluded. Animals were sacrificed both for blood sample collection and bone marrow studies by exposure to chloroform within a closed system.

Blood was collected via cardiac puncture into ethylene diamine tetra acetic acid (EDTA) anticoagulated bottles for all haematological analysis. Sacrificed animals were dissected and femoral marrow sample obtained and processed for bone marrow studies.

Haematological estimations

Complete blood count comprising of Hb, HCT, RBC count, MCHC, MCV, MCH, Total and Differential leucocyte count and platelet count were assayed using SYSMEX Automated Hematology Analyzer (KX-21) at Lautech teaching hospital, osogbo. Peripheral blood for reticulocyte count was incubated with new methylene blue, smeared and estimated manually at the Lautech teaching hospital osogbo.

Bone marrow analysis

Bone marrow smears were made on a clean grease free glass slide, fixed in methanol and stained with romanowsky stains (leishman) according to Dacie and Lewis) method [16] and perls prussian blue stain according to the mixed method by Awrioro, [17]. Stained smears were air dried and microscopically examined at X100 objectives. Results were obtained, documented and compared between test and control.

Statistical Analysis

Data obtained were analyzed using statistical package for social sciences version 15 (SPSS Inc., Chicago, IL) for windows and expressed as mean \pm 1 standard deviation. Test of significance comparing control and test was done using student T-Test and defined as $p < 0.05$.

Result

A total of fifty four (54) rats; 30 controls and 24 (80%) tests were utilized for this study. Two (6.6%) of the test rats were lost to death on day 2 and 3 post induction while the other four (13.3%) test rats showed no significant rise in plasma glucose when compared with their baseline plasma glucose and thus excluded.

Changes in body weight and plasma glucose with post-induction days in (Table 1) summarizes the effect of alloxan on the plasma glucose and body weight of the test rats compared to the control which received saline.

At day 3 post induction, plasma glucose was slightly increased in the test group compared to the control (102 vs. 82 mg/dl) with no observed difference in their body weight (216 ± 22 vs. 215 ± 30 g).

At the sixth (6) post induction day, significantly increased ($p < 0.05$) plasma glucose level (207 vs. 80 mg/dl) was observed and a slight reduction in body weight (211 ± 20 vs. 220 ± 30 g) of the test rats.

On day 9, significant reduction ($p < 0.05$) in body weight (202 ± 20 vs. 221 ± 30 g) and plasma glucose (267 vs. 80 mg/dl) were recorded in

hyperglycaemic test rats which continued at day 12 post induction day (188 ± 15 vs. 224 ± 30 g) and (308 vs. 79 mg/dl) respectively.

Control (Saline Induction)			Test (Alloxan Induction)	
Day	Body weight (g)	Plasma glucose(mg/dl)	Body weight(g)	Plasma glucose(mg/dl)
0	220 ± 30	79	220 ± 30	79
3	215 ± 30	82	216 ± 22	102
6	220 ± 30	80	211 ± 20	207
9	221 ± 30	80	202 ± 20	267
12	224 ± 30	79	188 ± 15	308

Table 1: The effect of alloxan on plasma glucose and body weight.

This table showed an inverse relationship between body weight and plasma glucose as induction days increases. (Table 2) shows the effect of hyperglycaemia on the red blood cell parameters and platelet count of test and control rats. A significantly reduced ($p < 0.05$) packed cell volume (24.40 ± 3.87 vs. $40.45 \pm 3.93\%$), also documented in haemoglobin concentration (7.81 ± 1.45 vs 13.39 ± 0.40 g/dl) and total red blood cell count (3.47 ± 0.29 vs. $7.16 \pm 0.25 \times 10^{12}$ /L) were observed in the test rats when compared to control rats. However, reticulocyte count (12.4 ± 1.87 vs. $3.69 \pm 0.47\%$) was significantly elevated ($p < 0.05$) in the test rats. The red cell indices of mean cell volume (70.55 ± 11.16 vs. 56.35 ± 5.18 fl) and mean cell haemoglobin (22.44 ± 3.97 vs 18.71 ± 0.96 /pg) also showed significant increase ($p < 0.05$). A moderate but not significant reduction was obtained in mean cell haemoglobin concentration (31.97 ± 2.55 vs 33.48 ± 3.71 g/dl) and platelet count (216.05 ± 26.37 vs. $203.95 \pm 23.05 \times 10^9$ /L). The hyperglycaemic effect on leucocyte (total and differential) parameters depicted in (Table 3) showed significant reduction ($p < 0.05$) in

neutrophil count (12.50 ± 2.72 vs. $24.90 \pm 5.34\%$) in the test group. Eosinophil count (13.35 ± 2.32 vs. $2.45 \pm 1.53\%$) was significantly increased ($p < 0.05$) alongside basophil count (2.45 ± 1.88 vs. $0.65 \pm 0.67\%$). No significant difference was found in the total white blood cell (5.50 ± 1.62 vs. $5.36 \pm 1.67 \times 10^9$ /L), lymphocytes (69.30 ± 3.28 vs. $70.15 \pm 4.17\%$) and monocytes (3.80 ± 1.5 vs. $2.60 \pm 0.8\%$) in both groups. An assessment of the leishman stained bone marrow film revealed an increased myeloid–erythroid ratio in the test group, (6:1) compared to control, (2.7:1) with myeloid series predominantly immature progenitor and precursor polymorphonuclear cells. In the red cell line, there was megaloblastic maturation with predominant polychromatic macrocytes. Adequate number of megakaryocytes was seen in both groups. An intense deep blue iron deposit of hemosiderin was observed on the perl prussian blue stained smear in the hyperglycaemic rats as compared to the scanty deposits observed in non-hyperglycaemic control rats.

