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Abbreviations

3-DG	3-deoxyglucosones.
8-OHdG	8-hydroxy-2-deoxyguanosine
8-OH-G	8-hydroxyguanine
ADA	American-Diabetes-Association
AGE	Advanced Glycation End product
ANDRS	Development of Health Research
AR	Aldose Reductase
C(60)HyFn	C(60) Fullerene
CASA	Computer Assisted Semen Analysis
CD-Vit E	Vit E encapsulated in CycloDextrin
CML	CarboxyMethyLlysine
CNS	Central Nervous System
CO_2	Carbon dioxide
DAG	Diacylglycerol
DM	Diabetes mellitus
EDTA	EthyleneDiamineTetracetic Acid
ELISA	Enzyme-Linked Immunoassay
F-6-P	Fructose-6-Phosphat
$Fe^{3+}O^{2-}$	Superoxo-Ferrihaem
FPG	Fasting Plasma Glucose
FSH	Follicle-Stimulating Hormone
G-3-P	Glyceraldehydes-3-Phosphate
G-6-P	Glucose-6-Phosphat
GAP	GTPase Activating Protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC-MS	Gas Chromatography-Mass Spectrometry
GLUTs	Glucose Transporters
GPx	Glutathione Peroxidase
GSH	Glutathione
H_2O_2	Hydrogen Peroxide
Hb	Hemoglobin
HbA_{1c}	Glycated Hemoglobin
HbA_{1c} H	High HbA_{1c} values
HbA_{1c} L	Low HbA_{1c} values
HbA_{1c} M	Moderate HbA_{1c} values
IDDM	Insulin-Dependent Diabetes Mellitus
IGF-I	Insulin-like Growth Factor-I
LDL	Low-Density Lipoproteins
LH	Luteinizing Hormone
low-HDL	Low levels of high-density lipoprotein
MCV	Mean Cell Volume
MDA	Malondialdehyde

metHb	Methemoglobin
MP	Moderate Progressivity
NAD	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric Oxide
O_2	Oxygen
O^{2-}	Superoxide anion radical
OGTT	Oral Glucose Tolerance Tests
OS	Oxidative Stress
PKC	Protein kinase C
PS	PhosphatidylSerine
PUFA	Polyunsaturated Fatty Acids
RAGE	Receptors for AGEs
RBCs	Red Blood Cells
RCS	Reactive chlorine species
RNS	Reactive Nitrogen species
ROS	Reactive Oxygen Species
RP	Rapid Progressivity
SCs	Sertoli Cells
SDH	Sorbitol Dehydrogenase
SGLTs	Sodium-coupled Glucose Transporters
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TBA	Thiobarbituric Acid
TBARS	Thiobarbituric Acid-reactive Substances
TCA	Trichloroacetic Acid
TG	TriGlyceride
UDP-GlcNAc	Uridine Diphosphate-N-AcetylGlucosAmine
VAP	Average Path Velocity
VCL	CurviLinear Velocity
Vit C	Vitamin C
Vit E	Vitamin E
VSL	Straight-Line Velocity
WHO	World Health Organization

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General Introduction

General introduction

Diabetes mellitus (DM) is emerging as a major global health problem with the number of people living with it expected to rise to 366 million by 2030 (Khalil, 2017). This metabolic disorder is characterized by hyperglycemia and develops by the involvement of several pathogenic processes (American-Diabetes-Association, 2009). Consequently, the carbohydrate metabolism abnormalities seen in diabetes are developed following to insulin deficiency or resistance to its action (Kaul *et al.*, 2013). Moreover, hyperglycemic patients have an increase incidence of endovascular alteration that is associated with blood cells destruction (Coleman, 2000).

Red cells are the most blood cells that are exposed to a huge carbonyl stress during diabetes (Coleman, 2000). This is mainly due to the high level of glucose consumption in red blood cells (RBCs) (Coleman, 2000), which is mostly ensured by the passive transport from the blood plasma through insulin-independent glucose transporter and GLUT1 (Gerich, 2010). They are continuously exposed to glucose in plasma during their circulatory life span of 120 days (Jeffcoate, 2004). Therefore, human erythrocytes are subjected to several abnormalities in their morphology, metabolism, and function under hyperglycemic conditions (Wang *et al.*, 2021). The glucose metabolism alteration inside cells increases enzyme glycation, lipid peroxidation, and as a consequence increases hemolysis (Jain *et al.*, 1989). Interestingly, DM-mediated RBCs disorders may alter other peripheral cells such as spermatozoa and they lead to premature organ and systems failure, namely reproductive system dysfunction (Ding *et al.*, 2015).

Mature sperm cells metabolize several substrates to ensure the energy supplies required for their motility and fertilization (Bucci *et al.*, 2011). However, DM is recognized for its ability to hinder the uptake and metabolism of glucose by sperm cells, primarily through changes in their glycolytic activity (Dias *et al.*, 2014). Clinically, this is evidenced by male sexual dysfunction. Significantly, research findings suggest that around 50% of male diabetic individuals exhibit subfertility and/or infertility (Dias *et al.*, 2014). Nevertheless, major studies have related the male reproductive dysfunction in diabetes to the disruption of endocrine control of spermatogenesis, spermatogenesis itself, or by impairing penile erection and ejaculation (Sexton *et al.*, 1997).

Regardless of the types of diabetes, the molecular mechanism responsible for these damages was initially an open question. Numerous investigations have highlighted pivotal

metabolic pathways that substantially contribute to cellular injury induced by hyperglycemia : 1) glucose autoxidation; 2) increased polyol pathway flux; 3) over-formation of advanced glycation end products (AGEs); 4) activation of protein kinase C (PKC); and 5) increased hexosamine pathway flux (Ighodaro, 2018). In addition, oxidative stress (OS) emerges as a significant pathogenic element in diabetic complications. Indeed, reactive oxygen species (ROS) are generated via glucotoxicity pathways and as a consequence of mitochondrial impairment (Baynes *et al.*, 1999). Therefore, these free radicals can concurrently assault lipids, proteins, and nucleic acids within living cells (Yang *et al.*, 2011). Consequently, This process is exacerbated by the inhibition of the antioxidant system, intensifying OS, promotes the progression of diabetes, and exacerbates complications development (Lipinski, 2001).

