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Research Article
The Haemolytic Changes During Progression of Pre-Diabetes to Type 2 Diabetes in a High-Fat High-Carbohydrate Diet-Induced Pre-Diabetic Rat Model

N.C. Mzimela, P.S. Ngubane and A. Khathi

School of Laboratory Medicine and Medical Science, College of Health Sciences, University of Kwa-Zulu Natal, Durban, South Africa

Abstract

Background and Objective: Reports show that type 2 diabetic (T2D) patients have changes in red blood cell (RBC) indices as well as decreased erythropoietin (EPO) levels and endothelial nitric oxide synthase (eNOS) activity. Some abnormalities have been reported to develop during the pre-diabetes stage. However, information on haemolytic changes during the progression of pre-diabetic stage has not yet been reported. Therefore, this study was sought to characterize the changes in RBC indices and concentration of eNOS and EPO in the pre-diabetic stage. **Materials and Methods:** Pre-diabetes was induced using a high-fat high-carbohydrate diet for 20 weeks. Rats were then divided into non-diabetic and prediabetic rats (n = 6 in each group). The pre-diabetes progressed to T2D over an additional 12-week experimental period. RBC indices were measured at the end of 20 and 32 week. Following sacrificed after 32 weeks, blood was collected for eNOS and EPO measurements. **Results:** The results showed significant increases in RBCs, hemoglobin (HGB), hematocrit (HCT) in the prediabetic group as compared to the non-prediabetic group. We further observed a significant decrease in white blood cell (WBC) concentration, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and red cell distribution width (RDW) in the prediabetic group as compared to the non-prediabetic group. **Conclusion:** Changes in blood cells indices indicate various haemolytic changes in RBCs morphology during progression towards overt T2D. However, EPO upregulation indicates the production of new RBCs, while decrease in eNOS activity indicated decrease in bioavailability of nitric oxide (NO).

Key words: Hemolysis, high-fat high-carbohydrate diet, pre-diabetes, pre-diabetic rat, type 2 diabetes, unhealthy diets

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Corresponding Author: Nomusa Mzimela, Department of Human Physiology, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Private Bag X54001, Durban 4000, South Africa Tel: (27) (31) 260 7585 Fax: (27) (31) 260 7132

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Type 2 diabetes (T2D), a condition often preceded by pre-diabetes, is characterized by metabolic and signaling abnormalities such as increased oxidative stress and formation of advanced glycation end products (AGEs)[1]. According to Taheri *et al.*[2], oxidative stress observed in T2D is caused by hyperglycemia which promotes lipid peroxidation. This results in an increase in reactive oxygen species (ROS) accompanied by a decrease in antioxidants such as superoxide dismutase (SOD) and glutathione peroxidase (GPx)[2]. Oxidative damage is associated with reduced function of red blood cells (RBC)[3]. The RBCs of people with diabetes have been shown to have depleted nicotinamide adenine dinucleotide phosphate (NADPH), which is necessary for its enzymatic activity[4]. Nada[3] reported that people withT2D has impaired erythropoiesis resulting in low levels of erythropoietin (EPO). Furthermore, these RBCs have been shown to have reduced deformability and have been reported to have a reduced life span[4,5]. Studies further indicate that red blood cell distribution width (RDW) in T2D is increased due to anisocytosis and RBC degradation[3]. Moreover, T2D increased RBC concentration causing an increase in blood viscosity and the development of high blood pressure[6]. This contributes to the development of cardiovascular complications due to the clogging of vessels[7-9]. Furthermore, according to Sharif *et al.*[10], anemia is the key indicator of chronic kidney diseases, cardiovascular factors and retinopathy. Several studies have shown that the metabolic perturbations often associated with T2D begin during the prediabeticstage[11-16]. Using a diet-induced animal model for pre-diabetes, we have demonstrated an increased blood pressure levels, impaired renal handling and impaired cardiovascular function in our previous study[13,15,16]. However, the changes that occur in RBC parameters during the progression from pre-diabetes (PD) to T2D are still unclear. Additionally, T2D causes changes in RBC indices such as mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin content (MCHC), haemoglobin (HGB) and hematocrit (HCT)[3,5,17]. However, the progressive changes in RBC structure and morphology during pre-diabetes have not been investigated. Therefore, this study sought to characterise the changes that occur in the RBC during the progression of pre-diabetes stage to T2D in a diet-induced pre-diabetic rat model.

