Effect of Glycohaemoglobin Adduct on Erythrocytes Osmotic Fragility in Nigerian Patients with Diabetes Mellitus

***Danladi SB,[1] Yusuf A,[1] AliyuIA,[1] Kumurya AS,[1] Usman MM,[3] Obazelu PA**

ABSTRACT

Anemia is one of the common complications in subjects with diabetes mellitus (DM); this could be attributed to a number of causes. One of which could be due to continuous intracellular influx of glucose resulting from non-enzymatic glycosylation of haemoglobin and this may alter erythrocyte membrane architecture or cell mass. We aimed to investigate the impact of haemoglobin glycosylation on erythrocytes osmotic fragility in subjects with DM attending Murtala Muhammad Specialist Hospital, Kano.To achieve this, fasting plasma glucose level, concentration of glycated haemoglobin (HbA_1C) in subjects with DM were assayed. The erythrocyte osmotic fragility of DM was compared with controls. Seventy-five participants were enrolled including 50(66.7%) DM and 25(33.3%) control subjects Participants were both males and females within the age of 23-72 years, with (Mean ± SD = 52.46±13.98 and 40.52±10.77years for DM and controls, respectively).The fasting plasma glucose (FPG), glycated haemoglobin (HbA_1C) and erythrocyte osmotic fragility (EOF) were determined colorimetrically using glucose oxidase-peroxidase, ion exchange resin and gradient hypotonic saline methods. In this study, there was significant difference (P<0.05) of FPG and HbA_1C of DM compared to controls. HbA_1C correlates positively with FPG in diabetic subjects. There were statistically significant differences (p < 0.05) between FPG, HbA_1C and EOF levels of DM subjects compared to controls. We inferred that high EOFin DM subjects reported in our study may contribute to chronic anaemia seen in this disease.

Keywords: Blood, Fasting, Glucose, Non-enzymatic, Participants

INTRODUCTION

In developing countries like Nigeria, diabetes mellitus (DM) is currently becoming a common problem at a time when the burden of diabetes is rising very quickly in wealthier countries. The prevalence of diabetes mellitus is increasing exponentially throughout the world.[1] The basic pathology in DM, involves hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of body organs, especially the eyes, kidneys, nerves, heart, and blood vessels.[2]

The American Diabetes Association (ADA) and world Health Organization (WHO) recommend that the sub-classification of diabetes based on insulin dependency diabetes mellitus(IDDM) and non-IDDM (NIDDM), should now be abandoned in favor of the etiologically based classification.[3] The most important sub-categories in public health terms are type 1 and type 2 diabetes, relies primarily on the presence (type 1 diabetes) or absence (type 2 diabetes) of autoantibodies against pancreatic islet β-cell antigens and age at diagnosis.[4]

Glycosylated haemoglobin Hb1C, minor components of total haemoglobins in erythrocytes of normal adult humans, is formed by post-translational non-enzymatic glycosylation and this process occurs continuously in vivo. Normal adult haemoglobin consists predominantly of HbA (2 2), HbA2 (2 2) and HbF (2 2) (97, 2.5 and 0.5% respectively). About 6% of total HbA is termed HbA1, which in turn is made up of HbA1a1, HbA1a2, HbA1b and HbA1c.These fractions are defined by their electrophoretic and chromatographic properties, which differ slightly from those of the major component HbA0, despite the

Department of Medical Laboratory Science,[1] Faculty of Allied Health Sciences, Bayero University, Kano, P.M.B. 3011 Kano- Nigeria.
Aminu Kano Teaching Hospital,[2] Kano P. M. B. 3452 Kano – Nigeria
Department of Medical Laboratory Science,[3] Faculty of Basic Medical Sciences, University of Benin, P. M. B. 1154, Benin city, Nigeria

*Corresponding author: dsbala.mls@buk.edu.ng

amino acid sequences of HbA1 and HbA0 being identical. HbA1c is the most abundant of these fractions and in health comprises approximately 5% of the total HbA fraction.[5]