This deficiency in cellular defense systems has received recently the consideration of many researchers. In addition, the antioxidant supplementation has been suggested to have the potential to improve these systems (Maritim *et al.*, 2003). Some antioxidants, namely vitamin E (Vit E) and vitamin C (Vit C) have the potential to reduce hyperglycemic disorders (Goh *et al.*, 2008). It's suggested that initiating antioxidant supplementation at the time of diagnosis could be more effective and advantageous. However, the monitoring method as well as the optimal combination and quantity of antioxidants are still under debate (Coleman *et al.*, 2001). Particularly, there are no sufficient data exploring the protective potential of combined Vit C and cyclodextrin-loaded Vit E (CD-Vit E) against OS triggered by diabetic plasma on human sperm cells.

The objective of this thesis is to determine the molecular mechanism responsible for RBCs alteration and mature sperm cells-damages mediated by diabetes. Also, it's conducted to examine the protective potential effect of the combination of Vit C and CD-Vit E against the alterations that occurred in poor glycemic control. Therefore, the first chapter of this thesis was carried out to summarize the literature review of diabetes and its pathogenic processes and complications, namely vascular and male reproductive impairment. The presentation of different strategies and future therapies such as antioxidant supplementation intending to minimize the risks of diabetic complications are also developed. In the second chapter, we assessed the effect of diabetic plasma at varying levels of glycated hemoglobin (HbA_{1c}) on RBCs to elucidate the mechanisms amplifying OS, particularly in relation to methemoglobin (metHb) production. In the third chapter, we investigated the direct impact of human diabetic plasma on mature spermatozoa by analyzing gametes motility and OS status in relation to HbA_{1c}. Finally, the fourth chapter was devoted to monitoring the protective potential of Vit C

and CD-Vit E, individually and in combination, against damages that occurred on mature spermatozoa in uncontrolled hyperglycemia conditions.

Chapter I:
Literature Review

CHAPTER I: Literature Review

I. Diabetes

I.1. Generalities

DM is defined as a metabolic disorder characterized by hyperglycemia. The deficiency of insulin action or its resistance results in altered metabolism of carbohydrates, fats, and proteins in diabetes. These interestingly range from autoimmune destruction of the β-cells of the pancreas (American-Diabetes-Association, 2010).

I.2. Glucose metabolism

In physiological conditions, The regulation of blood glucose levels involves a negative feedback loop mediated by the release of insulin and glucagon (Bansal *et al.*, 2008). Insulin, a 51-amino acid polypeptide comprising two chains (A and B) connected by disulfide bridges, is secreted in response to elevated blood glucose levels. This release is carried out by the β-cells located in the islet of Langerhans in the pancreas (Kaul *et al.*, 2013). Insulin binds to the tyrosine kinase insulin receptor, consisting of two extracellular subunits and two intramembrane subunits linked by disulfide bonds (Bansal *et al.*, 2008). This binding event, particularly to the B subunit of the receptor, stimulates autophosphorylation of the B subunit (Kaul *et al.*, 2013). Thus, the excess glucose in the blood was converted to glycogen for storage. Additionally, insulin prompts other cells in the body, such as adipose and skeletal muscle cells, to increase glucose uptake by facilitating the translocation of glucose transporter (GLUT4) to the cell surface (Chadt *et al.*, 2020). This mechanism helps to bring the circulating glucose concentrations to normal levels. Conversely, a decrease in blood glucose concentration stimulates the α-cells in the pancreas to release glucagon (Chadt *et al.*, 2020). This later signals the liver to convert stored glycogen into glucose which is then released into the bloodstream to restore homeostasis (Kaul *et al.*, 2013). In diabetes, abnormalities may manifest in insulin synthesis or secretion, pancreatic duct stenosis, or the emergence of insulin resistance, resulting in either decreased insulin production or impaired insulin function (Kaul *et al.*, 2013)

I.3. Classification

The clinical presentation observed at the time of diagnosis determines the classification of diabetes. Typically, individuals are categorized according to: 1) the age at the onset of diabetes; 2) the abruptness of hyperglycemia; 3) speed of hyperglycemia development; 3) ketosis presence; 4) obesity level; and 5) insulin requirement upon diagnosis (Alam *et al.*, 2014).

I.3.1. Type 1 Diabetes Mellitus

T1DM presents generally an aberration either in insulin synthesis or secretion, which can be caused by the autoimmune destruction of the β-cells of the pancreas (Knip *et al.*, 2017). Insulin-Dependent Diabetes Mellitus (IDDM) is developed by the activation of $CD4^+$, $CD8^+$ T cells, and macrophages that infiltrate the pancreatic islets (Knip *et al.*, 2017). However, TlDM only ensues when 90% of β-cells are lost. Approximately, 10% f diabetic patients are affected by Type 1 Diabetes Mellitus (T1DM), typically manifesting in childhood or early adulthood (<35 years) (Kaul *et al.*, 2013). Both genetic and environmental factors play significant roles in predisposing individuals to this form of diabetes (Kaul *et al.*, 2013).