MATERIALS AND METHODS

Drugs and chemicals: All chemicals and reagents were of analytical grade and sourced from the standard pharmaceutical supplier. The materials and kits were sourced as follows: EDTA tubes (Greiner bio-one, Monroe, USA); Heparinized containers (Greiner bio-one, Monroe, USA); Glucose sticks (Mzansi Medical Supplies, Durban, South Africa); Rat EPO ELISA kits (Elabscience, Texas, USA); Rat endothelial nitric oxide synthase (eNOS) ELISA kits (Elabscience, Texas, USA).

Animal studies: This study was accomplished using a group of male Sprague Dawley rats weighing 150-180 g, which were bred and kept at the Biomedical Research Unit (BRU) from the University of Kwa Zulu-Natal. Temperature (22±2°C) relative humidity (55±5%) and illumination (12 h light/dark circle) was maintained under standard laboratory condition. The level of noise was maintained at less than 65 decibels and food and fluids was provided *ad libitum*. Animal Research Ethics committee of the University of Kwa Zulu-Natal (ethics no.: AREC/035/016M) approved all animal procedures and housing conditions.

Induction of pre-diabetes: A well-established laboratory protocol was used for the induction of pre-diabetes[12]. Briefly, animals were exposed to a high-fat high-carbohydrate diet supplemented with 15% fructose for 20 weeks (induction period). Following this period, the experimental rats were checked for prediabetes according to the criteria given by the American Diabetes Association[18]. The control group was fed standard rat chow for 20 weeks.

Experimental design: At the end of the 20th-week induction period, the animals were then divided into the non-diabetic control (NC) and the high-fat high-carbohydrate diet-induced pre-diabetic group (PD) (n = 6 in each group). Both groups were monitored for a further 12 week experimental period while being kept on their respective diets. Blood indices (RBCs, RDW, MCV, MCH, MCHC, HGB, HCT and WBCs) were measured after 20th week (end of induction period) and at the end of the experimental period (32 week) using a hemocytometer (Beckman Coulter, Indianapolis, United States). At the end of the experimental period, Isoform (100 mg kg^{-1}) (Safeline Pharmaceuticals (Pty)Ltd, Roodepoort, South Africa) was used to anesthetize animals via the gas anesthetic chamber (Biomedical Resource Unit, UKZN, Durban, South Africa) for 3 min to sacrifice animals.

Blood and tissue collection: Blood was collected by cardiac puncture and then injected into individual pre-cooled heparinized containers while the rats were unconscious. The

blood was centrifuged for 15 min at 1000×g (2-8°C). EDTA tubes were used to collect plasma for EPO ELISA and eNOS ELISA.

EPO and eNOS concentration: EPO and eNOS were measured using their respective ELISA kits from Elabscience as per manufacturer instructions. The optical density of each well was determined using a Spectrostar nanoplate spectrophotometer (BMG Labtech, Ortenburg, Baden-Württernberg, Germany) at 450 nm.

Statistical analysis: Data was expressed as means±standard error of means. Graph Pad Prism Instat Software (version 5.00, Graph Pad Software, San Diego, California, USA) was used to conduct statistical analysis. Two-way analysis of variance (ANOVA) was used to analyze differences in blood indices between the controls and the experimental groups followed by the Bonferroni *post hoc* comparison test. Pearson's correlation coefficients were calculated to evaluate the relationship between EPO concentrations of NC and PD and then also calculated to evaluate the relationship between eNOS concentrations of NC and PD. Values of $p<0.05$ were considered statistically significant.

RESULTS

RBC, MCH, MCHC and Hb concentrations: At the end of the induction period (20 weeks) and the experimental period (32 weeks), HGB, MCH, MCHC and RBCs concentration were measured for NC and PD. The results showed that there was a significant ($p<0.05$) increase in RBCs and HGB concentration in pre-diabetic (PD) group compared to that of the non-diabetic control (NC)group at week 32, as shown in Fig. 1a and d. Figures 1b and 1c show a significant ($p<0.05$) decrease in MCH and MCHC concentration at week 32 in PD group when was compared to NC group.

