Sudan University of Science and Technology

College of Graduate Studies

Evaluation of Glucose-6 Phosphate Dehydrogenase level and Complete Blood count among Sudanese Diabetes Mellitus Type2 Patients in Khartoum State

تقويم مستوى انزيم الجلوكوز-6 نازع الهيدروجين وعدد الدم الكامل وسط المرضى السودانيين المصابين بالسكري النوع الثاني في ولاية الخرطوم

A Dissertation Submitted in Partial Fulfillment of the Requirements for the award M.Sc. Degree in Hematology and Immunohematology

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الاية

قال تعالى:

(أَفَمَن يَمْشِي مُكِبًّا عَلَىٰ وَجْهِهِ أَهْدَىٰ أَمَّن يَمْشِي سَوِيًّا عَلَىٰ صِرَاطٍ مُّسْتَقِيمٍ)

صدق الله العظيم

(سورة الملك)

(الاية ٢٢)

Dedication

To soul of my father,

To my beloved mother,

To my wife, my kids and all members of my family,

To my supervisor, teachers and colleagues…

I dedicate this study.

Acknowledgments

The greatest thank to my ALLHA. I would like to express my deepest appreciation and thanks to everyone who help me throughout this work at any step of it.

Firstly, most grateful to my supervisor Dr. Kawthar Abdalgaleil Mohammed Salih for her flexibility, expertise, supporting and guidance when I needed.

Secondly, my deepest appreciation to whole family members of Hematology and Immunohematology Department in Sudan University of Science and Technology for their valuable information and encouragement, throughout my research.

Finally, my thanks extend to all people whom the blood sample have been collected from, staff of Almoalem Hospital, staff of Alblsem Hospital and staff of Almarkaz Altebey Alhadeth Hospital for their contributions. To all of them and everyone helped me who was not mentioned very thanks with regards.

Abstract

This is Case Control study conducted between March and April, 2017 in Khartoum state among diabetic type 2 Sudanese patients who attended at the Almoalem Hospital to evaluate CBC and G6PD level.One hundred was confirmed diagnosis with Type 2 Diabetic Patients as Cases while the other hundred was healthy non Diabetic the controls. 5ml of anti coagulated venous blood was withdrawn from each participant for CBC analysis, G6PD activity testing and glucose test. Demographic characteristic of participants showed 61/200 of them were male (30.5%) and 139/200 were female (69.5%). Mean of age was 54 ± 14 year. Age group 46-65 years 98/200 (49%) was the highest frequency, followed by 25-45 years 52/200 (26%) and 66-85 years 50/200 (25%), while duration of disease was the highest frequency 1-10 years 60/100 (60%), 11-20 years 29/100 (29%) and 21-30 years 11/100 (11%). Frequency regard to medication of cases, injection was 57/100 (57%) and tabs were 43/100 (43%).

Comparison Means of G6PD level between Control and Case subjects showed no significant differences in means of G6PD activity (0.211).

Comparison of Means of CBC parameters between Control and Case, showed significant difference in PCV (0.039), MCV (0.000) and MCH (0.02).

Correlation between G6PD level and glucose level among study volunteers, showed no significant correlation (0.474), with intermediate negative Pearson Correlation (-0.51).

Comparison means of G6PD level among cases according to therapy, showed no significant differences in means of G6PD (0.364).

Comparison means of G6PD level among study volunteers according gender, showed no significant differences in means of G6PD (0.234).

Comparison means of G6PD activity Between Groups according to age group among study volunteers, showed no significant differences in means of G6PD(0.998) .

مستخلص الدراسة

هذه دراسة حالة تحليلية ضابطة في ولاية الخرطوم بمركز المعلم الطبي،في الفترة من مارس الى ابريل في عام٢٠١٧ م بولاية الخرطوم، وسط السودانيين المصابين بالسكري النوع الثاني الذين حضروا الى مركز المعلم ، وذلك لتقيم ارتفاع نسبة السكر بالدم واثره على مستوى انزيم الجلكوز ٦ فوسفيت نازع الهايدوجين ،وتعداد الدم الكامل.وتم تحليل النتائج لبواسطة برنامج الحزم الاحصائيةللعلوم الاجتماعيةاصدار٢١، حسب متوسط و الترددات وسجلت النتائج ذات الدلالة الإحصائية (>٠.٠٥). اختير مائين مشارك طوعا مائة شخص مشخصين كمرضى سكري النوع الثاني كحالة ، ومائة شخص من الاصحاء كحالة ضابطة . اخطر كل المشاركين في الدراسة شفاهه عن مفهومها واخذت موافقتهم ، اخذ ٥ مليلتر من الدم الوريدي من كل مشارك، ليحلل تعداد الدم الكامل ونشاط انزيم الجلكوز ٦ فوسفيت نازع الهايدوجينز ونسبة السكر بالدم . جمعت البيانات الديموغرافية للدراسة وكانت الترددات كالاتي: الاناث بنسبة٦٩.٥% (٢٠٠/١٣٩) والذكور فكانت بنسبة ٣٠.٥% (٢٠٠/٦١)،كما تم قسم منفي الدراسة على حسب الاعمار بمتوسط ٥٤ سنة الى ثلاث فئات عمرية، وكانت اعلاها (٤٦-٥٠) سنة بنسبة٤٩% ثم (٢٥-٤٥) سنة بنسبة (٢٦%) ثم (٦٦-٨٥) سنة بنسبة (٢٥%). كما وايضا قسمت الحالة علي حسب الفترة الزمنية منذ ظهور المرض وكان (١-١٠) سنة بنسبة ٦٠% وهي الأعلى تردد، و(١١-٢٠)سنةبنسبة٢٩%و(٢١-٣٠)سنةبنسبة١١%. ثم قسم المرضي على حسب نوع الدواء وكانت الحقن ٥٧% والحبوب٤٣%.

مقارنة انزيم الجلكوز ٦ فوسفيت نازع الهايدوجين بين الحالة والحالة الضابطة، اشارت النتائج الى عدم وجود فرق ذو دلاله إحصائية.

مقارنة متوسط تعداد الدم بين الحالة والحالة الضابطة، اشارت النتائج الى وجود فرق واضح ذو دلاله إحصائية في حجم الخلية المعبئة(٠.٠٣٩) وحجم الخلية الوسطي (٠.٠٠٠) و خضاب الخلية الوسطي(٠.٠٢) تباعا.

العلاقة بين انزيم الجلكوز ٦ فوسفيت نازع الهايدوجين ومستوى سكر الدم اشارت النتائج الى وجود فرق واضح ذو دلاله إحصائية (٠.٤٧٤) وعلاقة بيرسون كانت متوسطه وسالبة (-٠.٥١).

مقارنة متوسط انزيم الجلكوز ٦ فوسفيت نازع الهايدوجين بين أفراد الحالة علي حسب نوع العلاج (حبوب ام حقن) اشارت النتائج الى وجود فرق واضح ذو دلاله إحصائية في تعداد الصفائح الدموية(٠.٠٣٢) .

مقارنة متوسط انزيم الجلكوز ٦ فوسفيت نازع الهايدوجين بين الحالة و الحالة الضابطة علي حسب نوع الجنس ،اشارت النتائج إلي عدم وجود فرق واضح ذو دلاله إحصائية (٠.٢٣٤).

مقارنة متوسط انزيم الجلكوز ٦ فوسفيت نازع الهايدوجين بين الحالة والحالة الضابطة علي حسب الفئات العمرية لم تشير النتائج بوجود فرق واضح ذو دلاله إحصائية ونشاط الانزيم (٠.٩٩٨).

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List of Abbreviations

ADP	: Adenosinediphosphate.
AGEs	: Advanced Glycation End-products.
AHA	: Acute heamolatic aneamia.
ATP	: Adenosine triphosphate.
β-cell	: Beta-cell.
cAMP	: Cyclicadenosine monophosphate.
CBC	: Complete blood count.
CNSHA	: Congenital non spherocytic haemolytic anaemia
CO groups	: Carbonyl groups.
DKA	: Diabetic ketoacidosis.
D.M	: Diabetes mellitus.
eNOS	: Endothelial NitroOxide synthase.
FBG	: Fasting blood glucose.
fL	: Femtolitre (10^{-15})
GPX	: Glutathione peroxidase.
GR	: Glutathione reductase.
GSH	: Glutathione.
GSSG	: Oxidized glutathione.
G6PD	: Glucose-6-Phosphate Dehydrogenase .
HbA1C	: Hemoglobin A1C.
HCT	: Hematocrit value.
HGB	: Hemoglobin.
MCH	: Mean Red Blood Cell hemoglobin.
MCHC	: Mean Red Blood Cell hemoglobin concentration.
MCV	: Mean Red Blood Cell volume.