Non-enzymatic glycosylation (NEG) refers to the covalent binding of carbonyl groups of reducing sugars to amino groups of proteins, lipids, and nucleic acid.[6,7] Structural and chemical investigations elucidated that glucose, in the open chain format, binds to the N-terminal to form an aldimine (Schiff base) before undergoing an Amadori rearrangement to form a more stable ketoamineform, amino-1– deoxyfructose.[8] Endogenous glycations occur mainly in the bloodstream to a small proportion of the absorbed simple sugars: glucose, fructose, and galactose. It appears that fructose has approximately ten times the glycosylation activity of glucose, the primary body fuel.[9]

Dysfunction of auto regulatory glucose concentration leads to biochemical alterations in plasma and erythrocytes in diabetes mellitus and have direct influence on the haemorheological properties of cells. Excessive availability of glucose within the cell leads to formation of HbA1c at a higher rate.[10] Persistent hyperglycemia and haemoglobin glycosylation induces changes in the red cell membrane and its cytoplasm milieu leading to alteration in the red cell deformability.[11,12]

In vitro, critical assessment of the erythrocyte deformability by incubation of normal erythrocytes in high concentration of glucose (50mmol/l), reduction in deformability was noticed.[13] In related study, Petit *et al.*[14] reported high percentage of rigid cells to be responsible for decrease in deformability in diabetes, as detected by nickel filters compared to that of polycarbonate filters. Other observations using various techniques indicate that there is a part of the cell population which is affected by the diabetes process and these erythrocytes are contributing to their decrease deformability.[15]

Until recently, it was thought that exogenous glycations and advanced glycation end-product (AGEs) were negligible contributors to inflammation and disease states, but recent work has shown that they are important as AGEs accumulate in erythrocytes.[16,17] Similar to the formation haemoglobin A1c (HbA1C), AGEs are sugar-derived substances (reducing sugars with free amino groups of proteins, lipids, and nucleic acids) produced by Maillard reaction.[18] The AGEs form at a constant but slow rate in the normal body, starting in early embryonic development, and accumulate with time. These initial reactions are reversible depending on the concentration of the reactants. A lowered glucose concentration will unhook the sugars from the amino groups to which they are attached; conversely, high glucose concentrations will have the opposite effect. In case of chronic hyperglycaemia, AGEs are actively produced and accumulate in circulating blood and various tissues.[19] A series of subsequent reactions, including successions of dehydrations, oxidation-reduction reactions, and other arrangements lead to the formation of AGEs. Several compounds, e.g., N-carboxymethyl-lysine, pentosidine, or methylglyoxal derivatives, serve as examples of well-characterized and widely studied AGEs.

In spite of the fact that, AGEs are produced endogenously by oxidative stress or hyperglycemia, they can be consumed exogenously through food. About 6% to 7% of AGEs derived from meals are known to be present in the body for a certain period of time.[20] Elevated serum AGEs were found in anaemic patient with type 2 diabetes.[21]

AGEs accelerate the expression of receptors for advanced glycation end-products (RAGEs). The constant activation of the AGE-RAGE system is presumed to create the long-term metabolic memory or legacy effect.[22] Upon the recognition of AGEs by RAGE in endothelial cells, the production of oxidative stress is accelerated in the cells, and various cytokines and growth factors are secreted.[23]

In patients with diabetes, RAGE expression is accelerated in atherosclerotic lesions in proportion to aggravation of blood sugar regulation.[24] A large body of evidence

suggests that AGEs are important pathogenetic mediators of almost all diabetes complications, conventionally grouped into macro- or microangiopathies. For instance, atherosclerosis is significantly accelerated in diabetic patients and is associated with greater risk of cardiovascular and cerebrovascular mortality. Animal and human studies have shown that AGEs play a significant role in the formation and progression of atherosclerotic lesions. Increased AGE accumulation in the diabetic vascular tissues has been associated with changes in endothelial cell, macrophage, and smooth muscle cell function.[18]

In this study, we aimed to evaluate the impact of haemoglobin glycosylation on erythrocytes osmotic fragility in subjects with diabetes mellitus.

MATERIALS AND METHODS

Study Area

The study was conducted at Murtala Muhammad Specialist HospitalKano, a secondary health care facility situated within Kano metropolis. The state is a cosmopolitan state, located in the Northwest geopolitical zone of Nigeria. Kano state adjoins with states like Bauchi, Jigawa, Kaduna and Katsina.[25,26]

Study Design

The study was prospective cross-sectional and lasted for three months (fromAugust to October, 2018).