I.3.2. Type 2 Diabetes Mellitus

T2DM results through the development of tissue resistance to insulin at one or more points in the complex pathways of hormone action (Kaul *et al.*, 2013). This is the most prevalent form of diabetes, comprising 90–95% of cases (American-Diabetes-Association, 2010). It is characterized by a relative insulin deficiency stemming from insulin resistance as the primary defect (Alam *et al.*, 2014). The mean time from onset to diagnosis of T2DM is 4–7 years (Harris et al., 1992). The obese T2DM patients commonly develop resistance to endogenous insulin due to changes in cell receptors, notably associated with abdominal fat distribution (Castro *et al.*, 2014). In non-obese T2DM patients, there is often insulin resistance at post-receptor levels, coupled with deficiencies in insulin production and release (Kaul *et al.*, 2013). Patients with T2DM may present some neurologic symptoms and signs as decreased level of consciousness (Alam *et al.*, 2014). Additionally, the blood glucose level exceeding 33 mmol/L (600 mg/dL) and a plasma osmolarity greater than 320 mOsm/L. Polyuria, polydipsia, thirst, fatigue, and generalized weakness are common symptoms in T2DM (Alam *et al.*, 2014).

I.3.3. Other secondary types of diabetes

Gestational diabetes is considered as another entity of T2DM (Zhu *et al.*, 2016). Additionally, other secondary types of diabetes can be induced by conditions like pancreatitis, Cushing's syndrome, Klinefelter's syndrome, and hyperthyroidism. Certain medications and chemicals such as thiazide diuretics, 3-blockers, calcineurin inhibitors, protease inhibitors, and atypical antipsychotic drugs can also trigger secondary diabetes (Kaul *et al.*, 2013).

Table 1 : Types of Diabetes (Who, 2019).

Type of diabetes	Brief description	Change from previous classification
Type 1 diabetes	β-cell destruction (mostly immune-mediated) and absolute insulin deficiency; onset most common in childhood and early adulthood	Type 1 sub-classes removed
Type 2 diabetes	Most common type, various degrees of β-cell dysfunction and insulin resistance; commonly associated with overweight and obesity	Type 2 sub-classes removed
Hybrid forms of diabetes		New type of diabetes
Slowly evolving, immune-mediated diabetes of adult	Similar to slowly evolving type 1 in adults but more often has features of the metabolic syndrome, a single GAD auto-antibody and retains greater β-cell function	Nomenclature changed – previously referred to as latent autoimmune diabetes of adults (LADA)
Ketosis-prone type 2 diabetes	Presents with ketosis and insulin deficiency but later does not require insulin; common episodes of ketosis, not immune-mediated	No change
Other specific types		
Monogenic diabetes - Monogenic defects of β-cell function - Monogenic defects in insulin action	Caused by specific gene mutations, has several clinical manifestations requiring different treatment, some occurring in the neonatal period, others by early adulthood Caused by specific gene mutations; has features of severe insulin resistance without obesity; diabetes develops when β-cells do not compensate for insulin resistance	Updated nomenclature for specific genetic defects
Diseases of the exocrine pancreas	Various conditions that affect the pancreas can result in hyperglycaemia (trauma, tumor, inflammation, etc.)	No change
Endocrine disorders	Occurs in diseases with excess secretion of hormones that are insulin antagonists	No change
Drug- or chemical-induced	Some medicines and chemicals impair insulin secretion or action, some can destroy β-cells	No change
Infection-related diabetes	Some viruses have been associated with direct β-cell destruction	No change
Uncommon specific forms of immune-mediated diabetes	Associated with rare immune-mediated diseases	No change
Other genetic syndromes sometimes associated with diabetes	Many genetic disorders and chromosomal abnormalities increase the risk of diabetes	No change
Unclassified diabetes	Used to describe diabetes that does not clearly fit into other categories. This category should be used temporarily when there is not a clear diagnostic category especially close to the time of diagnosis	New types of diabetes
Hyperglycaemia first detected during pregnancy		
DM in pregnancy	Type 1 or type 2 diabetes first diagnosed during pregnancy	No change
Gestational DM	Hyperglycaemia below diagnostic thresholds for diabetes in pregnancy	Defined by 2013 diagnostic criteria
Diagnostic criteria for diabetes: fasting plasma glucose ≥ 7.0 mmol/L or 2-hour post-load plasma glucose ≥ 11.1 mmol/L or Hba1c ≥ 48 mmol/mol. **Diagnostic criteria for gestational diabetes**: fasting plasma glucose 5.1–6.9 mmol/L or 1-hour post-load plasma glucose ≥ 10.0 mmol/L or 2-hour post-load plasma glucose 8.5–11.0 mmol/L		

I.4. Diagnosis

The diagnosis of DM is easily recognized when a patient presents the classic symptoms of hyperglycemia and has a random blood glucose value of 200 mg/dL (11.1 mmol/L) or higher (American-Diabetes-Association, 2010). Interestingly, the family history of disease is also an autosomal dominant condition. This must be confirmed by the basic diagnosis, as evidenced by fasting plasma glucose (FPG ≤126 mg/dl; 7.0 mmol/l) and an oral glucose tolerance test (OGTT), which measures blood glucose levels after fasting for at least 8 hours and 2 hours after drinking a glucose-containing beverage. The installation of a diabetes will be validated if its value ≤ 2-h post-load glucose 200 mg/dl (11.1 mmol/l) (Report of a WHO Consultation, 1999). These two tests are associated with metabolic syndrome, which includes obesity (especially abdominal or visceral obesity), hyper dyslipidemia (TG ≥ 150 mg/dL; low-HDL <40 mg/dL), and hypertension (≥systolic_130 or diastolic ≥85 mmHg) (American-Diabetes-Association, 2005). The American Diabetes Association (ADA) now considers HbA_{1c} not only as a measure of hyperglycemia control and intervention efficacy, but also as a diagnostic test for diabetes when the value exceeds 48 mmol/mol (6.5%) (American-Diabetes-Association, 2010).

I.5. Complications

Hyperglycemia plays a crucial role in the development of long-term complications in diabetic patients. Poor glucose control is particularly detrimental (American-Diabetes-Association, 2014). Furthermore, complications appear to affect organs where cells do not require insulin for glucose uptakes, such as nervous system, heart, kidneys, large (macrovascular) and small (microvascular) vessels (Figure 1) (Kaul *et al.*, 2013). Patients with diabetes have an increased incidence of endovascular alteration associated with blood cells destruction affecting the eyes and blood vessels (Brownlee *et al.*, 1984).