NADPH : Nicotinamide adenine dinucleotide phosphate.
NO : Nitric oxide.
OS : Oxidative stress.
PDR : Proliferative diabetic retinopathy.
Pg : Picogram (10^{-15}).
PKA : Protein kinase A.
PKC : protein kinase C.
PLT : Platelet.
PPP : Pentose phosphate pathway.
RBCs : Red blood cells.
RNS : Nitrosative species.
ROS : Reactive Oxygen Species.
RPE : Ribulose-5-phosphate-3-epimerase.
RPI : Ribulose-5-phosphate isomerase.
SH : Sulphydryl groups.
SOD : Superoxide dismutase.
TKT : Transketolase.
T1DM : Type 1 diabetes mellitus.
T2DM : Type 2 Diabetes mellitus.
UCP-2 : Uncoupling protein 2.
UV : Ultra violet.
6PGD : 6-Phosphogluconate dehydrogenase.
2,3-DPG : 2,3-Diphosphoglycerate.

Chapter I

1. Introduction:

Glucose-6-Phosphate Dehydrogenase (G6PD) is a critical housekeeping enzyme in all body cells(red blood cells) that supports protective systems against oxidative challenge (Fig.A) by producing the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) (Domingo *et al.,* 2013). G6PD is critical for RBCs, it is particularly critical to the integrity and functioning of red blood cells (RBCs) by protects the cells and prevents them from being damaged. The entire antioxidant (reductant) system relies on an adequate supply of NADPH which is the principal intracellular reductant for all cells (Xu *et al.,* 2005).

Oxidative stress plays a pivotal role in the development of diabetes complications, both microvascular and cardiovascular. Metabolic abnormalities of diabetes cause mitochondrial superoxide overproduction in endothelial cells which activation of five major pathways involved in the pathogenesis of complications: polyol pathway flux, increased formation of advanced glycation end-products (AGEs), increased expression of the receptor for AGEs and its activating ligands, activation of protein kinase C (PKC) isoforms, and overactivity of the hexosamine pathway. Beside inactivates two critical antiatherosclerotic enzymes, eNOS and prostacyclin synthase, that lead to increase intracellular ROS cause defective angiogenesis in response to ischemia, activate a number of pro-inflammatory pathways, and cause long-lasting epigenetic changes which drive persistent expression of proinflammatory genes after glycemia is normalized (‘hyperglycemic memory’) (Giacco and Brownlee, 2010). Atherosclerosis and cardiomyopathy in type 2 diabetes are caused in part by pathway selective insulin resistance, which increases mitochondrial ROS production

from free fatty acids and by inactivation of anti atherosclerosis enzymes by ROS. Over expression of superoxide dismutase in transgenic diabetic mice prevents diabetic retinopathy, nephropathy, and cardiomyopathy (Giacco and Brownlee, 2010).

1.1. Glucose-6-Phosphate Dehydrogenase (G6PD):

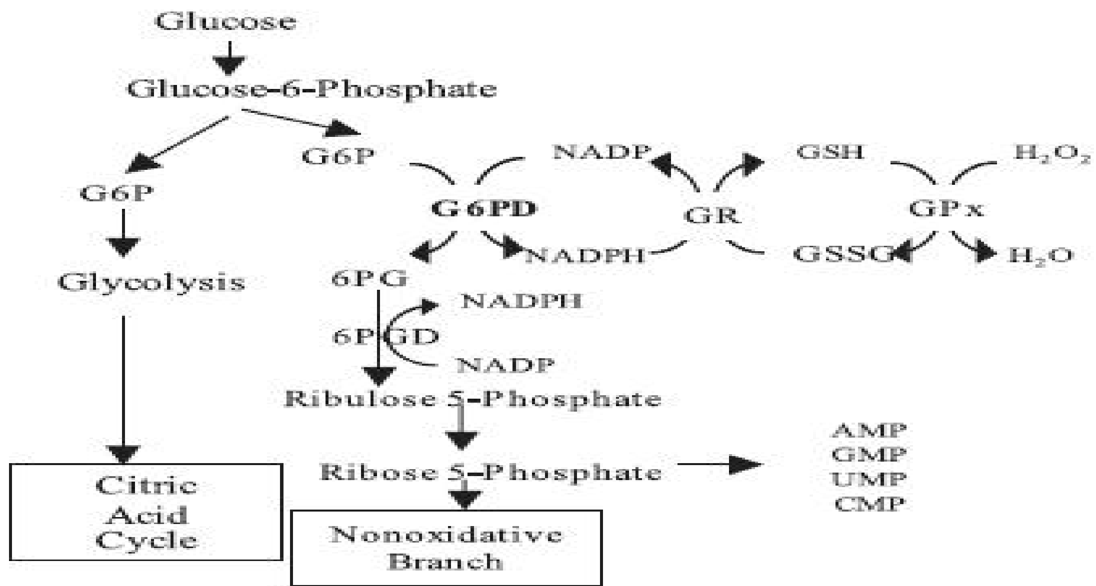
1.1.1. G6PD is an important enzyme that catalyses the first reaction in the pentose-phosphate pathway, G6PD is the sole source of enzymatic activity that protects against the build-up of super-radicals and thus, oxidative stress (Monteiro *et al.*, 2014). It is the first and rate limiting enzyme of the pentose phosphate pathway which results in the production of ribose-5 phosphate and produces NADPH to fuel glutathione recycling which has a housekeeping role in all cells (Xu *et al.*, 2005). Key role in metabolism is to provide reducing power in the cytoplasm in the form of NADPH. This role is particularly important in red blood cells where NADPH serves as an electron donor for detoxification of hydrogen peroxide via reduced glutathione, and its production is crucial for the protection of cell from oxidative stress (Nantakomol *et al.*, 2013).

1.1.2. Structure of G6PD:

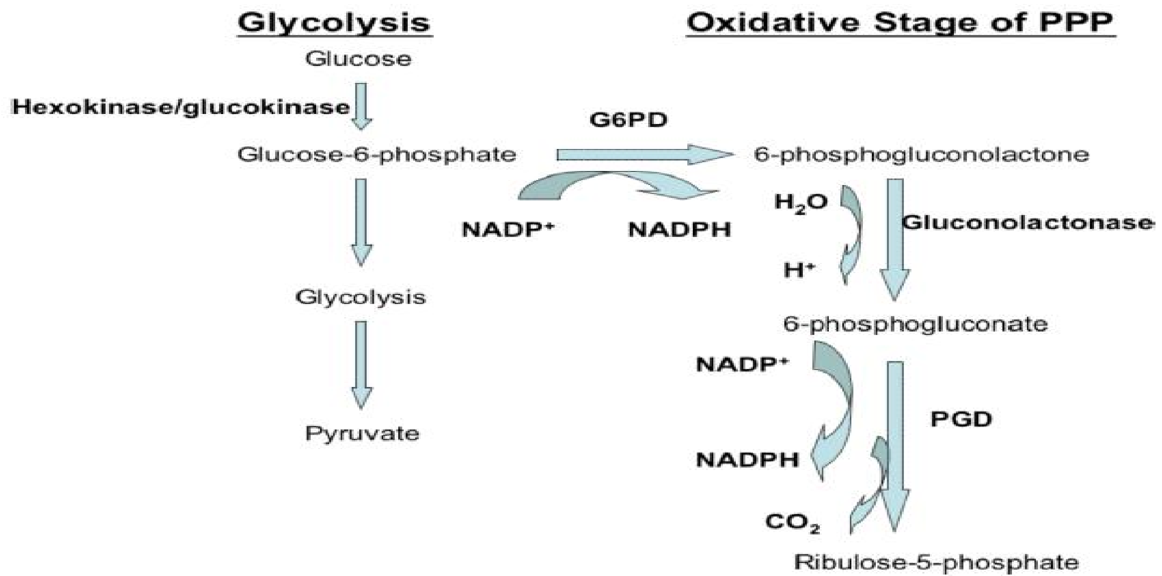
is glucose-6-phosphate: NADP oxidoreductase enzyme is present in all cells (Fig.E). It is found in its dimeric and tetrameric forms. The monomer has a molecular weight of 59,256 daltons and counts with 515 amino acids. The catalytic activity is only initiated when an association is established, in balance status, between the dimeric and tetrameric forms. Such an association requires the NADP presence, strongly tied by the enzyme which makes that NADP performs a dual role not only as structural component but as a coenzyme (Bonilla *et al.*, 2007).