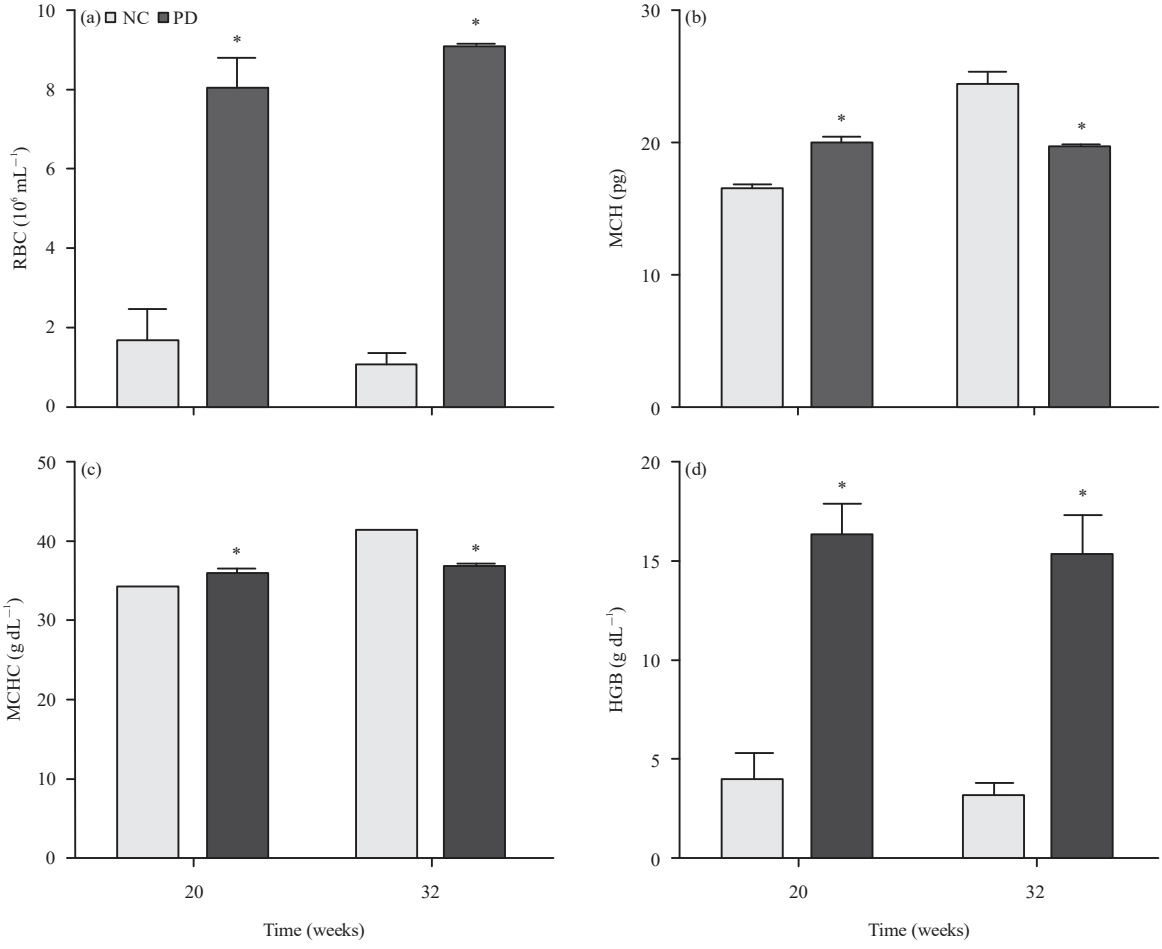

Fig. 1(a-d): RBCs, MCH, MCHC and HGB concentration of non-diabetic control (ND) and pre-diabetic group (PD) at week 20 (end of induction period) and week 32 (end of experimental period). Values are represented as the standard error of means (±SEM). * = $p<0.05$ vs non-diabetic control