I.5.1. Macrovascular complications

Macrovascular complications refer to damage in the circulatory system's large blood vessels (Figure 1). Individuals with this condition face a 2 to 4- fold increase in susceptibility to stroke (cerebrovascular), coronary, heart disease, and peripheral vascular disease. These complications may culminate in gangrene, ulceration, and necessitate lower extremity amputations (Kaul *et al.*, 2013).

I.5.2. Microvascular complications

Microvascular complications entail injury to the small blood vessels (Figure 1) (Cade, 2008). This is caused by several factors, including erythrocytes alterations (Korol *et al.*, 2013). Hyperglycemia increases the glycation phenomenon and enhances OS in RBCs (Grossin *et al.*, 2009). Consequently, modifications are observed in macromolecules and metabolites inside RBCs (Grossin *et al.*, 2009). It's caused a change in the cell's hemorheological characteristics, lipid membrane peroxidation, reduced deformability, and increased cell aggregation (Babu *et al.*, 2004). These can reduce RBCs' lifespan and impair O_2 delivery to the tissues (Cho *et al.*, 2008). Furthermore, erythrocyte alterations have been linked to microvessel integrity damage and an increased accumulation of erythrocytes in the microvessel, which is considered the first step in vascular complications in diabetes (Wang *et al.*, 2021). These complications include retinopathy (potential loss of vision), nephropathy (leading to renal failure), peripheral neuropathy (with a risk of foot ulcers, amputations, and Charcot's joints), and autonomic neuropathy (causing gastrointestinal, genitourinary, and cardiovascular symptoms)(American-Diabetes-Association, 2005).

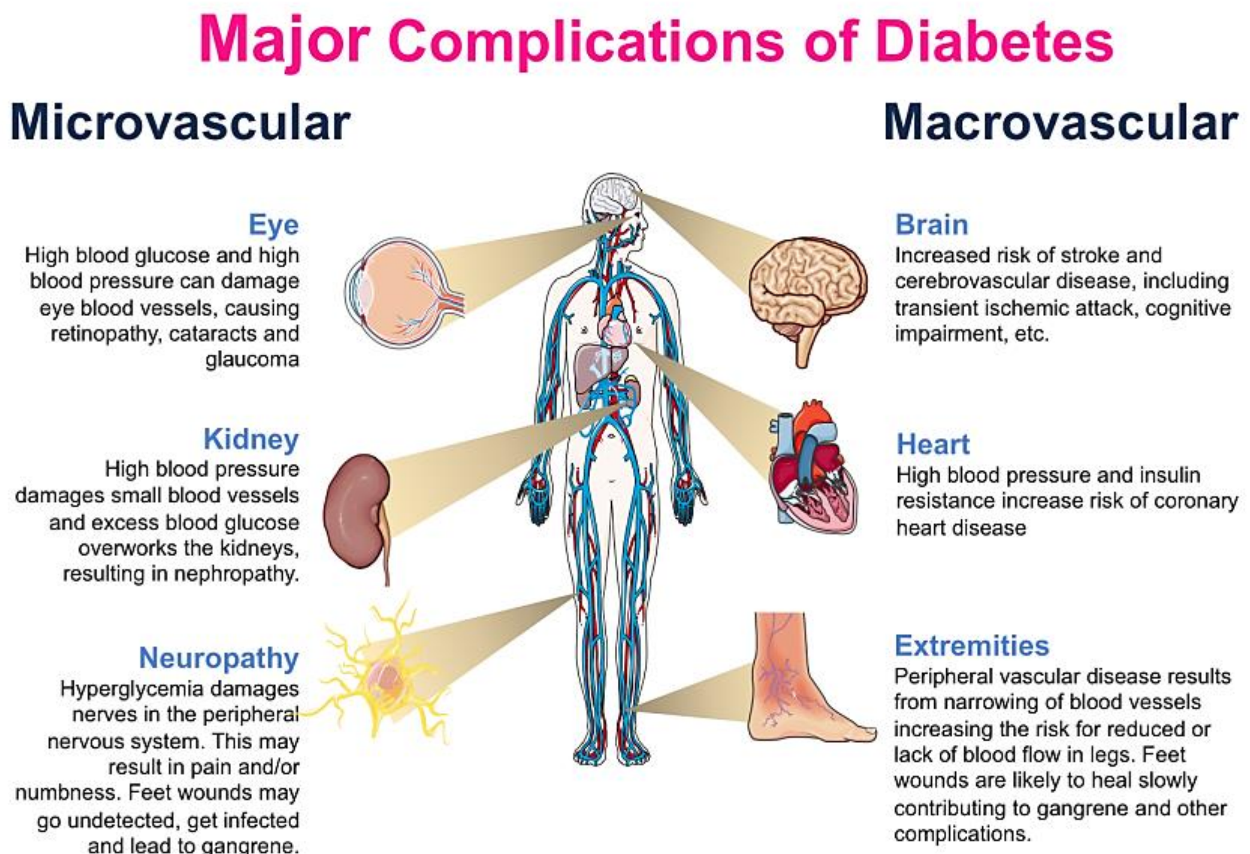


Figure 1: Diabetes complications.

I.6. Pathogenesis of diabetic complications

Diabetic complications have multiple factors contributing to their development, but they are particularly linked to the duration of high blood sugar levels. Additionally, they are inversely related to the level of glycemic control achieved in the previous years (Goh *et al.*, 2008). Hyperglycemia accelerates the pathological effects of the biochemical process of advanced glycation and increased OS in chronic hyperglycemia (Wolff *et al.*, 1991).