Study subjects

The study participants were subjects with diabetes mellitus attending Murtala Muhammad Specialist Hospital Kano along with apparently healthy control subjects.

Sample collection and Processing

Five millitre(5ml)venous blood samples was collected aseptically using sterile disposable syringe from each participant, and transferred into labeled sodium fluoride anticoagulant container for fasting plasma glucose estimation and dipotassium ethylene diamine tetracetic acid (K_2EDTA) anticoagulant container for determination of HbA1c level and EOF test, respectively.

Ethical Consideration

The study was approved in accordance with institutional guidelines set forth by research ethics committee of Kano state Ministry of Health. DM and control subjects were well informed about the study per declaration of Helsinki 1975 and 2008 revised ethical principles for medical research involving human subjects and consent to participate.

Inclusion and Exclusion criteria

Outpatient adult male and female diabetes mellitus subjects who consented to participate were included into the study. While, those that did not consent and inpatients were excluded from the study.

Laboratory Analyses

Measurement of Plasma Glucose Level

Reagent: FPG was estimated by glucose oxidase - peroxidase (GOD-PAP) method, (RANDOX LABORATORIES LTD. UK, CAT. NO. GL364).

Method: Oxidase-peroxidase Method

Principle: Glucose oxidase catalyzes the oxidation of glucose to give hydrogen peroxide (H_2O_2) and glucronic acid. The enzyme peroxidase catalyzed hydrogen peroxide and the oxygen released reacts with 4- aminophenazone and phenol to give pink color.[27]

Reagents (ml)	Test	Standard	Blank
Glucose Reagent	1.0	1.0	1.0
Plasma/Standard/Distilled-Water	0.01	0.01	0.01

The preparations were mixed separately and incubated at 37°C in a water-bath for 10 minutes. The tubes were shakes occasionally to ensure adequate aeration of the samples. The absorbance of the color produced was measured with spectrophotometer at 515nm wavelength.

Calculations: The results were obtained as below

$$\text{Glocose conc.} = \frac{AT}{AS} \times \text{Conc. of Standard}$$

Where:
A_T = Absorbance of test
A_S = Absorbance of standard (mmol/l)

Measurement of Glycated Haemoglobin
Reagents: MISPA-i3HbA1c, 25T lot number: 11018001, Expiry date: Nov.2020-a product of AGAPPE Diagnostics, Switzerland GmbH was used for the quantitative determination of glycated haemoglobin.

Method: Micro Column ion exchange resin (Agappe Diagnostic, Inc).

Materials and Reagents

Reagent	Composition
Resin	25×3ml-Tubes with ion exchange resin
Lysing Reagent	10ml of lysing reagent
Resin Separators	25 nos of porous resin separators
HbA_{1C} Control	1×0.5ml

Principle: from prepared haemolysate. The $HbA_{1C}$is specifically eluted after washing away the HbA_{1C}fraction and is quantified by direct photometric reading at 415nm.

Assay Procedure:
Haemolysate preparation:
Into a chemically clean test tube, 250µl of the lysing reagent was added. 50µl of mixed-whole blood was also added. It was mixed and then left for 5 minutes at room temperature.

Test for total haemoglobin: (Thb)
Five (5ml) of deionized water was placed in to a clean test tube. 20µl haemolysate was then added and mixed well. Absorbance of the test was read at 415 nm against distilled water as blank.

Test for glycated haemoglobin: (HbA_{1C})
The resin tubes were brought to room temperature and 100µl of the haemolysate was added into each tube. The resin separator was positioned inside the tube ensuring that the rubber sleeve was approximately 3cm above the resin level. The contents were mixed by vortexing for 5 minutes. The resin was allowed to settle at specified assay temperature for 5 minutes. The resin separator was pushed down into the tube until the resin was firmly packed. The supernatant was poured directly into a cuvette and the absorbance was read against deionized water as blank at 415nm.