1.1.3 Biochemistry and Molecular Biology:

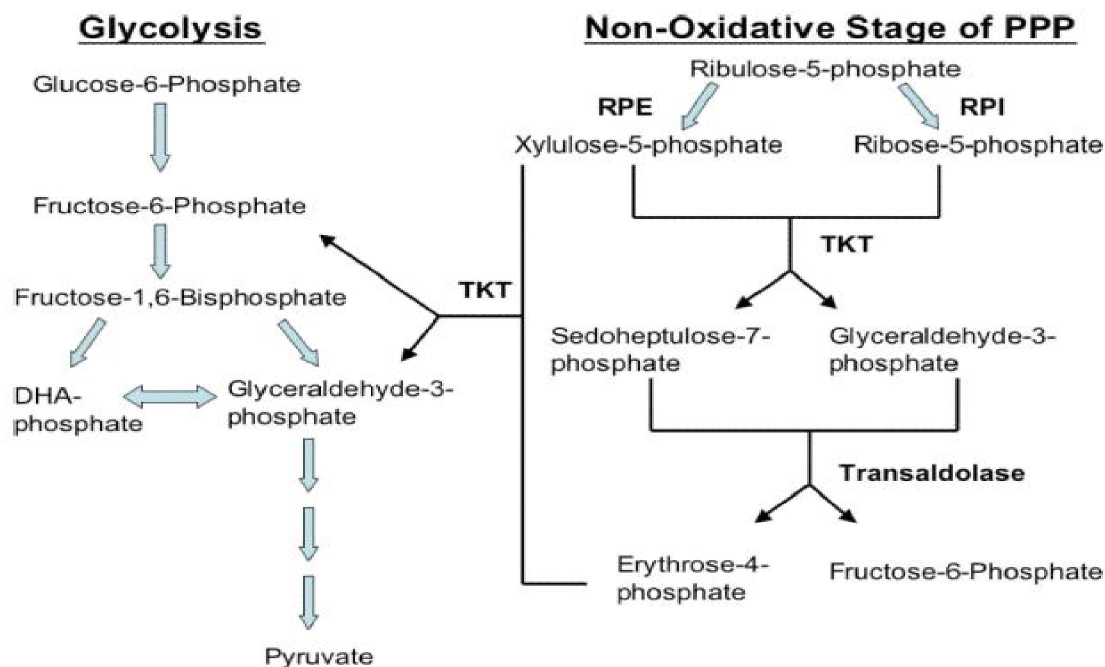
G6PD is a cytosolic protein with a key role in the pentose phosphate pathway that produces nicotinamide adenine dinucleotide phosphate (NADPH), a very important reducing agent that confers protection against cellular oxidative stress and helps in the regeneration of oxidized glutathione (GSSG) (Manzo *et al.*, 2017). Human glucose-6-phosphate dehydrogenase is an X-linked housekeeping enzyme that catalyses the first and rate-limiting step of the pentose phosphate shunt. It converts glucose 6-phosphate (G6P) to 6- phosphoglucono -lactone with the reduction of NADP+ to NAPDH, providing cells with pentoses for nucleic acid synthesis and NADPH as the principal modulator of intracellular redox potential (Fig.B and C). It has been identified as the only NADPH producing enzyme that is activated during oxidative stress. In erythrocytes, the role of G6PD is particularly important as the sole source of NADPH (Kotaka *et al.*, 2005). G6PD activity is post- translationally regulated by SIRT2, a cytoplasmic NAD+ dependent deacetylase, thereby linking NAD+ levels to DNA repair and oxidative defences and identifying potential new approaches to treating this common genetic disease (Wu and Sinclair, 2014).



(Fig.A): Pentose phosphate pathway and glutathione coupling (Xu *et al*., 2005).



(Fig.B):Oxidative Branch of the Pentose Phosphate Pathway (PPP)(Stanton, 2012).



(Fig. C): Non-oxidative Branch of the PPP (Stanton, 2012).

1.1.2. Genetic aspects of G6PD gene:

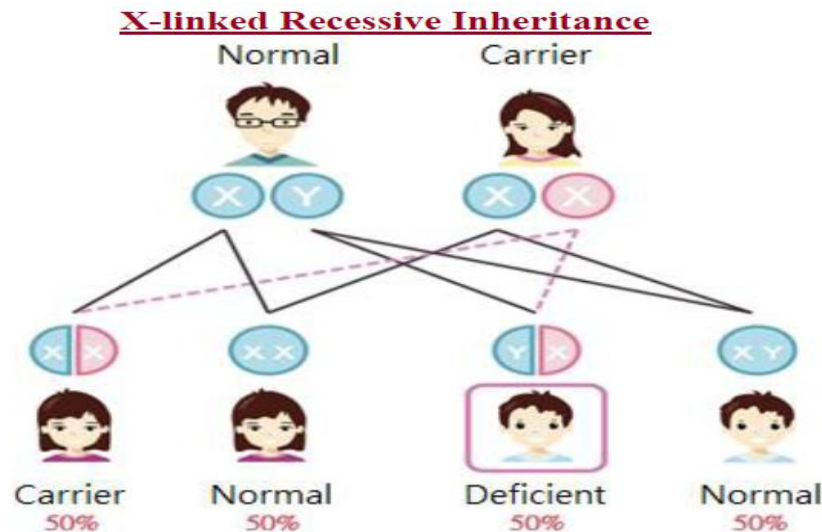
1.1.2.1 Genetic map and Mode of inheritance of G6PD gene :

G6PD gene is located on the long arm of the X chromosome (Xq28.2)(Bakhtiari *et al.*, 2016). The enzyme is a dimer (predominantly) or tetramer (pH dependent) in the active form composed of identical subunits, 515 amino acids long and weighs about 59 kDa (Burtis *et al.*, 2008). The G6PD enzyme gene is spread over 18.5 Kb and 13 exons (Domingo *et al.*, 2013). The completed sequence of the human G6PD gene is 18.5 Kb in size and consists of 13 exons and 12 introns encoding a product of 1545 bp (Manzo *et al.*, 2017). Because the inheritance is sex linked, affecting males and carried by females (Fig.D). The degree of deficiency varies with ethnic group, often being mild (10–60% of normal activity) in black African people, more severe in Middle Eastern and South East Asian people and most severe in Mediterranean people (<10% of normal activity). Severe deficiency

occurs occasionally in white people (Hoffbrand and Moss, 2016).

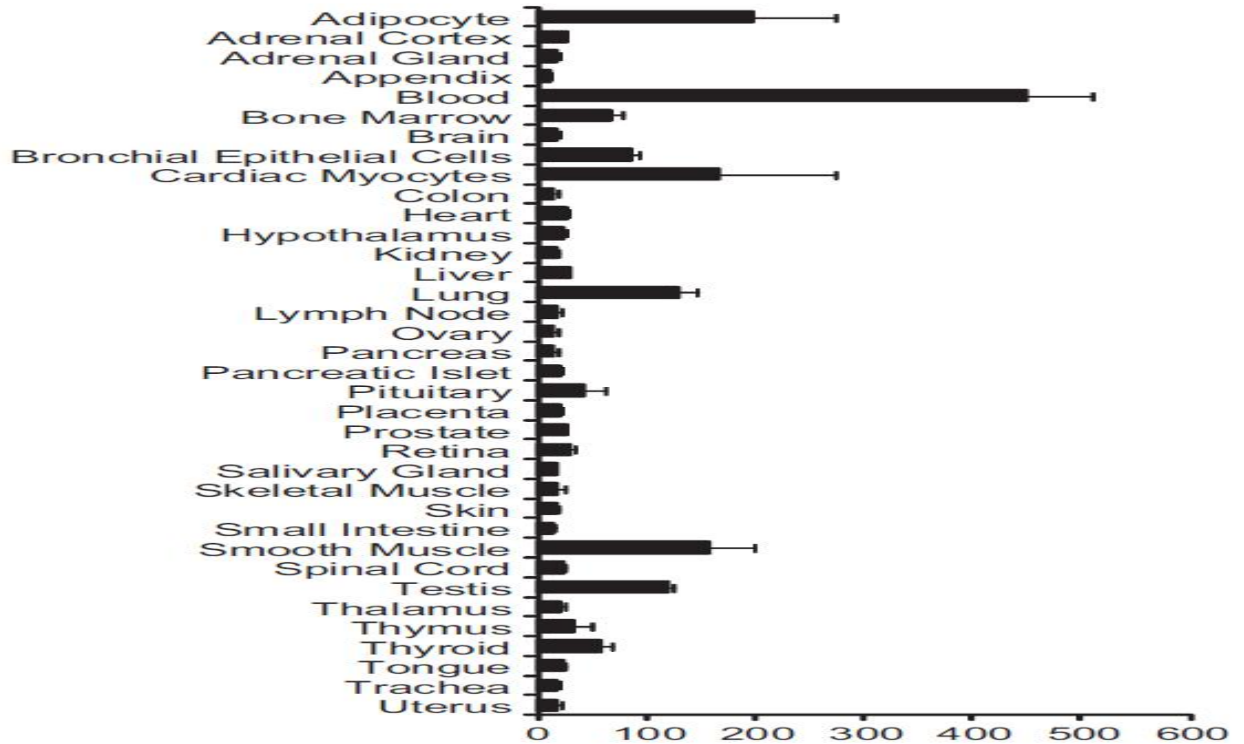
1.1.2.2 Effect of each mutation in G6PD gene:

G6PD deficiency is globally distributed it is more prevalent in malaria endemic countries. Several mutations have been identified in the G6PD gene, which alter enzyme activity (Amoah *et al.,* 2016). The majority of mutations disrupts the enzyme structural stability and thus reduces its overall activity. The effect of each mutation on enzyme structure and function depends on the location of the substituted amino acid. For example, many of the most severe mutations map to exon 10 (El-Khawaga *et al*., 2010). Recently G6PD has been implicated in the regulation of cell growth and survival, replicative senescence and development. G6PD-deficient cells undergo premature senescence upon serial passage and show an increased propensity for oxidant-induced senescence. In addition, these cells are more susceptible to oxidant-induced apoptosis and display altered growth responses to signalling factor(Chiu *et al*., 2008).