I.6.1. Glucotoxicity

The evidence of glucose toxicity is based on the ability of glucose to glycate proteins and the gradual build-up of AGEs, in parallel to the ability of glucose to oxidize (Wolff *et al.*, 1991). These AGEs form on intra- and extracellular proteins, lipids, nucleic acids, and possess complex structures (Brownlee, 1992). Thereafter, there is considerable interest in receptors for AGEs (RAGE) that are present in many cell types, particularly those affected by diabetes. The interaction of AGEs results in altered intracellular signaling, gene expression, and the release of pro-inflammatory molecules and free radicals. These changes contribute to the development of diabetic complications (Brownlee, 1992). Moreover, these may be accelerated and be reinforced by the involvement of the five major pathways of glucose toxicity described below (Figure 2).

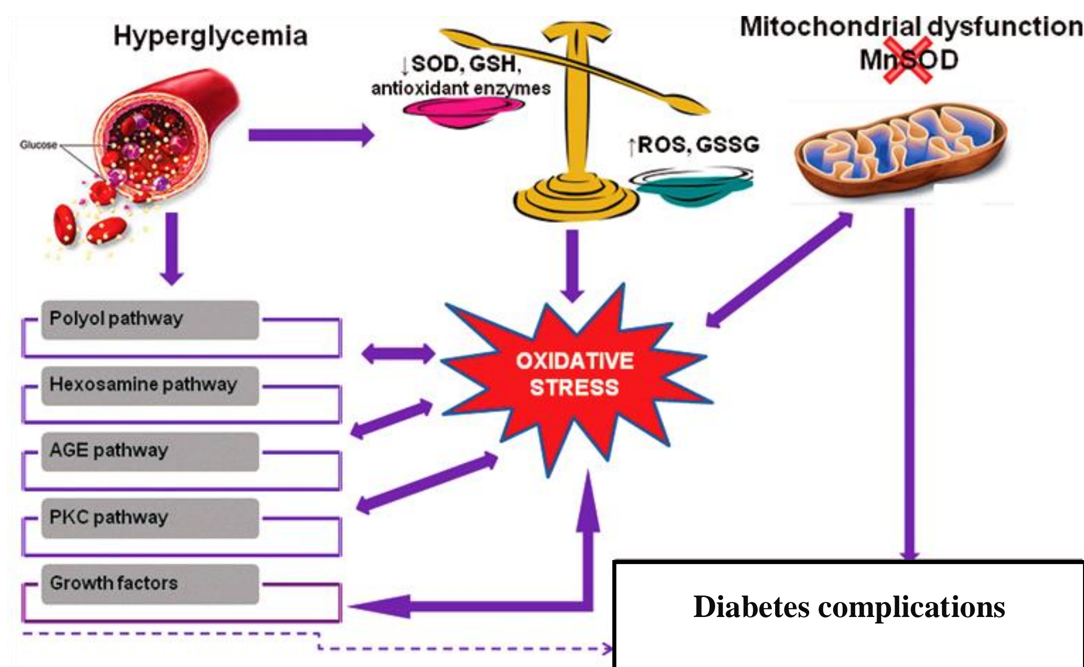


Figure 2: Major pathways implicated in the development of diabetes-mediated oxidative stress *(Cilensek* et al.*, 2016).*

I.6.1.1. Glycoxidation or autoxidation of glucose

It's known that glycolysis is the initial process by which glucose oxidized in body cells (Figure 3). Briefly, it is a ten-step pathway catalyzed by enzymes, and it is as the foremost among several other metabolic pathways, including the Krebs cycle and the electron transport chain. All these pathways are collectively involved in generation ATP (energy) from glucose (Hinzpeter, 2018). However, there is an overproduction of the superoxide anion radical (O^{-2}), which disrupts the body's antioxidant defenses. This, in turn, results in the damage of critical cellular biomolecules like DNA (Makino *et al.*, 2010; Robertson, 2004; Rolo *et al.*, 2006; Styskal *et al.*, 2012) that leads to the downregulation of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Du *et al.*, 2003; Easterday *et al.*, 2007; Giacco *et al.*, 2010). This results in the increased levels of some glycolytic intermediates such as GAP, Fructose-6-Phosphat (F-6-P), and Glucose-6-Phosphat (G-6-P) as well as glucose (Figure 3). The buildup of these molecules within cells activates other pathways that promote OS and causes autooxidation of glucose. Therefore, these molecular alterations increase in the generation of hydrogen peroxide (H_2O_2), which further exacerbates OS (Chung *et al.*, 2003; Rolo *et al.*, 2006). This typically leads to glyoxal formation and serving as a precursor for AGEs (Cho *et al.*, 2007).