Calculation: The results were obtained as below

$$HbA_{1C}\text{ percentage} = \frac{HbA_{1C}\text{ Absorbance}}{\text{THB Absorbance}} \times 30 \times \text{Temperature Factor (TF)}$$

Where,
Temperature Factor: 30^0C = 0.9
The HbA_{1C} assay can also be done at 24^0C with a TF of 1.0

Measurement of Erythrocyte Osmotic Fragility:
Reagent:
Stock solution of sodium chloride osmotically equivalent to 10% was prepared as follows:

Sodium chloride (BDH)	90.0g
Disodium hydrogen phosphate	13.65g
Sodium dihydrogen phosphate	2.34g
Distilled water	1000ml

Method:

1. The stock solution was diluted 1/10 with distilled water to obtain a 1% solution
2. 12 test tubes were arranged to prepare dilutions as follows:

Test tube number	Volume of saline (ml)	Volume of distilled water (ml)	Conc. of saline (%)
1	4.50	0.50	0.90
2	3.75	1.25	0.75
3	3.25	1.75	0.65
4	3.00	2.00	0.60
5	2.75	2.25	0.55
6	2.50	2.50	0.50
7	2.25	2.75	0.45
8	2.00	3.00	0.40
9	1.75	3.25	0.35
10	1.50	3.50	0.30
11	1.00	4.00	0.20
12	0.50	4.50	0.10

The 1st tube in the series serves as blank (0% lysis) as isotonic saline (0.9%), while 12th tube contained the lowest concentration (0.1%)hypotonic saline that gave 100% lysis.

Calculation: percentage lysis was calculated as below

$$\% \text{ Lysis} = \frac{\textit{Absoebance of test}}{\textit{Absorbance of 100\% lysis tube}} \text{ X } 100$$

Statistical analysis

The unprocessed data was stored in Microsoft Excel 2010 worksheet. Statistical package for social sciences (SPSS) for Windows (version 20.0.) was used for all analyses. Unpaired Student's t-test was the inferential statistics used to compare differences between variables and expressed as mean ± standard deviation (M ± SD). A value of $p<0.05$ was considered statistically significant.

RESULTS

In this our prospective cross sectional study, which was undertaken between the months of August to October, 2018. Seventy-five participants were enrolled, including 50 (66.7%) diabetes mellitus subjects and 25 (33.3%) apparently healthy controls. The participants were both males and females within the age of 23-72 years (Mean ± SD = 48.48±14.11), Table 1.

Table 1: Distribution of Study participants by gender

Participants	Number (n)	Percentage (%)
DM subjects		
Males	25	(33)
Female	25	(33)
Control subjects		
Males	15	(20)
Females	10	(14)
Total	**75**	**(100)**

Key: n= number of subjects, %= Percentage

Table 2: Distribution of DM subjects by duration of disease

Duration (years)	Number (n)	Percentage (%)
1-5	35	70
6-10	13	26
>10	2	4
Total	**50**	**100**

Key: n= number, %= Percentage

Table 3: Distribution of FPG, HbA_{1C} and EOF of DM and Control subjects

Parameter	Subjects DM (M±SD)	Control (M±SD)	P-value
FBG (mmol/L)	7.17±2.09	4.38±1.04	0.000
HbA 1C(%)	8.67±0.71	5.97±0.91	0.000
EOF (%)	0.45±0.04	0.42±0.01	0.001

Key: FPG= Fasting plasma Glucose, HbA_{1C}= Glycated haemoglobin, EOF= Erythrocyte Osmotic fragility,M±SD= Mean±Standard Deviation

Table 4: Relationship between different parameters in Diabetic Mellitus subjects

Parameters	r-value	P-value
HbA 1Cand FPG	0.06	0.02
HbA 1Cand EO F	0.09	0.00
FPG and EOF	0.08	0.00

Key: HbA_{1C}= glycated haemoglobin, EOF= erythrocyte osmotic fragility, r-value = Pearson correlation, p-value = probability value

The distribution of DM subjects based on the duration diagnosed with disease is presented in Table 2, in the following order 1-5 years (35), 6-10 years (13), above 10 years (2), respectively. Thirty-five subjects diagnosed with the disease between 1-5 years were the highest while two that were diagnosed with the disease (>10 years) were the least. In Table 3, results of fasting plasma glucose, glycated haemoglobin and osmotic fragility of DM and control subject were presented. The values are for FPG = 7.17± 2.09mmol/l and 4.38 ±1.04mmol/l, for HbA_{1C}=8.67±0.71% and 5.97±0.91% and for EOF= 0.45±0.04 and 0.42±0.01 for DM and control groups, respectively. And results were statistically significant ($p < 0.05$) in all the three parameters.