(Fig.D): Offspring from normal father and carrier mother (Website: http://www.cgs.dh.gov.hk, 2017).

1.1.2.3. G6PD expression tissues:G6PD is expressed in all cells and catalyzes the first step in the hexose monophosphate pathway (Burtis *et al.*, 2008).



(Fig.E) G6PD expression sites in the body .

(website:http://www.ajpheart.org,2012).

1.1.3. Glucose-6-phosphate dehydrogenase (G6PD) deficiency:

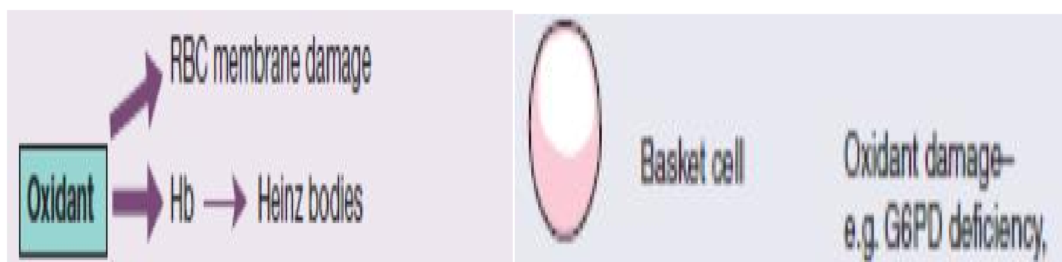
It is a disease of the red blood cell (RBC) which exposes patients to intravascular haemolysis, which predisposes the cells to oxidative injury (Emeka *et al.*, 2015). G6PD deficiency is the most common enzymopathy, affecting 400 million people worldwide. More than 400 different types of G6PD variants, leading to different enzyme activities associated with a wide range of biochemical and clinical phenotypes. The variants are grouped into five categories according to the amount of enzyme activity and clinical phenotype (Burtis *et al.*, 2008).G6PD deficiency is the most common gene mutation in the world and the numerous mutations have been classified by

the World Health Organization according to the activity as: Class I is <1% of wild type activity, Class II is <10%, Class III is 10–60% (the most), Class IV is 60–90% (this is considered normal G6PD activity) and Class V(Hektoen) is >110% (Stanton, 2012).

1.1.3.1 The clinical expression of the disease: It is heterogeneous and five different clinical syndromes have been recognized (Drugs induced hemolysis, infection induced hemolysis, Favism, Neonatal jaundice and Chronic nonspherocytic hemolytic anemia) but the majority of G6PD-deficient individuals develop hemolysis only when oxidative stress occurs (Burtis *et al.*, 2008). Outside these causes, they are usually asymptomatic. However, G6PD deficiency also leads to mild to severe chronic hemolysis, exacerbated by oxidative stress. The reference interval for G6PD in erythrocytes is 8 to 14 U/g Hb. *value*s greater than 18 U/g Hb are often encountered in any condition associated with younger RBCs than normal (Burtis *et al.*, 2008).

1.1.3.2 Role of G6PD in RBCs survive:

RBC is 8µm in diameter, so in order to carry haemoglobin and for successful gaseous exchange must be able to pass repeatedly through the microcirculation (3.5µm). Haemoglobin in a reduced (ferrous) state and to maintain osmotic equilibrium. RBC is a flexible biconcave disc with an ability to generate energy. G6PD deficiency particularly affects RBCs due to they have not been able to synthesis the G6PD enzyme, while G6PD level is decreasing with ageinng during their 120 days lifespan and RBCs are susceptible to oxidizing agents in the blood continuously, which generated from deoxygenated and oxygenated of haemoglobin. Majority of G6PD deficient peopel are subclinical and remain asymptomatic until they are exposed to a haemolytic trigger (Fig.F). Acute heamolatic aneamia (AHA) is the most manifestation of the deficiency (Hoffbrand and Moss, 2016) .



(Fig. F): Effect of oxidant on RBC membrane and Hb (Hoffbrand and Moss, 2016).

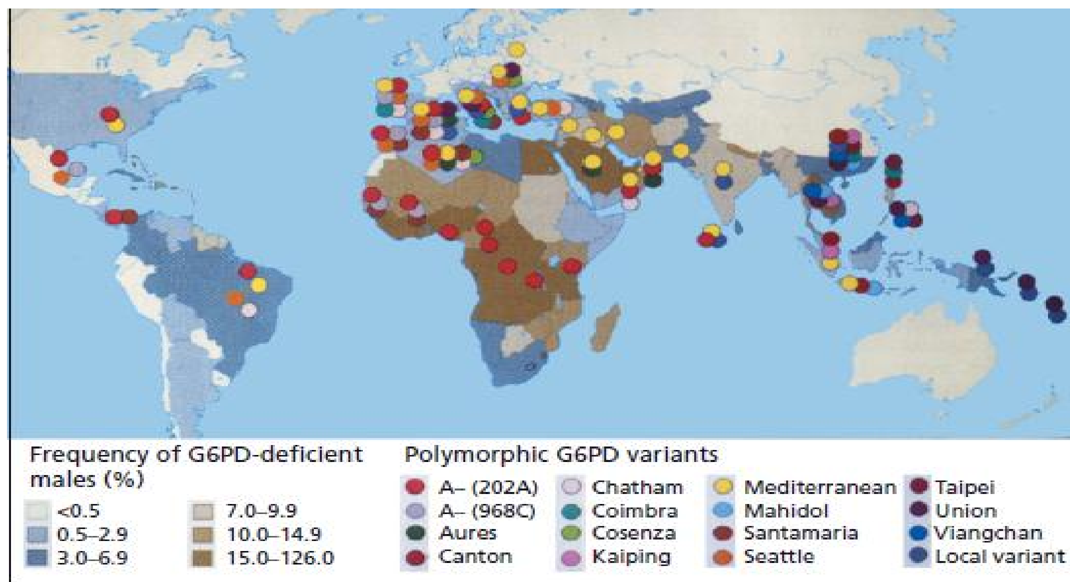
1.1.3.3. Role of G6PD in Red cell metabolism:

Embden–Meyerhof pathway: In this series of biochemical reactions, glucose that enters the red cell from plasma by facilitated transfer is metabolized to lactate. For each molecule of glucose used, two molecules of ATP and thus two high energy phosphate bonds are generated (Hoffbrand and Moss, 2016). ATP drives mechanisms that slow the destruction of protein and iron by environmental peroxides and superoxide anions, maintaining hemoglobin's function and membrane integrity. However Oxidation eventually takes a toll, limiting the RBC circulating life span to 120 days, whereupon the cell becomes disassembled into its reusable components globin, iron, and the phospholipids and proteins of the cell membrane, while the protoporphyrin ring is excreted as bilirubin (Keohane *et al.*, 2016). The Embden–Meyerhof pathway also generates NADH, which is needed by the enzyme methaemoglobin reductase to reduce functionally dead methaemoglobin containing ferric iron (produced by oxidation of approximately 3% of haemoglobin each day) to functionally active, reduced haemoglobin containing ferrous ions. The Luebering–apoport shunt (side arm) of this pathway generates 2,3-DPG. Approximately 10% of glycolysis occurs by

this oxidative pathway (Fig.B) in which glucose-6-phosphate is converted to 6-phosphogluconate and so to ribulose-5-phosphate. NADPH is generated and is linked with glutathione which maintains sulphydril (SH) groups intact in the cell(Hoffbrand and Moss, 2016).