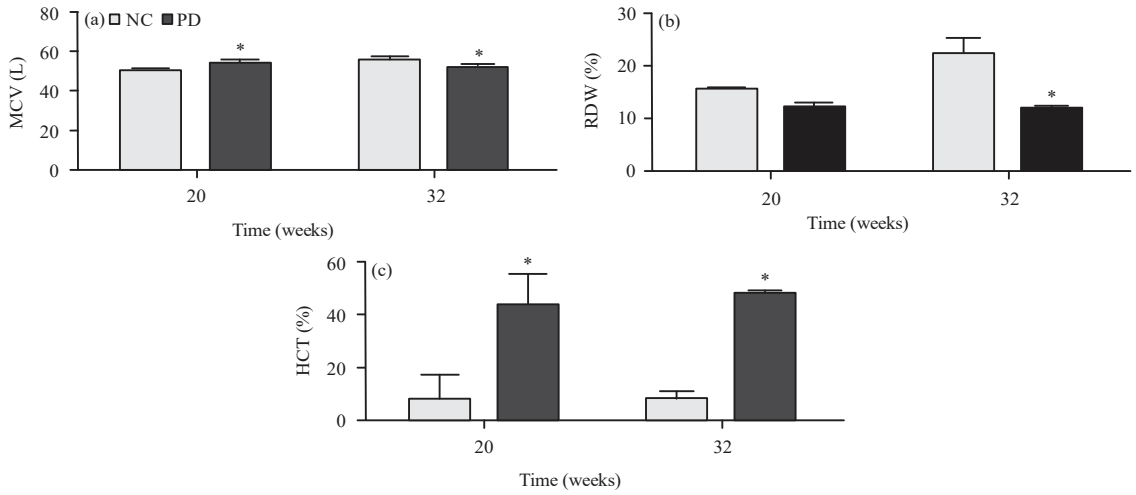

Fig. 2(a-c): Mean corpuscular volume concentration, red cell distribution and hematocrit percentage count of non-diabetic control (ND) and pre-diabetic group (PD) at week 20 and 32. Values are represented as the standard error of means (±SEM). * = p<0.05 vs non-diabetic control

MCV concentration, hematocrit and RDW percentage count: Figure 2 shows MCV concentration and percentage count of RDW and HCT in both NC and PD group measured during 20-32 weeks. The results showed a significant (p<0.05) increase in MCV concentration and HCT percentage count at week 32 in PD group compared to NC, (Fig. 2a and 2c). However, there was a significant (p<0.05) decrease in RDW percentage count in PD group compared to NC group at week 32, (Fig. 2b).

White blood cell concentration: During 20-32 weeks, the concentration of white blood cells was measured in both NC and PD group. Figure 3 shows that there was a significant (p<0.05) increase in white blood cell concentration in PD compared to the NC group after 20 weeks. However, there was a decrease in white blood cell concentration after 32 weeks in PD group compared to the NC group (Fig. 3).

Erythropoietin and endothelial nitric oxide concentration: Figure 4 shows the correlation coefficients between EPO concentrations measured at the end of the experimental period (32-week) in both NC and PD group. It also shows the correlation coefficients between eNOS concentrations in both NC and PD group at the end of the experimental period (32-week). The results show that there was a positive correlation between EPO concentrations of the pre-diabetic group (PD) and non-diabetic control (Fig. 4a). There was also a negative correlation between eNOS concentrations in PD and NC at the end of the experimental period (32-week), (Fig. 4b).

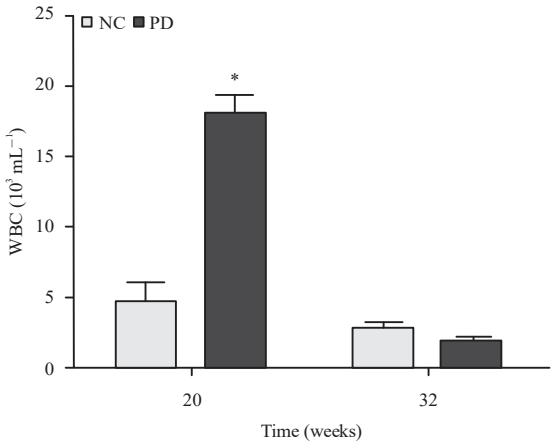

Fig. 3: WBCs concentration of non-diabetic control (ND) and pre-diabetic group (PD) at week 20 and week 12. Values are represented as the standard error of means (±SEM). * = p<0.05 vs non-diabetic control

DISCUSSION

Constant consumption of unhealthy diets such as High-Fat High-Carbohydrate (HFHC) along with a sedentary lifestyle has been reported to be the leading causes of T2D[19]. Furthermore, the onset of T2D has been reported to be preceded by pre-diabetes which has been reported to last for a long period in human subjects[20]. In our laboratory, a diet-induced pre-diabetes rat model was created using HFHC-diet and this model was found to mimic the human condition[15]. Furthermore, Gamede *et al.*[13] reported that these pre-diabetic