The relationships between levels of FPG and concentration of HbA_{1C}, concentration HbA_{1C} and EOF, and FPG and EOF were r-value 0.06, 0.09, and 0.08, respectively. The results were statistically significant ($p<0.05$), Table 4.

DISCUSSION

Our study shows majority of studied subjects were type 2 DM aged 40 and above years. This is in keeping with the previous reports, where it was documented that type 2 diabetes mellitus (T2DM) to be adult onset disease.[28] The fasting plasma glucose levels of DM and control reported in this our present study was statistically significant ($p < 0.05$), this also agrees with results of several studies done elsewhere.[29-31]

We also observed that, the concentration of glycated haemoglobin was higher in DM compared to control subjects and was statistically significant ($p < 0.05$). These could be attributed to influx of plasma glucose across erythrocyte membranes and non-enzymatic post-translational glycosylation of haemoglobin. This is in consonant with reports of several studies; Arora *et al.*[32] reported increased glycosylation of both spectrin and haemoglobin in DM as compared to controls. Other studies done by Aaron *et al.*[2, 33-35] in which all reported an increased glycation of a number of proteins including haemoglobin and this increase is directly proportional to the fasting glucose levels of diabetes.

The corpuscular fragility in this study was found to be statistically significant among DM and control subjects ($p < 0.05$); this was in accord with the study conducted in Egypt, by Arora *et al.*[32] who reported percentage of red blood cells haemolysis of fourteen females and sixteen males T2DMsubjects increased compared to five healthy controls with hypo-tonicity of saline.

The EOF in fourteen females and sixteen males T2DM subjects were greater than EOF of five healthy controls. Equally, Chien-Min *et al.*[36] in a cross sectional study documented that EOF was greater in T2DM subjects compared to non-diabetic controls and EOF was positively correlated with HbA_{1C}. Harika *et al.*[35] demonstrated that erythrocyte osmotic fragility is greater in T2DM subjects compared to nondiabetic controls and erythrocyte fragility was positively correlated with increased duration of exposure of the disease for 10 years. Similarly, Arun,[37] in a study conducted in India established that erythrocyte osmotic fragility was greater in diabetic subjects when compared to non-diabetic controls and erythrocyte osmotic fragility was higher in diabetic patients with duration of exposure greater than 5 years.

Our report also indicates positive relationship between HbA_{1C} and EOF of diabetic subjects and was statistically significant ($p < 0.05$). This is in accord with the report of Padmini *et al.*[1] in a review article "Monitoring Glycosylated Haemoglobin and Osmotic Fragility with Respect to Blood Glucose Level in Type 2 Diabetes Mellitus" indicates that the diabetic patients with poor glycemic control have increased EOF compared to non-diabetic individuals. More so, according to Sharma,[38]who reported positive correlation between HbA_{1C} and EOF in diabetic patients. As the HbA_{1C} was increased, so also EOF also increases. And these can alter the haemodynamic status in diabetes patients, which can cause both macrovascular and microvascular disease.[39]

CONCLUSION

In this our present study, the increased levels of fasting plasma glucose, glycated haemoglobin and erythrocyte osmotic fragility of DM reported in this our study coupled with positive correlation between glycated haemoglobin and erythrocyte osmotic fragility may play an important role in the pathogenesis of diabetes. Though, we did not classified our subjects with related complications like renal failure, which could be a confounding factor and often predisposes patient to anaemia consequent to reduced red cell survival.

ACKNOWLEDGEMENTS

The authors acknowledged the assistance of staff of Metabolic Clinic, Murtala Muhammad Specialist Hospital, Kano. Our gratitude also goes to all participants in this study.