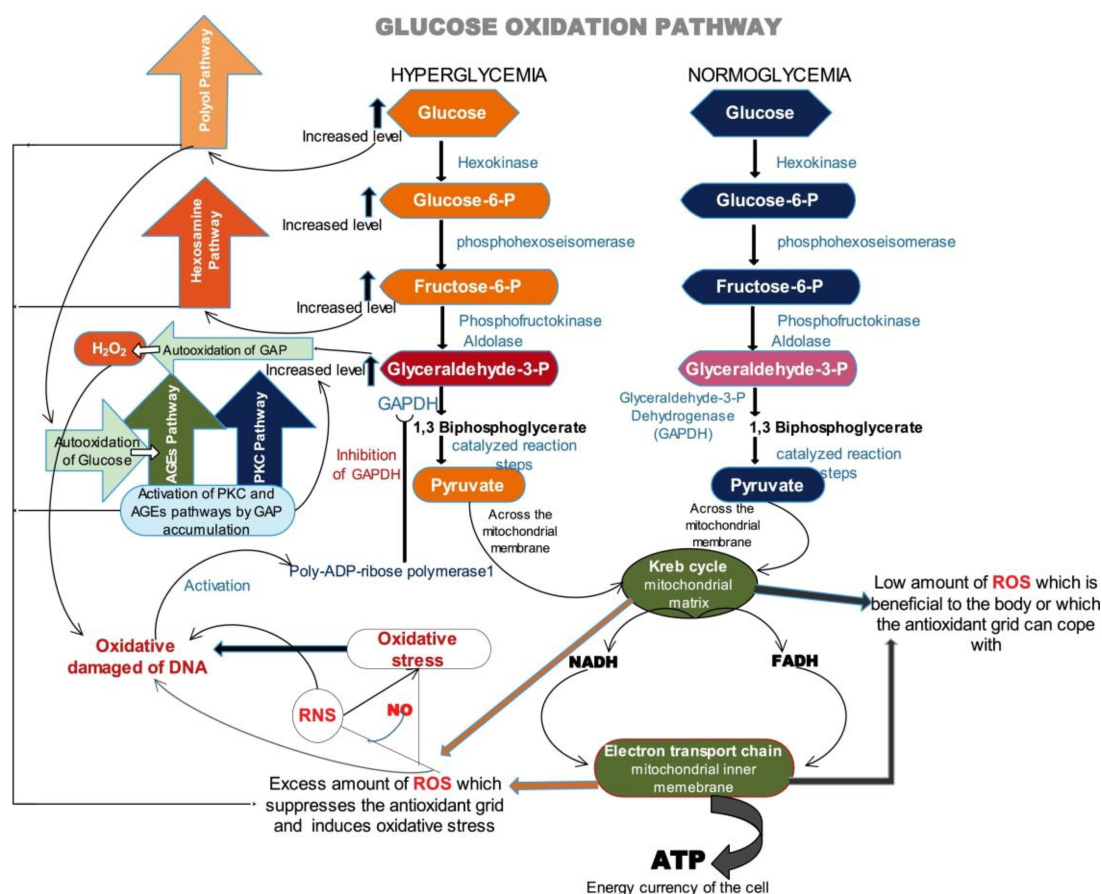


Figure 3: Glucose oxidation and induced-oxidative stress in hyperglycemic conditions *(Ighodaro* et al.*, 2018)*

I.6.1.2. Polyol pathway

The polyol pathway is a less prominent metabolic pathway for glucose, predominantly involving the family of aldo-keto reductase enzymes (Chung *et al.*, 2003). Briefly, glucose is reduced to sorbitol by the enzyme aldose reductase (AR) using reduced Nicotinamide adenine dinucleotide phosphate (NADPH). Then, sorbitol is oxidized to fructose by the enzyme sorbitol dehydrogenase (SDH), with Nicotinamide adenine dinucleotide (NAD) as a cofactor (Hwang *et al.*, 2003). In hyperglycemia, the increased activity of AD in the polyol pathway results in a disproportionate depletion of NADPH reserves, culminating in reduced intracellular levels of glutathione (GSH) and glutathione peroxidase (GPx). Consequently, this sequence suppresses antioxidant defenses, and amplifying the susceptibility of biomolecules to oxidative damage mediated by OS (Giacco *et al.*, 2010; Halliwell, 2007). In parallel, the activity of SDH is increased, leading to the formation of a large amount of fructose (Figueroa-Romero *et al.*, 2008), which is easily phosphorylated and hydrolyzed into G-3-P and dihydroxyacetone-3-phosphate. These triose phosphates accumulate and increase NADH/ NAD^+ ratio (Carracedo *et al.*, 2013). The enhanced formation of AGE precursors occurs due to the bioaccumulation of triose phosphates through auto-oxidation, while activation of the PKC pathway is initiated by the de novo synthesis of diacylglycerol (DAG) (Figure 4). This phenomenon collectively creates an oxidative environment within the cell (Ighodaro, 2018).

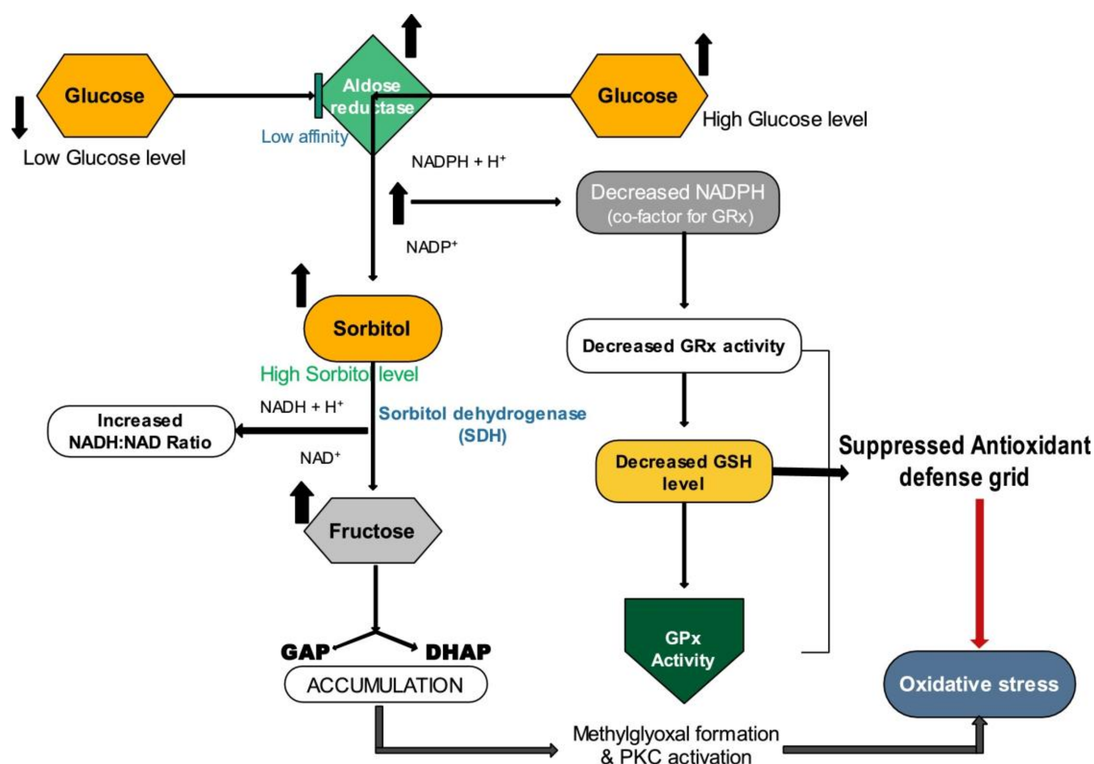


Figure 4: Hyperglycemia-induced hyperactivity of the Polyol pathway *(Ighodaro* et al.*, 2018)*