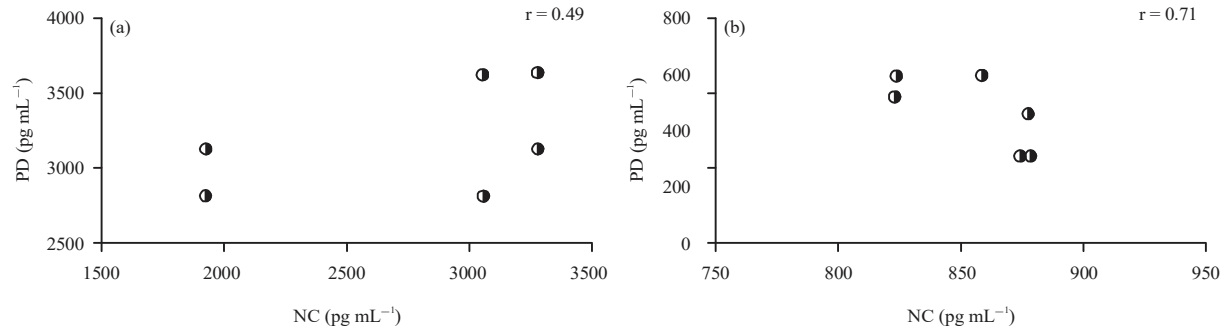

Fig. 4(a-b): Correlation analysis of erythropoietin concentration and endothelial nitric oxide synthase of non-diabetic control (ND) and pre-diabetic group (PD) at the end of the experimental period (a) EPO concentration and (b) eNOS concentration

animals further developed T2D after an additional 12 weeks of continued ingestion of the HFHC diet. We have reported on changes that occur in the white blood cells in pre-diabetic animals but no information has been shown in red blood cell changes in the progression from pre-diabetes to overt T2D[11]. This study, therefore, aimed to characterize the changes in RBC indices during the progression of pre-diabetes to T2D.

It has become evident that persistent hyperglycemia is the leading cause of excessive generation of free radicals in T2D that lead to oxidative stress[21]. Additionally, excessive free radical production causes an imbalance in the anti-oxidant defense system causing oxidative damage[21]. Interestingly, Mabuza *et al.*[16]. reported that the pre-diabetic rat model demonstrated the same characteristic of free radicals in the pre-diabetic stage. Jabeen *et al.*[21]. reported that hyperglycemia-induced oxidative damage in T2D is caused by the excessively produced highly reactive free radicals which contribute to structural and functional abnormalities of RBCs. According to Kim *et al.*[22]., the MCHC, which is used as a marker of cytoplasm viscosity in the RBCS is decreased due to loss of deformability.

Moreover, Kim *et al.*[22]. reported that loss of deformability was due to the loss of water from the hypertonic media which then contributes to the local increase in cytoplasmic viscosity. This has also been reported to contribute to the increase in MCHC[22]. However, literature has also reported that RBC deformability and shape maintenance depends on ATP generated by RBC[22]. Therefore, reduction in ATP concentration causes a reduction in RBC deformability[4,22]. Kim *et al.*[22] further mentioned that the deformability of the aged cells also correlates with the increased hemoglobin concentration. This correlates with the increase in MCHC in the pre-diabetic group compared to the non-diabetic control at week 20. However, a positive correlation between EPO concentrations in the pre-diabetic and non-diabetic control group at week 32 indicates that erythropoiesis occurred, where the new young reticulocytes replace the senescent RBCs[23]. There was a decrease in MCHC on the pre-diabetic group by comparison with the non-diabetic control at week 32, which also correlates with the positive correlation between EPO concentrations in the prediabetic group and non-diabetic control at week 32.

According to Marsden[24], EPO is mainly produced in the kidneys when supply of oxygen is inadequate at tissue level (hypoxia) and is responsible for controlling the production of RBCs. EPO released by renal EPO-producing cells during stimulus bind to EPO receptor (EPOR) at the surface of erythroid progenitor cells in the bone marrow which triggers the formation and production of RBCs[24-26]. The increased EPO levels also trigger the increase in O_2 delivery on working muscles due to increased RBCs since they have oxygen-carrying capacity[24,27]. In rodents, EPO has been reported to stimulate megakaryocyte proliferation and maturation[23]. Interestingly, circulating EPO has been reported to bind to EPO receptor extracellular which then activate EPO to cause the sequential activation of intracellular mechanisms[23]. These mechanisms include the phosphorylation of Janus tyrosine-protein kinase 2 (JAK2) and phosphorylation and nuclear translocation of STAT5 (signal transducer and activator of transcription 5) pathways[23]. However, Magri and Fava[28] reported that impaired erythropoiesis and RBCs degradation are related to elevated levels of RDW in T2D. Literature reports also indicate that the low levels of EPO in T2D are due to the oxidative damage to the kidneys which lead to the development of chronic kidney diseases such as nephropathy[29,30].