1.1.3.4. Epidemiology of G6PD deficiency:

There is more than 400 variants caused by point mutations or deletions which show less activity than normal and worldwide over 400 million people are G6PD deficient. The most common being type B (Western) and type A in Africans (Hoffbrand and Moss, 2016).

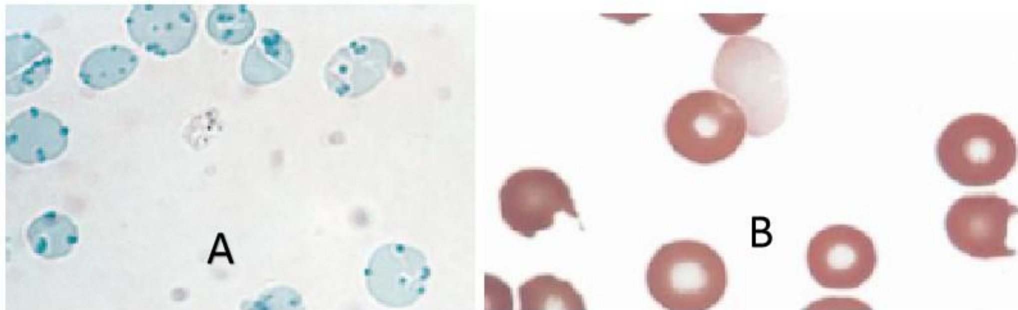


(Fig.G): Global distribution of G6PD deficiency. It Show distribution of G6PD among Sudanese between 7 to 9.9% (Hoffbrand *et al* ., 2011).

1.1.3.5 Clinical features of G6PD deficiency:

Presentation of disease variants are associated with different mutations: Class I (rare) is caused by mutations that lead to severe enzyme deficiency (<10% of normal). It is associated with chronic and nonspherocytic hemolytic anemia. Class II (G6PD Mediterranean) and class III(G6PD-A−)

variants are associated with severe and moderate enzyme deficiency, generally associated with new-onset intravascular hemolysis and anemia in the setting of an inciting exposure oxidizing stress (Fig.H). Some patients (particularly with class I variants, but also II and III) may present with neonatal hyperbilirubinemia, highest incidence at 2-3 days after birth (Aster *et al.*, 2012).



(Fig.H): Blood film in G6PD deficiency with acute haemolysis after an oxidant stress. (A-*Heinz bodies* and B- *Shows bite cells*) (Aster *et al.*, 2012).

1.1.3.6 Diagnosis of G6PD deficiency:

The diagnosis of G6PD deficiency is made by a quantitative spectrophotometric analysis or more commonly, by a rapid fluorescent spot test detecting the generation of NADPH from NADP. The test is positive if the blood spot fails to fluoresce under ultraviolet light (in field research quick screening of a large number). Tests based on polymerase chain reaction detect specific mutations and are used for population screening, family studies, or prenatal diagnosis. In patients with acute hemolysis, testing for G6PD deficiency may be falsely negative because older erythrocytes with a higher enzyme deficiency have been hemolyzed. Young erythrocytes and reticulocytes have normal or near normal enzyme activity. Female heterozygotes may be hard to diagnose because of X-chromosome mosaicism leading to a partial deficiency that will not be detected reliably

with screening tests (Frank, 2005).

1.1.3.7 Agents that may cause haemolytic anemia in G6PD deficiency :

Oxidant stress is induced by exposure to a variety of chemicals or medications. Acute viral or bacterial infection, acidosis, and liver disease may also precipitate acute hemolysis (Schmaier and Lazarus, 2012).

1.1.3.8 Laboratory findings in G6PD deficiency haemolytic anaemia:

Acute intravascular haemolysis raises the suspicion of G6PD deficiency. The blood film shows red cells with contracted haemoglobin (Fig.H) in ghost membranes. Haemoglobinuria may be gross, producing almost black urine without red cells in the centrifuge deposit. Several screening tests have been devised to identify G6PD deficiency in red blood cells. The most widely used tests have been the brilliant cresyl blue decolorization test, the methaemoglobin reduction test and an ultraviolet spot test. These tests can reliably distinguish between deficient and non deficient individuals, but are not reliably quantitative. Hemizygous deficient males and homozygous deficient females will be identified, the threshold being a G6PD activity of about 30% of normal. If a screening test indicates deficiency or is doubtful, the ideal follow up test for definitive diagnosis is quantitation of G6PD activity by spectrophotometric assay. First, during a haemolytic attack, the oldest red cells (with the least G6PD activity) are destroyed selectively and therefore the surviving red cells have a relatively higher (but still deficient) G6PD activity. These increases further as the reticulocyte response sets in over the following days (Hoffbrand *et al.*, 2011).

1.1.3.9 Management of G6PD deficiency:

The main treatment for G6PD deficiency is avoidance of oxidative stress agents. Rarely, anemia may be severe enough to warrant a blood transfusion. Splenectomy generally is no recommended. Folic acid and iron potentially

are useful in hemolysis, although G6PD deficiency usually is asymptomatic and the associated hemolysis usually is short-lived. Antioxidants such as vitamin E and selenium have no proven benefit for the treatment of G6PD deficiency (Frank, 2005).

1.1.4. Antioxidants system and Reactive oxygen species (ROS):

1.1.4.1 Antioxidants function and examples:

They act as free radical scavengers to protect cells against Reactive oxygen species ROS. These antioxidants are superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPX). Beside to a variety of non enzymatic antioxidant: vitamin C, vitamin E, pyruvate and carnitine (Agarwal, 2005).

1.1.4.2 Reactive oxygen species and sources :

ROS are chemically reactive molecules which formed as a natural consequence of the usual metabolism of oxygen and play a major role in cell signaling and homeostasis. ROS are produced by two major routes either Exogenous from smoke, drugs, radiation and pollutants or Endogenous (intracellularly) by several mechanisms, depending on the type of cell and tissue, however the main sources are the seven isoforms of NADPH oxidase that are located in the membranes, mitochondria, and endoplasmic reticulum. ROS agents such as, the superoxide radical, the hydroxyl radical, and singlet oxygen (Bakhtiari, 2016).

1.1.4.3 ROS toxicity (ROS) and Oxidative stress (OS) :

ROS are biologically toxic which are considered injurious to cell survival and function when present in abundance. Depending on the type and concentration, ROS can have adverse or beneficial effects (Bakhtiari *et al* ., 2016). OS occurs when there is an excessive production of free radicals or impaired antioxidant defense mechanisms, which is harmful to cell. OS has been proposed as the root cause underlying the development of insulin

resistance, b-cell dysfunction, impaired glucose tolerance, T2DM, and implicated in the progression of long-term diabetes complications (Wright *et al.,* 2006).

1.2. Diabetes mellitus (D.M.)

1.2.1. Overview and Historical back ground of D.M.

It has been described as Polyuric diseases for over 3500 years. Apolyuric state was described in an Egyptian papyrus dating from. The name “diabetes” comes from the Greek word for a syphon, the sweet taste of diabetic urine was recognized at the beginning of the first millennium, but the adjective “mellitus” (honeyed) (Holt *et al.*, 2010). It is one of metabolic diseases characterized by hyperglycemia. If the hyperglycemia of diabetes is not managed properly, it causes long-term damage, dysfunction, and failure of different organs. Notably the eyes, kidneys, nerves, heart and blood vessels (Mahmoud and Nor El-Din, 2013). Diabetes is a complex multisystemic disorder characterized by a relative or absolute insufficiency of insulin secretion and disturbances in carbohydrate, protein and lipid metabolism (Govindappa, 2015). Type 2 diabetes mellitus (T2DM) accounts for approximately 90% of all cases of diabetes mellitus, which is one of the most common chronic diseases. It was estimated that about 285 million people were living with diabetes mellitus all over the world. Pathologically, T2DM is characterized by hyperglycemia which may be caused by the combination of insulin resistance and inadequate insulin secretion (Zhang *et al.*, 2015).

1.2.2. Clinical Presentations of Diabetes:

classic symptoms are Polyuric state and Sugary urine. Presentation may be asymptomatic and discovered on routine examination or laboratory test, especially in T2DM. Complications are Diabetic ketoacidosis (Diabetic coma and Acidotic breathing), impotence, Hyperlipidemia (Lipemia retinalis), Retinopathy (Microaneurysms, New vessels, beading of retinal veins and lesions specific to diabetes), Neuropathy (Glomerulosclerosis

associated with heavy proteinuria) and foot disease (gangrene or Perforating foot ulcers). Patients with type 1 diabetes usually present with classic symptoms and occasionally diabetic ketoacidosis. Patients with type 2 diabetes mellitus (T2DM) may be asymptomatic or present with classic symptoms. With advancing age, the renal threshold for glucose increases and thirst perception diminishes. T2DM may present with complications of diabetes which may be either microvascular or macrovascular. Initial diagnosis of T2DM during acute myocardial infarction or stroke is common (Holt *et al.*, 2010).