Subject	PCV (%)	Hb (g/dl)	RBC ($\times 10^{12}$ /L)	Retics %	MCV (fl)	MCH (pg)	MCHC (g/dl)	Platelets ($\times 10^9$ /L)
Test Mean n=24	24.40 ± 3.87	7.81 ± 1.45	3.47 ± 0.29	12.4 ± 1.87	70.55 ± 11.16	22.44 ± 3.97	31.97 ± 2.55	216.05 ± 26.37
Control Mean n=30	40.45 ± 3.93	13.39 ± 0.40	7.16 ± 0.25	3.69 ± 0.47	56.35 ± 5.18	18.71 ± 0.96	33.48 ± 3.71	203.95 ± 23.05
T-Test	-13.04	-16.61	-42.77	20.19	5.16	4.09	-1.5	1.55
P-Value	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	>0.05	>0.05

Table 2: Hyperglycemia effect on red cell parameters and platelets.

Subject	WBC ($\times 10^9$)	Neutrophils	Eosinophils	Basophils	Lymphocytes	Monocytes
Test Mean n=24	5.50 ± 1.62	12.20 ± 2.72	13.15 ± 2.32	2.45 ± 1.88	69.30 ± 3.28	2.60 ± 0.8

Control Mean						
n=30	5.36 ± 1.67	24.60 ± 5.34	2.10 ± 1.53	0.65 ± 0.67	70.15 ± 4.17	3.80 ± 1.5
T-Test	0.28	-9.25	17.5	4.04	-4.64	0.19
P-Value	>0.05	<0.05	<0.05	<0.05	>0.05	>0.05

Table 3: The effect of hyperglycemia on leukocyte parameters.

Discussion

A high grade haematological derangement is associated with uncontrolled or poorly controlled diabetes mellitus. Michota and Frost described the complete blood count as a commonly ordered test in medicine providing an overview of an individual's general health status as well as information for infection, inflammation and inflammatory diseases, deficiencies in the immune system, bone marrow diseases and other health related conditions. In this study, we observed that the packed cell volume, haemoglobin concentration and red blood cell count, as indices of anaemia were significantly lowered in the hyperglycaemic test rats.

This finding might be due to medullary red cell destruction (dserythropoiesis), in vivo destruction of megaloblastic matured red cell [18-20], or premature intravascular destruction of the circulating red blood cell secondary to oxidative stress [21,22] derived from increased lipid peroxidation of erythrocyte membrane.

The anemia reported here is a regenerative type as indicated by statistically significant ($P<0.05$) elevated reticulocyte count [17]. This may further explain the significantly elevated ($P<0.05$) mean cell volume and mean cell haemoglobin obtained in the study. Our findings on the red cell adaptive response is in consonance with other studies such as Bersch et al., who reported that hyperglycaemia tends to increase the concentration of hypoxia-inducible factor -1- α which promotes the synthesis of erythropoietin resulting in an accelerated production of reticulocytes.

A hypercoagulable state has been hypothesized in diabetes, the elevated platelet count observed in this study in conjunction with coagulation proteins status not estimated may predispose a patient to developing venous thrombosis leading to necrosis and subsequent gangrene of distal tissue [23]. We also observed in this study a significant reduction in neutrophil in hyperglycaemia. This testifies that depressed myelomonophagocytic system (MNPS) immune status has been developed, ultimately results in unhealed wounds experienced by the diabetic patient when exposed to pathogenic challenges [24].

Significantly increased eosinophil and basophil counts may be indicative of allergies resulting from vasoactive amines, also accompanying hyperglycaemic complications of diabetes mellitus [11].

The observed increased myeloid-erythroid ratio in the test bone marrow micro environment suggest that in hyperglycaemia there could be diminished marrow erythroid series production due to medullary dyserythropoiesis, and hypercellularity on the myeloid blast and progenitor cell (leukaemoid reaction) as a reaction to toxic agent ROS (Reactive oxygen species) produced in hyperglycaemic state such as the Superoxide [20].

The medullary dserythropoeisis is attributable to lack of insulin in the peripheral and bone marrow circulation which is a vital co-factor

for blast and progenitor cells of the erythroid series for proliferation and maturation [25,26]. Skudelski however proposes that destruction of the pancreatic beta cells of the experimental animal following alloxan induction results in sudden outburst of insulin and transient hypoglycaemic effect [2]. This episode coupled with other documented immediate cytotoxic effect of alloxan may account for the loss in two (6.6%) of the experimental animals.

This study hence proposes that hyperglycaemia will result in regenerative hemolytic anaemia with reduced immune status and contributory hypercoagulable status which may ultimately end up in thrombosis leading to gangrene of distal tissue.

It is hereby evidenced that untreated or poorly controlled diabetes mellitus may end up with manifestations of hemolytic anaemia, reduced immune status, hypercoagulability of the venous blood, thrombosis, gangrene and allergic reactions.

Conclusion

Taking all these mechanism into consideration, it is evident that destruction of insulin producing pancreatic cells may impair erythroid proliferation and maturation resulting in medullary dserythropoiesis and peripheral regenerative anaemia; along with myeloid leukaemoid reaction resulting from reactive oxidative substances. Neutropaenia verifying reduction in myelomonophagocytic immune system leading to the unhealed wounds in diabetic patient; while the non-significant thrombocytosis contribute to hypercoagulable states resulting in thrombosis and gangrene.

Competing Interest

There is no financial or non-financial competing interest as regards funding or sponsorship from any organization, corporate bodies or groups.

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