CONFLICT OF INTEREST: none

REFERENCES

1. Padmini H, Bhagwat K. Monitoring Glycosylated Haemoglobin and Osmotic Fragility with Respect to Blood Glucose Level in Type II Diabetes Mellitus. *International Journal of Health Sciences and Research* 2015;5:171-2.
2. American Diabetes Association (ADA). Diagnosis and Classification of Diabetes Mellitus. *Diabetes Care,* 37: 581-582. Www.academicresearchjournals.org/ ADA-diabetic-careAccessed on 25th February, 2018.
3. WHO Expert Committee on Definition. Diagnosis and Classification of Diabetes Mellitus and its Complications, *Geneva* 1999;1-59.
4. Emma A, Petter S, Annemari K, Mats MM, Dorkhan A, Carlsson PV, *et al.* Novel Subgroups of Adult-onset Diabetes and Their Association with Outcomes: A Data-driven Cluster Analysis of Six Variables, *The Lancet Diabetes & Endocrinology* 2018;6:361-9 .
5. Al-Ansary L, Farmer A, Hirst J, Roberts N, Glasziou P, Perera R, *et al.* Point of Care Testing for HbA1c in the management of Diabetes: a systemic review and meta-analysis. *Clinical Chemistry* 2011; 57:568-76.
6. Vasudevan DM, Sreekumari S, Vaidyanathan K. *Textbook of Biochemistry for Medical Students.* (sixth edition), Jaypee Brothers Medical Publishers (P) Ltd, New Dehli, India 2011; pp 290-1.
7. Alin S, Thomas G, Michael R. Effects of Advanced Glycation Endproducts:

Clinical Effects and Molecular Mechanisms, *Molecular Metabolism* 2014:3:94-108.

8. Robert S, Michael JM, Clyde Z, Franklin BH. Sites of Nonenzymatic Glycosylation of Human Haemoglobin A. *The Journal of Biological Chemistry* 1980 255: 3120-7.
9. Alison G, Joshua AB, Ann MS, Mark AC. Advanced Glycation End Products: Sparking the Development of Diabetic Vascular Injury, *Circulation* 2006;114:597-605.
10. Goldstain DE, Little RR, Wiedmeyer HM, England JD, McKenzie EM. Glycated Haemoglobin: Methodologies and Clinical Application. *Clinical Chemistry* 1986;32: B64.
11. Iwata H, Ukeda H, Maruyama T, Fujino T, Sawamura M. "Effect of carbonyl compounds on red blood cells deformability". *Biochemistry and Biophysics Research Communication* 2004;321:700-6.
12. Shin S, Ku Y, Babu N, Singh M. Erythrocyte Deformability and its Variation in Diabetes Mellitus. *Indian Journal of Experimental Biology 2007;*45:121-2.
13. Bareford D, Jennings AE, Stone PCW, Baer S, Barnett AH, Stuart J. effects of hyperglycaemia and Sorbitol accumulation on erythrocyte deformability in Diabetes Mellitus. *Journal of Clinical Pathology* 1986; 39:722.
14. Petit KL, Hunt WB, George SJ, Barnes AJ. Is impaired red cell filtration in Diabetes due to small abnormal population of cells? *Clinical Haemorheology* 1991;16:479.
15. Megha S, Sehyan S. Changes in erythrocyte aggregation and deformability in diabetes mellitus: A brief review. *Indian Journal of Experimental Biology* 2009;47:7-16.
16. Ando K, Beppu M, Kikugawa K, Nagai R, Horiuchi S."Membrane proteins of human erythrocytes are modified by advanced glycation end products during aging in the circulation". *Biochemical and Biophysical Research Communication* 1999;258:123-7.
17. Vlassara H."Advanced glycation in Health and Disease: Role of the Modern Environment". *Annals of the New York Academy of Sciences* 2005;1043: 452-60.
18. Melpomeni P, Jaime U, Helen V. Glucose, Advanced Glycation End Products and Diabetes Complications: What is New and What Works, *Clinical Diabetes* 2003;21:186-7.
19. Yamagaishi S, Imaizumi T. Diabetic Vascular Complications: Pathophysiology, Biochemical basis and Potential Therapeutic Strategy. *Current Pharmaceutical Design* 2005;11:2279-99.
20. Uribarri J, Cai W, Sandu O, Peppa M, Goldberg T, Vlassara H. Diet-derived advanced glycation end products are major contributors to the body's AGEpool and induce inflammation in healthy subjects. *Annals New York Academy of Sciences* 2005;1043:461-6.
21. Thomas MC, Tsalamandris C, MacIsaac R."Low molecular weight AGEs are associated with GFR and anemia in patients with type 2 diabetes". *Kidney International* 2004;66:1167-72
22. Bierhaus A, Hofmann MA, Ziegler R, Nawroth PP. AGEs and their interaction with AGE-receptors in vascular disease and diabetes mellitus 1: the AGE concept. *Cardiovascular Research* 1998;37:586-600
23. Sang YR, Young SK. The role of advanced glycation end products in diabetic vascular complications. *Diabetes and Metabolism Journal* 2018;42:188-95