1.2.3. The mechanism responsible for the pathogenesis of diabetic:

Oxidative stress is an important pathophysiologic factor in DM (Agrawal *et al.*, 2009). It is not fully understood but many studies imply blood glucose and its oxidant derivatives have indicated to a key role in pathogenesis of DM. Activity of G6PD enzyme, an anti-oxidant system is important in preventing its complications. Unsuitable control of blood glucose decreases G6PD activity and increases DM complications, by inhibition of G6PD which occurred in part via phosphorylation caused by high glucose induced protein kinase A (PKA) activation (Zhang *et al.*, 2000 and Xu *et al.*, 2005) . Advanced glycation end products (AGEs), activation of protein kinase C (PKC), activation of aldose reductase and others have been shown to play important roles (Xu *et al.*, 2005). Hyperglycemia increase oxidative stress which affects the normal function of cellular proteins and enzymes. High glucose level was found to activate adenylate cyclase which increases cAMP levels. cAMP activates PKA, an inhibitor of G6PD (Mahmoud and Nor El-Din, 2013). G6PD is the major source of NADPH, the main intracellular reductant, hence the decrease in its activity increases the oxidative stress. In addition, hyperglycemia induces mitochondrial superoxide production which

in turn activates four damaging pathways in cells through inhibiting the activity of the key glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase. Increased levels of glyceraldehyde-3-phosphate activate advanced glycation end product pathway, protein kinase C pathway, hexosamine pathway, and polyol pathway. It is also found that hyperglycaemia induces effects within the cell nucleus through reactive oxygen species (ROS). Hyperglycaemia initiates a cascade of transcription events, ultimately leading to changes in the levels of NO, cytokines, acute phase reactants, and cellular adhesion molecules. Generation of ROS can be reduced by avoiding hyperglycaemia and by minimizing fluctuations in blood glucose levels. Finally The introduction of CO groups into proteins causes alterations in protein conformations leading to increased aggregation, fragmentation, distortion of secondary and tertiary structure, and susceptibility to proteolysis, and decrease of normal function (Mahmoud and Nor El-Din, 2013). Hyperglycemia induced diabetic vascular damage focus on five major mechanisms: increased flux of glucose and other sugars through the polyol pathway, increased intracellular formation of advanced glycation end-products (AGEs), increased expression of the receptor for advanced glycation end products and its activating ligands, activation of protein kinase C (PKC) isoforms and overactivity of the hexosamine pathway (Giacco and Brownlee, 2010). Diabetes mellitus leads to important morbidity and mortality, consequence of macro and micro vessels complications. Type 2 DM is characterized by insulin resistance, with or without insulin deficiency that induces organ dysfunction. Persistent hyperglycemia in DM generates reactive oxygen species (ROS) and nitrosative species (RNS), both are considered an essential factor for DM macro and micro vessels complications. Along with overproduction of ROS

and RNS, a reduction of the activity of antioxidant enzymes is known to cause endothelial dysfunction, insulin resistance, and DM complications. Furthermore, oxidative stress inhibits insulin secretion in pancreatic beta cells by activation of uncoupling protein 2 (UCP-2) which in turn reduces the ATP/ ADP ratio and thus reduces the insulin secretory response. ROS and RNS are responsible for structural derangement of carbohydrates, proteins, lipids, and nucleic acids (Pintos *et al.*, 2016).

1.3. Hypothesis :

- Null Hypothesis (H0) : there is no significant difference in G6PD activity between diabetic patients and healthy individuals.

- **Alternative Hypothesis (H1) :** there is significant difference in G6PD activity between diabetic patients and healthy individuals.

1.4. Justification:

Diabetes mellitus is a common and growing public health problem. Oxidative stress is an important pathophysiologic factor in DM. G6PD is an important enzyme that catalyses the first reaction in the pentose phosphate pathway, it is the sole source of enzymatic activity that protects against the buildup of super radicals (a major free radical scavenger in the RBC) (Galtrey and Pathansali, 2008). Different studies and cases reports have indicated there is relationship between G6PD deficiency and hyperglycemia ((Rashidi *et al*., 2013.; Xu *et al*., 2005.; Heymann *et al*., 2012.; Pommelet *et al*., 2006.; Gu *et al*., 2010. and Silvestri *et al*., 2015.). Also we have lack of Data in Sudan, so this study may highlight the relationship of Hyperglycemia with G6PD activity and give some Data about this serous problem about patient of Hyperglycemia in Sudan.

1.5 Objectives (General and Specific) :

1.5.1 General objective:

To evaluate quantitatively G6PD level and CBC parameters between diabetic and non diabetic among Sudanese in Khartoum state.

1.5. 2 Specific objectives:

1. To measure quantitatively G6PD level between cases and controls subjects.
2. To measure CBC parameters between cases and controls subjects.
3. To compare means of G6PD level between cases and controls subjects.
4. To compare means of CBC parameters between cases and controls subjects.
5. To compare means of G6PD level with in other factors (therapy among Cases, gender and age group between volunteers) .

Chapter II

Previous studies:

Study had done in Iran, obtained results revealed that: G6PD activity was significantly higher in non diabetic subjects ($P<0.01$) (Rashidi *et al.*, 2013).

Study had done in USA, obtained results revealed that: G6PD inhibition in diabetic kidneys may partly be due to decreased G6PD expression and increased phosphorylation of G6PD ($P< 0.001$) caused by PKA activation (Xu *et al.*, 2005).

Study had done in Israel, obtained results revealed that: significantly higher proportion of patients with G6PD deficiency among the diabetic population aged 45–64 years ($P< 0.002$). Also results showed a significantly increased association between prevalence of diabetes in the 45–65 years old age group among patients with G6PD deficiency when compared with the general population (Heymann *et al.*, 2012).

Study had done in Ghana, obtained results revealed that: Prevalence of G6PDD status of diabetics and non diabetics Among the 211 type 2 patients studied, there was a significantly ($p< 0.001$) higher prevalence of G-6-PD severely defective (35.1%) compared to 12.8% in nondiabetes individuals (Adinortey *et al.*,2011).

Study had done in USA, obtained results revealed that: high glucose causes inhibition of G6PD activity. Concentrations as low as 10 mM glucose as early as 15 min after exposure are sufficient to suppress G6PD activity. Data were normalized by cell number and expressed as means $\pm$ S.E. of five separate experiments, each run in triplicate ($p < 0.001$ *** $p < 0.0001$ compared with control) (Zhang *et al.*, 2000).

Study had done in Italy, obtained results revealed that: a higher prevalence of G6PD deficiency in controls compared to diabetic patients with PDR, but

it was not significant ($P = 0.09$) (Pinna *et al*., 2013).

Case study had done in France: haemolytic anemia after a diabetic ketoacidosis led us to diagnose G6PD deficiency in a 38-year old male patient. (Pommelet *et al*., 2006).

Case study had done in China: 59-year-old Chinese male patient was admitted at diagnosis of type1 diabetes with ketoacidosis. The factors predisposing to hemolysis were not found, except the significantly diminished activity of G6PD. DNA analysis did not show any coding or intronic mutation in the G6PD gene (Gu *et al*., 2010).

Case study had done in Italy: Haemolysis due to G6PD deficiency in patients with type 1 diabetes mellitus (T1DM) has been principally reported in males, but is uncommon. During the last 10 years 2 girls with an unknown G6PD deficiency showed haemolysis during DKA (Errico *et al*., 2009).

Case study had done in Italy: A 14-year-old male patient was admitted to emergency department with hyperglycemia. He was treated initially with fluid therapy, after two hours with subcutaneous ultra rapid insulin. After five days from hospitalization he showed scleral and skin jaundice were made diagnosis of hemolytic anemia by G6PD deficiency. This case wants to emphasize that the G6PD deficiency has been unmasked by hyperglycemia until now unknown without signs and symptoms (Silvestri *et al*., 2015).

Case study had done in UK: A 54 year old Kenyan man presented with a 3 day history of reduced appetite, weakness and reduced level of consciousness as a result of a hyperglycaemic diabetic crisis with both hyperosmolarity and ketoacidosis. The patient then developed haemolysis and a raised creatine kinase level. A diagnosis of G6PD deficiency and rhabdomyolysis was made (Galtrey and Pathansali, 2008).

Chapter III

3. Materials and Methods

3.1 Study Design:

This is Case-Control conducted study.

3.2 Study Area and Duration:

This study was conducted in Almoalem hospital in Khartoum state during the period from march to April 2017.

3.3 Study Population:

The population unit (study unit) whom were diagnosed with type 2 Diabetic patients was taken as cases and matched group of apparently healthy (non diabetic) as controls after their verbal consent, who attended to Almoalem Hospital were included.