I.6.1.3. Hexosamine pathway

The hexosamine pathway is involved in the process of glycolysis that metabolizes F-6-P to glucosamine 6-phosphate (Figure 5), an interim compound, undergoes subsequent conversion to Uridine diphosphate-NAcetylglucosamine (UDP-GlcNAc) through the catalytic action of UDP-NAcetylglucosamine synthase (Inoguchi *et al.*, 2000). However, in hyperglycemic conditions, increased amounts of F-6-P are diverted into the hexosamine pathway, leading to elevated levels of UDP-GlcNAc and upregulated activity of O-Glucosamine-N-Acetyl transferase. This hyperactivity has been linked to changes in gene expression and elevated expression of transcription factors which is associated with complications such as nephropathy in diabetes (Edwards *et al.*, 2008; Fernández-Mejía, 2013; Figueroa-Romero *et al.*, 2008).

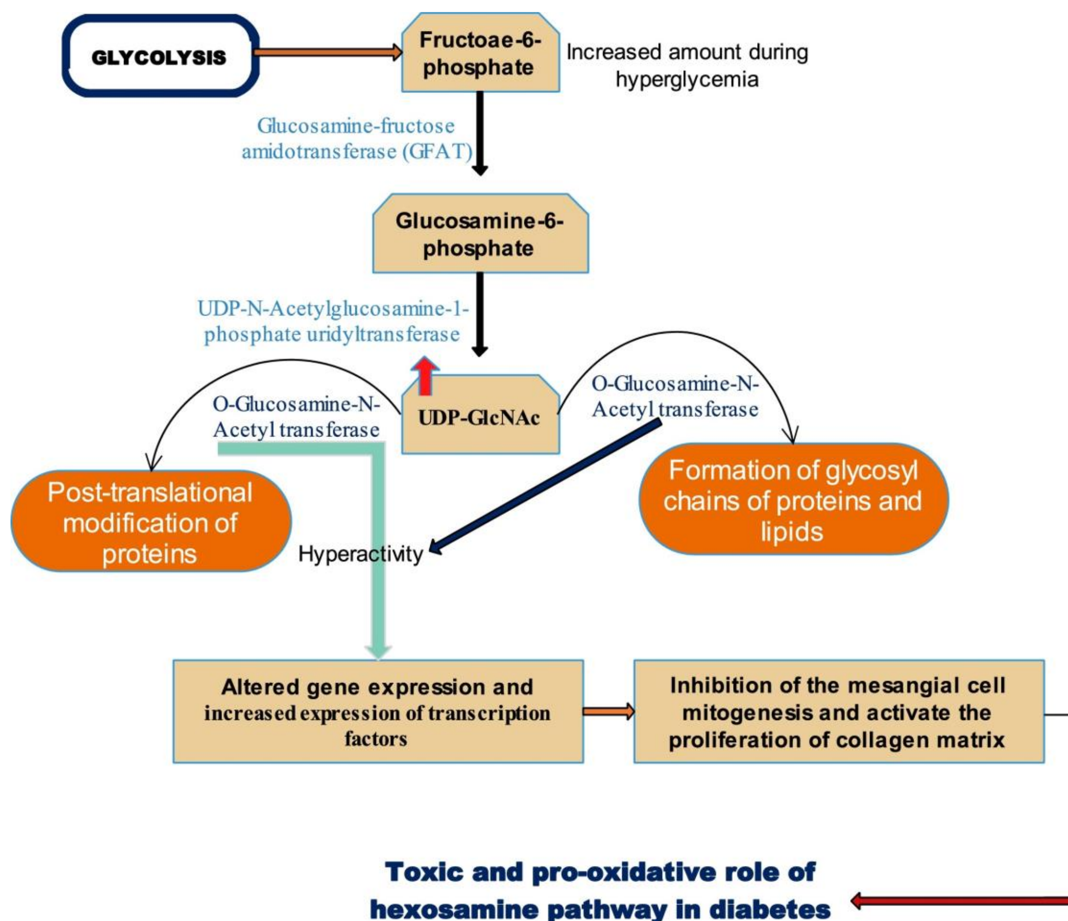


Figure 5: Hyperglycemia-induced hyperactivity of the Hexosamine pathway *(Ighodaro* et al.*, 2018)*

I.6.1.4. Activation of protein kinase C (PKC)

PKC is a family of proteins that modulates the activities of other proteins through the process of phosphorylation in a cascade of reactions involving DAG, phosphatidyl serine (PS), and calcium (Nishizuka, 1995). In diabetes, the accumulation of G-3-P, due to the inhibition of G-3-P dehydrogenase, leads to an elevated level of dihydroxyacetone-3-Phosphate (DHA-3-P), which is subsequently reduced to glycerol-3-Phosphate (Figure 6). This leads to an increase in the de novo synthesis of DAD that upregulates the PKC pathway/isoforms. The PKC pathway is also stimulated by the interaction of AGEs with their extracellular receptors RAGE, resulting in increased cellular levels of DAG. The elevated activity of the PKC pathway stimulates ROS-generating enzymes such as NADPH-oxidases and lipooxygenses, leading to an exacerbation of the cellular oxidative environment (Inoguchi *et al.*, 2000).