24. Schmidt SE,Schmidt AM. RAGE axis: animal models and novel insights into the vascular complications of diabetes. *Arteriosclerosis Thrombosis Vascular Biology* 2004;24:1342-6
25. Ado-Kurawa I. Geography and History of Kano in Three Years of Good Governance. Shekarau Stewardship in Kano State. Research and Documentation Directorate, Government House, Kano,2006.
26. Usman AK, and Ahmed M. Distribution of Primary Health Care Facilities in Kano Metropolis Using GIS (Geographic Information System), *Research Journal of Environmental and Earth Sciences* 2013;5:167-76
27. Cheesbrough M.*District Laboratory Practice in Tropical Countries*. Part 1. *Cambridge University Press, United Kingdom* 2000, pp 343-5
28. Ahlqvist E, Storm P, KäräjämäkiA, Martinell M, Dorkhan M, Carlsson A.*et al.* Clustering of adult-onset diabetes into novel subgroups guides therapy and improves prediction of outcome in the bioRxiv Data set, 2017. Available online:www.https://doi.org/10.1101/186387 *Accessed on* 22th August, 2019.
29. Maitra A. Abbas AK. Endocrine System in Robbins and Coctran Pathologic Basis of Disease (7thedition) Saunders, *Philadelphia* 2005;Pp 1156-226
30. Baynest HW. Classification Pathophysiology Diagnosis and Management of Diabetes Mellitus. *Journal of Diabetes and Metabolism* 2015;6:1-9.
31. Lehninger A. Nelson D, Cox M. Lehininger Principles of Biochemistry. New York: *W.H. Freedom* 2017;Pp 931-4.
32. Arora M, Mahat, RK, Kumar S, Tyagi S, Batra J. Oxidative stress and its relation to glycemic control in patients of type 2 diabetes mellitus. *International Journal of Medical Science and Public Health* 2016;5:1173-7.
33. Aaron I, Vinik TE, Tae S. Platelet Dysfunction in Type 2 Diabetes. *Diabetes Care* 2001;1476-85.
34. Demirtunc R, Duman D, Basar M, Bilgi M, Teomete M, Garlip T. The Relationship between Glycemic Control and Platelet Activity in Type2 Diabetes Mellitus. *Journal of Diabetes Complications* 2009;89- 94.
35. Harika PK, Asha LP, Pradnya S, Ayesha J, Samatha P, Mani RK. Comparative Study of Erythrocyte Fragility in Diabetes Mellitus and Non-diabetes Mellitus, *International Journal of Medical Research and Health Sciences* 2015;4:183-5.
36. Chien-Min K, Zu-Lin T, Hai-Lung W. Erythrocyte Fragility Increases with Level of Glycosylated Haemoglobin in Type 2 Diabetic Patients, *Clinical Hemorheology and Microcirculation* 2009;43:345-51.
37. Arun PM. Erythrocyte Fragility Increases with the Duration of Diabetes in Indian Population, *International Journal of Basic and Applied Medical Sciences 2013;3:172-7*
38. Sharma M, AroraM, Mustafa I, KumarS, Mittal A, Soam SS, *et al.* Correlation of Glycated Haemoglobin with Oxidative Stress and Erythrocyte Fragility in Type-2 Diabetes Mellitus. *International Journal of Contemporary Medical Research* 2017;4:1909-10.
39. Ye S, Ruan P, Yong J, *Shen H, Liao Z, Dong X.* The impact of the HbA1c level of type 2 diabetics on the structure of haemoglobin *Scientific Reports* 2016;6:33352.