3.4 Inclusion and Exclusion Criteria:

Cases included only fully diagnosed with type 2 D.M. patients. Control included only apparently healthy (non diabetic) individual, with aged 25-80 years and after their verbal consent acceptance. All non type 2 D.M. patients and other metabolic disease patients are excluded from case group. All individual with age less than 25 years or more than 80 years, pregnant women's and verbal consent refuse were excluded from control and case groups.

3.5 Sample Size and Sampling technique:

Sample Size: Included 200 (100 cases and 100 controls individuals) .

Sampling technique: Sampling frame was individuals with fully diagnosed with type 2 D.M patients as cases and only apparently healthy (non diabetic) individuals as control. Sampling technique selecting sampling units from sampling frame was convenient, from individuals who attended to Almoalem Hospital. Blood sample was collected from each patient and control using

5ml sterile syringe after disinfecting with 70% alcohol, it had divided in 2.5 ml in tri potasium EDTA container for direct CBC analysis (Sysmex KX-21) then stored at 2-4 C (with in five days from collection) for G6PD activity test (Mindary BS 480). EDTA blood centrifuged for 5 minutes at around 3000 rpm, suspended 20ul of centrifuged erythrocyte in 1 ml of distilled water and mix fully for 3 minutes, while the other 2.5 ml in fluoride oxalate container for glucose test.

3.6. Principles, Procedures and Normal values of tests :

3.6.1 G6PD assay (Mindary BS 480 instrument,UV Enzymatic Method):

Principle of G6PD assay : Nictotinamide adenine dinucleotide phosphate (NADP) is reduced by G6PD in the presence of G-6-P. The enzyme activity is determined by measurement of the rate of absorbance change at 340 nm due to reduction of NAPD.

Procedure of G6PD assay : Mindary BS 480 calculates the test result automatically and displays G6PD activity of the tested sample as u/L.The linearity of is up to 3000 u/l. If the activity become more than 3000u/l, the sample should be diluted by normal saline 1:1 and the result multiply by dilution factor.

Steps: Stored (2-4 C) EDTA blood centrifuged for 5 minutes at 3000 rpm, suspended 20ul of centrifuged erythrocyte in 1 ml of distilled water and mix fully for 3 minutes. Mindary calculates Then result automatically as u/L.

Normal values of G6PD assay (according to manufacturer)**:**S.I unit >1300 u/l.When result does not fall in range calculate : U/gm of Hb = S.I(u/l) / Hb (gm/l). Normal range >3.8 U/g (Errico *et al.*, 2009).

3.6.2 CBC analysis (Sysmex KX-21 instrumen**):**

Principle of CBC analysis (Whole blood mode)**:** This instrument analyzes

the following parameters using three detector blocks and two kinds of reagents: WBC, RBC and PLT are detected by DC detection method, then it counts Cells in 1mL of whole blood. HGB is measured by Non-Cyanide hemoglobin analysis method, then calculate gram of hemoglobin in 1 dL of whole blood.

HCT is RBC pulse height detection method, then calculate Ratio (%) of whole RBC volume in whole blood. MCV (fL) in whole blood, which is calculated by Hct/RBC. MCH: Mean hemoglobin volume (pg) per RBC, which is calculated by Hgb/RBC. MCHC (g/dL), which is calculated by Hgb/Hct.

Procedure of CBC analysis (Whole blood mode): The collected blood sample in the whole blood status in EDTA tube. The tube cap is opened and the sample is aspirated through the sample probe one after another. And Procedure Flow as: Inspection before Turning ON the Power, Turning ON the Power and Self-Check, Execution of Quality Control, Display and Printing of Analysis Result.

3.6.3. Glucose test :

Principle of Glucose test: Glucose oxidase (GOD) catalyses the oxidation of glucose to gluconic acid. The formed hydrogen peroxide (H2O2) is detected by achromogenic oxygen acceptor ,phenol,4-aminophenazone (4-AP) in the presence of peroxidase (POD):The intensity of the color formed is proportional to the glucose concentration in the sample.

Procedure of Glucose test:

Wavelength 505 nm (490-550) , I used 520 nm. The instrument adjusted to zero with distilled water. I pipetted in three test tubes as follow :

	Blank	Standard	Sample
R(ml)	1	1	1
Standard(ul)	-	10	-
Sample(ul)	-	-	10

I mixed and incubated for 20 min at room temperature (15-25C).I read the absorbance (A) of sample and standard, against the blank.The color is stable for at least 30 minutes.

Calculation :{(A)sample-(A)blank/(A)standard-(A)blank}*100 = mg/dl glucose in sample. Conversion factor : mg/dl*0.0555 = mmol/l

Normal values of glucose (according to manufacturer): Fasting glucose of Serum or plasma 60-110 mg/dl (3.33-6.10 mmol/l) .

3.7 Ethical Considerations:

Participants were informed verbally in private, in their simple language about the research, its benefits, confidentiality and method of sample collection.

3.8 Statistical analysis:

Data were collected using self administered questionnaires. The questionnaires was specifically designed to collect demographic data information's about age, sex, onset, type of medication, level of blood glucose test (fasting or random), CBC and G6PD activity. Data entered, checked and analyzed using Statistical Package for Social Sciences (SPSS) version 21, using T-test for testing difference significance and Pearson correlation test (*r value* as the coefficient), *P. value* ≤ 0.05 was considered statistically significant and frequency.

Chapter IV

4. Results:

4.1 Demographic characteristic of study participants:

This study Included 200 participants,100 cases (D.M. Type 2) and 100 controls (healthy individuals). Frequency of sex was 61/200 male (30.5%) and 139/200 female (69.5%), with mean of age was 54 ± 12 year, age grouped to three. Age 46-65 years 98/200 (49%) was the highest frequency, followed by 25-45 years 52/200 (26%) and 66-85 years 50/200 (25%), while highest frequency of disease duration 1-10 years 60/100 (60%), 11-20 years 29/100 (29%) and 21-30 years 11/100 (11%). Among Cases frequency of medication: injection was (57%) and Tabs was (%43). Among Cases frequency of subjects had glucose level with in Renal threshold was (60%) and more than Renal threshold was (40%). Among Cases frequency of subjects had glucose level with in normal range was (44%) and more than normal range was (54%). (Table 4.1).

4.2 Comparison means of G6PD level between cases and controls subjects: Showed no significant differences in means of G6PD activity (0.211). (Table 4.2).

4.3 Comparison means of CBC parameters between cases and controls subjects: Showed significant differences in means of PCV(0.039), MCV (0.000) and MCH (0.020) ,while no significant differences in means RBCs (0.507), Hb (0.218), WBCs (0.204), MCHC (0.539) and Plts (0.609). (Table 4.3).

4.4 Correlation between G6PD level and glucose level among study volunteers: Showed no significant correlation (0.474), with intermediate negative Pearson Correlation (-0.51). (Table 4.4).

4.5 Comparison means of G6PD level among cases according to therapy: Showed no significant differences in means of G6PD (0.364). (Table 4.7).

4.6 Comparison means of G6PD level among study volunteers according gender: Showed no significant differences in means of G6PD (0.234). (Table 4.7).

4.7 Comparison means of G6PD activity Between Groups according to age group among study volunteers: Showed no significant differences in means of G6PD activity (0.998). (Table 4.8).

Table 4.1: Demographic characteristic of study participants:

Variable	**Volunteers catogre**	**Case**	**Control**
Sex	Male (61/200) 30.5%	(33/100) 33%	(28/100) 28%
	Female (139/200) 69.5%	(67/100) 57%	(72/100)72%
Age grouping (Years)	25-45(52/200)	(11/100) 11%	(41/100) 41%
	46-65(98/200)	(58/100) 58%	(40/100) 40%
	66 < (50/200)	(31/100) 31%	(19/100) 19%
Medication	Injection	(57/100) 57%	
	Tabs	(43/100)43%	

Table 4.2:Comparison means of G6PD level between cases and controls subjects

G6PD	No	Mean	SD	P.*value*
Case	100	1182.067	709.0583	0.211
Control	100	1310.260	736.5769	