Furthermore, Barbieri[27] reported that low EPO contributes to decreased bone marrow response and reduced erythropoiesis in T2D. Barbieri[27] further reported that in T2D, reduced bone marrow response is also related to activation of macrophages and release in inflammatory cytokines, particularly interleukin 1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α), interferon-gamma (INF-γ) and interleukin

6 (IL-6)[27]. These inflammatory cytokines inhibit erythropoiesis by inhibiting the proliferation of erythroid precursors[27]. Additionally, IL-6 has been reported to be elevated in T2D and has been shown to promote apoptosis of immature erythrocytes, thus causing a decrease in the number of circulating RBCs and thus causing a reduction of circulating hemoglobin[27,31].

A previous study by Khathi *et al.*[15] reported that the diet-induced pre-diabetic rat model showed elevated markers of renal dysfunction. Further research conducted by Mzimela *et al.*[11], using the same animal model, demonstrated that during pre-diabetes, there is increased mean arterial pressure (MAP) in diet-induced pre-diabetes. The study further reported significant upregulation of inflammatory markers such as TNF-α and IL-6[11]. Ironically, these factors in the pre-diabetic stage do not contribute to the decrease in EPO levels. This is confirmed by the positive correlation between EPO concentrations in the pre-diabetes and non-diabetic control group. It can be hypothesized that since it is still pre-diabetic stage, renal failure is still at the acute phase and the upregulation of TNF-α and IL-6 is not yet chronic to cause damage that can damage the kidney's renal EPO-producing cells.

Moon *et al.*[32] mentioned that the RBC's of type 2 diabetics are stiffened by chronic hyperglycemia causing them to have a harmful effect on vasculature. The study further mentioned that these stiff RBCs had been shown to damage the endothelium in addition to the damage caused by oxidative stress[32,33]. Additionally, the change in composition and function of RBCs in T2D can increase their endothelium aggregation ability and adherence[32]. This is also considered as an effect that also facilitates the adherence of platelets, which result in thrombus formation in T2D[32,34]. Gutierrez *et al.*[35]*.* mentioned that the rigid RBCs affect the blood flow and which also result in WBC adhesion change. An increase in hematocrit is exacerbated by the flowing rigid RBCs therefore causing a decrease in adhesion of WBC to the damaged endothelium[36]. This correlates with the increase in hematocrit in the pre-diabetic group compared to the non-diabetic control during the progression of prediabetes, indicating that RBCs disturbed the blood flow and their rigidity affected the damaged endothelium. Additionally, it is further explained by the increase in fibrinogen levels in the pre-diabetic stage which was reported by Mzimela *et al.*[11] suggesting the increased risk of clot formation at pre-diabetes stage.

According to Natali[36], in T2D and hypertension, endothelial function is impaired which results in increased hematocrit values. This increase in hematocrit value is also due to the decreased bioavailability of NO[36]. According to literature, under normal conditions, NO reacts with Hb through oxy Hb oxidation to produce nitrate and metHb, S-nitrosylation and production of NO-Fe[37] through the addition of Fe[37]-Hb[36]. The spilling of NO produced from the blood stream alters this to the arterial compartments, which enable it to reach Hb in the RBCs[36]. However, in hypertension and T2D, these reactions are disturbed due to decreased NO bioavailability[36]. This can be confirmed by the negative correlation between eNOS concentrations in the pre-diabetic and non-diabetic control group at week 32, indicating that there is a decrease in bioavailability of NO during the pre-diabetic stage. This has also been reported to cause an increase in hematocrit[32]. This correlates with the increase in hematocrit in pre-diabetic group compared to the non-diabetic control group during progression of pre-diabetes stage suggesting that there is alteration of the RBCs function. Several studies have shown that T2D develop hypertension, in part, due tohyperglycemia[38,39]. This indicates that T2D patients are at risk of developing cardiovascular diseases such as heart failure, stroke, tissue inflammation and atherosclerosis[34,38,39]. However, according to Magri and Fava[28]., RDW has been clinically used as a marker for morbidity and mortality caused by cardiovascular complications. RDW has been clinically reported to reflect chronic inflammation and oxidative stress in subjects who suffer from cardiovascular disease[28]. In T2D, the increase in RDW is also associated with nephropathy since it results from RBCs fragmentation[28]. Due to oxidative stress in T2D, RBCs anisocytosis occurs resulting from degraded and distorted erythropoiesis and increased RDW, which is a measure of the degree of anisocytosis[3,5]. However, in the progression of pre-diabetes to T2D, the RDW was decreased in the pre-diabetic group compared to the non-diabetic control group during the progression of the stage. We speculate that as much as there is oxidative damage caused by oxidative stress in prediabetic patients, the damage is not sufficient to degrade and distort erythropoiesis[15].