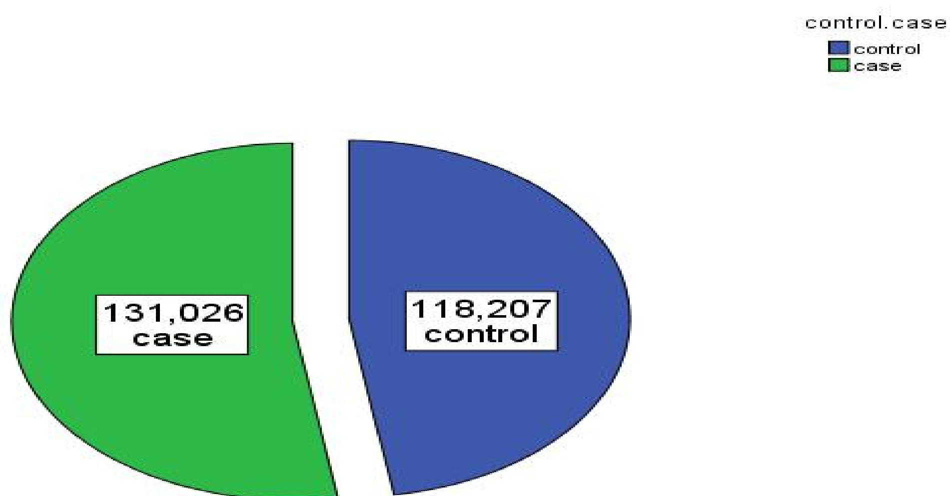
Table 4.3:Comparison means of CBC parameters between cases and controls subjects					
CBC parameter	Participants	No	Mean	SD	P.*value*
WBCs	Case	100	5.7680	3.17417	0.204
	Control	81	6.2827	1.96467	
RBCs	Case	100	4.6805	0.53784	0.507
	Control	100	4.7373	0.66352	
Hb	Case	100	13.0710	1.44804	0.218
	Control	100	12.7840	1.81246	
PCV	Case	100	41.007	4.3869	0.039
	Control	100	39.469	5.9575	
MCV	Case	100	87.698	5.4618	0.0000
	Control	100	84.741	5.6845	
MCH	Case	100	27.996	2.3395	0.020
	Control	100	27.141	2.8099	
MCHC	Case	100	31.850	1.5664	0.539
	Control	100	32.005	1.9840	
Platelets	Case	100	267.62	101.563	0.609
	Control	100	274.29	78.585	

Table 4.4: Correlation between G6PD level and glucose level among study volunteers.			
Participant		G6PD	Glucose
G6PD	Pearson Correlation	1	-0.51
	Sig. (2-tailed)	0.474	
	Number	200	200
Glucose	Pearson Correlation	-0.051	1
	Sig. (2-tailed)	0.474	
	Number	200	200

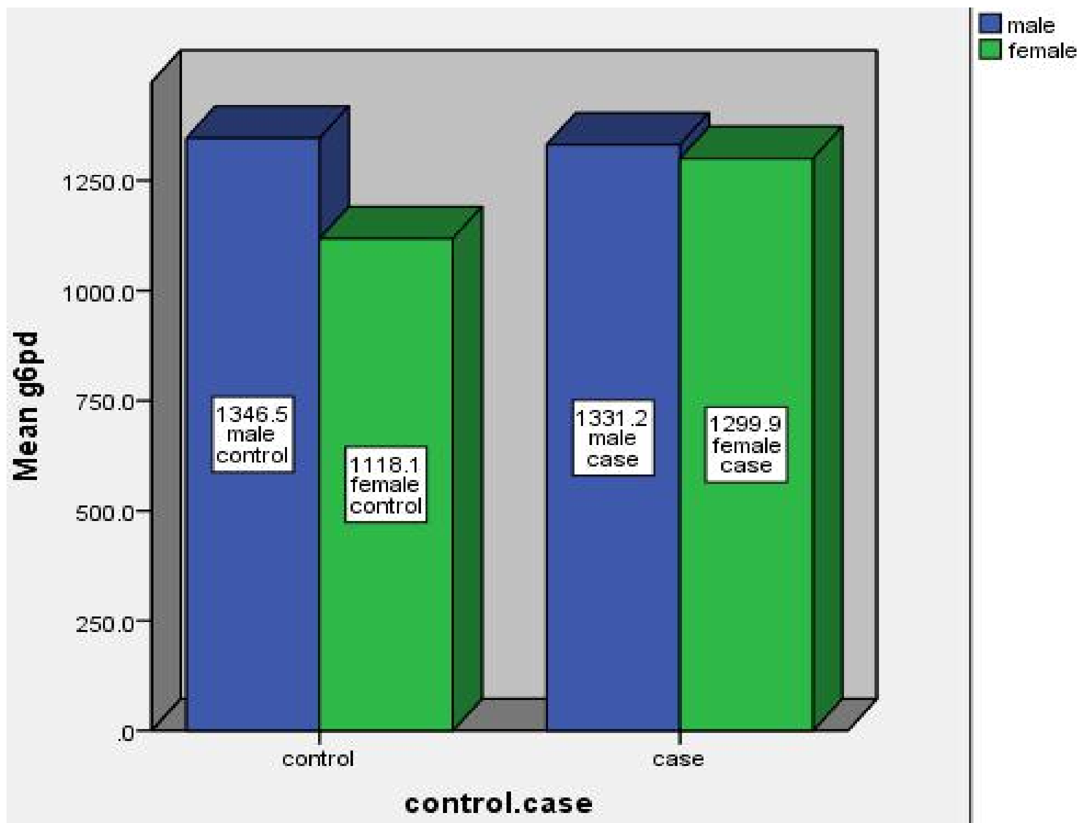
Table 4.5 Comparison means of G6PD level among cases according to therapy					
G6PD	Variable	Number	Mean	SD	P. value
Medication	Injection	43	1232.781	779.0910	0.364
	Tabs	57	1368.709	704.1444	

4.6 Comparison means of G6PD level among study volunteers according gender					
Sex	Female	139	1205.755	708.1806	0.234
	Male	61	1338.241	756.6751	

Table 4.7 Comparison means of G6PD activity Between Groups according to age group among study volunteers	
Age groups	P. value
25-45 years 46-65 years =<66 years	0.998



(Fig.1) mean of G6PD activity among study participants



(Fig.2) mean of G6PD activity among controls and cases according to sex

Chapter V

5.1 Discussion

This was Case Control study which conducted between March to April, 2017 in Khartoum state among DM Type 2 Sudanese patients, at the Almoalem Hospital to find out subsequent effect of hyperglycemia on G6PD activity and CBC, for tries to establish policies to be considered in the management of patients with Diabetes Mellitus Type2. It include 200 volunteered participants 100 Diabetic patients and 100 age and sex matched group of apparently healthy control.

The present study showed clear significant (p.*value*< 0.05) difference between control and case in comparison Means of CBC parameters, show significant increase in mean of PCV(*p. value* 0.039), MCV (p.*value* 0.000) and MCH (*p. value* 0.02),which approximately agree with studies of (Jaman*et al.*, 2018 and Biadgo *et al.*, 2016).

5.2. Conclusion

Hyperglycemia may causes inhibition of G6PD level but it dose not effect any of CBC parameters.

5.3. Recommendations

- Further studies should be done with more sample size to determine the cause of G6PD activity and CBC parameters among diabetic patients, with excluded G6PD gene mutations that disrupt the enzyme structural stability and thus reduce its overall activity.
- CBC analysis dose not need as follow up for diabetic patients to overcome complications due to oxidative stress.

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Appendixes

Parameter	Sex	N.R
RBCs count	Male	5 ± 0.5 million/cmm
	Female	4.3 ± 0.5million/cmm
Hb concentration	Male	15± 2 g/dl
	Female	13.5± 2 g/dl
PCV	Male	0.45 ±0.05L/L
	Female	0.41± 0.05 L/L
MCV	Male	92 ± 9 Fl
	Female	
MCH	Male	29.5± 2.5Pg
	Female	
MCHC	Male	33 ±1.5 g/dl
	Female	
WBCs count	Male	(4-10)*1000 cell/cmm
	Female	
Plts count	Male	280 (±130)*1000/cmm
	Female	

Normal *values* of CPC parameters :(Bain *et al* ., 2011).

BS-480

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Questioner

Sudan University of Science and Technology
College of Graduate Studies

Evaluation of Glucose-6-Phosphate Dehydrogenase level Enzyme and Complete Blood count among Sudanese Diabetes Mellitus Type2 Patients in Khartoum State.

تقييم مستوى الجلوكوز نازع الهيدروجين السداسي وعدد الدم الكامل وسط المرضى السودانيين المصابين بالسكري النوع الثاني في ولاية الخرطوم.

Name:……………………………………………………………………………

No( ).

Age:…………year. Gender :Male( ) Female( ).

Are you diabetic?Yes( )No( ). If yes, treatment ?Tabs( ) Inject ( ).

level of blood glucose ?……...mg/dl. Activity of G6PD enzyme?..……..u/l.

CBC		
Variables	Result	Unit
RBCs		Cell*10^6/cmm
Hb		G/dl
PCV		%
MCV		fl
MCH		pg
MCHC		G/dl
WBCs		Cell*10^3/cmm
Plts		Cell*10^4/cmm
G6PD		U/l