In clinical practice, an increase in RDW in T2D has been used together with MCV to differentiate between the causes of anemia[3]. Nada[3] reported that MCV is decreased in diabetics. This is due to the structural deformation of the RBCs due to oxidative stress caused by hyperglycemia[32]. This correlates with MCV results of the pre-diabetic group in comparison with non-diabetic control at week 32 of the progression of the pre-diabetes stage, indicating that the RBCs structure is affected by oxidative stress. However, the RDW level in the prediabetic group is not increased and we hypothesize that in the pre-diabetes stage, the RBCs abnormalities are still not

enough to cause anemia. The decrease in EPO levels in patients with T2D is another factor contributing to anemia as a secondary disorder[5]. Interestingly, the positive correlation between EPO concentrations was observed in the pre-diabetic and non-diabetic control group at the end of the experimental period. (32 weeks). These results indicate that the abnormalities in RBCs during the prediabetic state are not yet enough for developing anemia. According to Nada[3], in patients withT2D, hyperglycemia causes activation of caspase-3 which then impairs RBC function and morphology. These impairments have been reported to contribute to a reduced life span of RBCs thus causing excessive variability of RBCs volume[3]. Additionally, Bizjak *et al.*[40] reported that in aging RBCs, their hemoglobin is also glycated and carbamylated. This leads to excessive denature of hemoglobin at the inner membrane sites[40].

Furthermore, Nada[3] showed that hyperglycemia in T2D causes the formation of glycated hemoglobin through a process called glycation. This correlates with the increase in hemoglobin concentration in the prediabetic animals after32weeks. However, a study reported that Hb is the major catabolic sink for the nitric oxide (NO) that is produced byendothelium[36].

A previous study reported decreased MCH levels in T2D patients[21]. This was hypothesized due to the small size of the RBCs, which makes it difficult for the cells to take up hemoglobin as they should due to shrinkage or deformation. The results obtained correlate with the literature indicating that the change in size of RBCs cause decreased MCH in the prediabetic group at week 32. According to Biadgo *et al.*[41], the inflammatory process that could lead to atherosclerosis progression and cardiovascular disease is indicated by the increase in WBC. It can be hypothesized that during the prediabetic phase, there is chronic low-grade inflammation. It can also be hypothesized that low-grade inflammation is not severe enough to contribute to cardiovascular disease and atherosclerosis. Biadgo *et al.*[41]*.* further mentioned that increased WBCs count in T2D patients could be due to the increase in oxidative stress, which is caused by hyperglycemia. This suggests that the moderate hyperglycemia observed in prediabetes causes activation of WBCs by the cytokines and AGEs[41].

CONCLUSION

The findings of this study indicate that there is progressive deterioration in the structural properties of RBCs during the prediabetic state. This was confirmed by changes observed in RBC indices such as HGB, sMCV, MCH, MCHC and RDW. We suggest that more studies should be done on RBC integrity during the prediabetic state as these changes could be implicated in the development of cardiovascular complications.

SIGNIFICANCE STATEMENT

This study discovered the changes in RBCs indices and concentration of eNOS and EPO at the pre-diabetes stage in a diet-induced pre-diabetic rat model. This can be beneficial for future research on pre-diabetic human subjects. Findings from this research will pave the way for the pre-diabetic subjects to understand the changes that might possibly occur at this stage, as it mimics the diet consumed by human beings even though the animals were in a controlled environment. This study will help the researchers and clinicians to uncover the areas of the pre-diabetes stage that many researchers were not able to explore. Thus, a new theory on pre-diabetic human subjects can be documented.

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