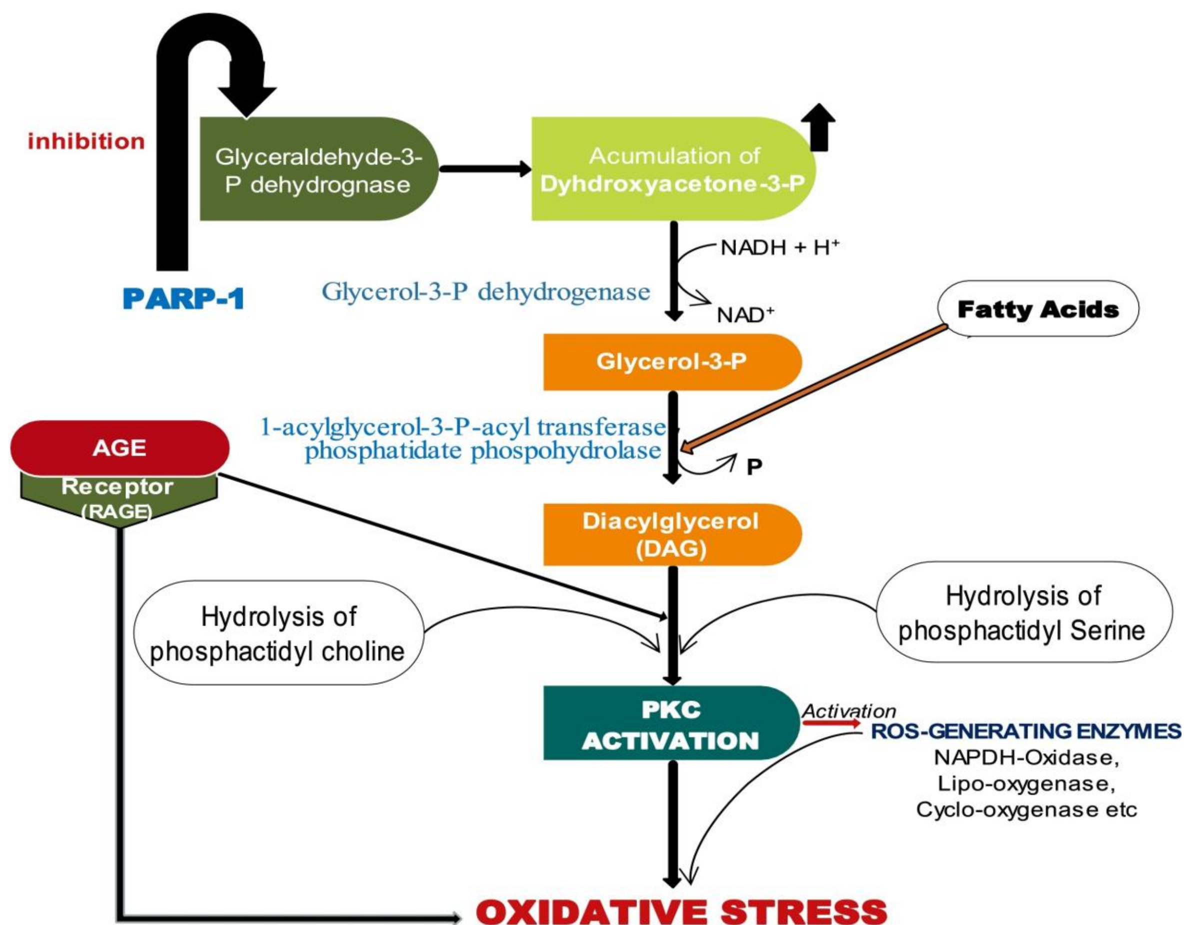


Figure 6: Protein Kinase C Activation and induced –Oxidative stress in hyperglycemia *(Ighodaro* et al., *2018)*

I.6.1.5. Advanced glycation end products formation pathway

AGEs are a complex group of compounds that are formed when reducing sugars react with proteins, lipids, or nucleic acids. AGEs are formed through a process called glycoxidation, which involves a series of chemical reactions (Ahmed, 2005). First, a freely reversible Schiff base is formed via nonenzymatic glycosylation of amine residues on proteins with reduced sugars. The labile Schiff base rearranges irreversibly to a more stable ketoamine or Amadori product, the best known is HbA_{1c} (Chandalia *et al.*, 2002). Furthermore, glycated proteins can undergo further reactions, involving dicarbonyl intermediates, such as 3-deoxyglucosones (3-DG). These secondary compounds can react with proteins to form cross-links, as well as chromo/fluorophoric adducts called Maillard products or AGE (Figure 7) (Wolff *et al.*, 1991)

Three key factors increase the formation of AGEs including the rate of turnover of proteins for glycoxidation, the degree of hyperglycemia, and the extent of oxidant stress in the environment (Goldin *et al.*, 2006). However, the level of AGEs does not decline when hyperglycemia is corrected. Instead, these products continue to accumulate at varying rates over the lifetime of the diabetic tissues and organs component (Brownlee, 1992; Hogan *et al.*, 1992). The presence and the accumulation of AGEs in many different cells affect extracellular and intracellular structure, signaling pathways as well as modifying the function of intracellular proteins via engagement of RAGE (Goldin *et al.*, 2006). As result, they increase inflammation, promote the formation of atherosclerotic plaques, and enhance the fragmentation and the oxidation of nucleic acids and lipids (Ahmed, 2005; Goh *et al.*, 2008). The persistence of accumulated AGEs during periods of normal glucose homeostasis may explain the phenomenon of hyperglycemic memory (Brownlee, 1992). Recently, there are some sophisticated and expensive laboratory techniques such as mass spectrometry, gas and/or liquid chromatography, which are required for the measurement of specific AGEs. However, it does not exist a universal accepted method with no internationally recognized standard unit to measure them (Goh *et al.*, 2008).

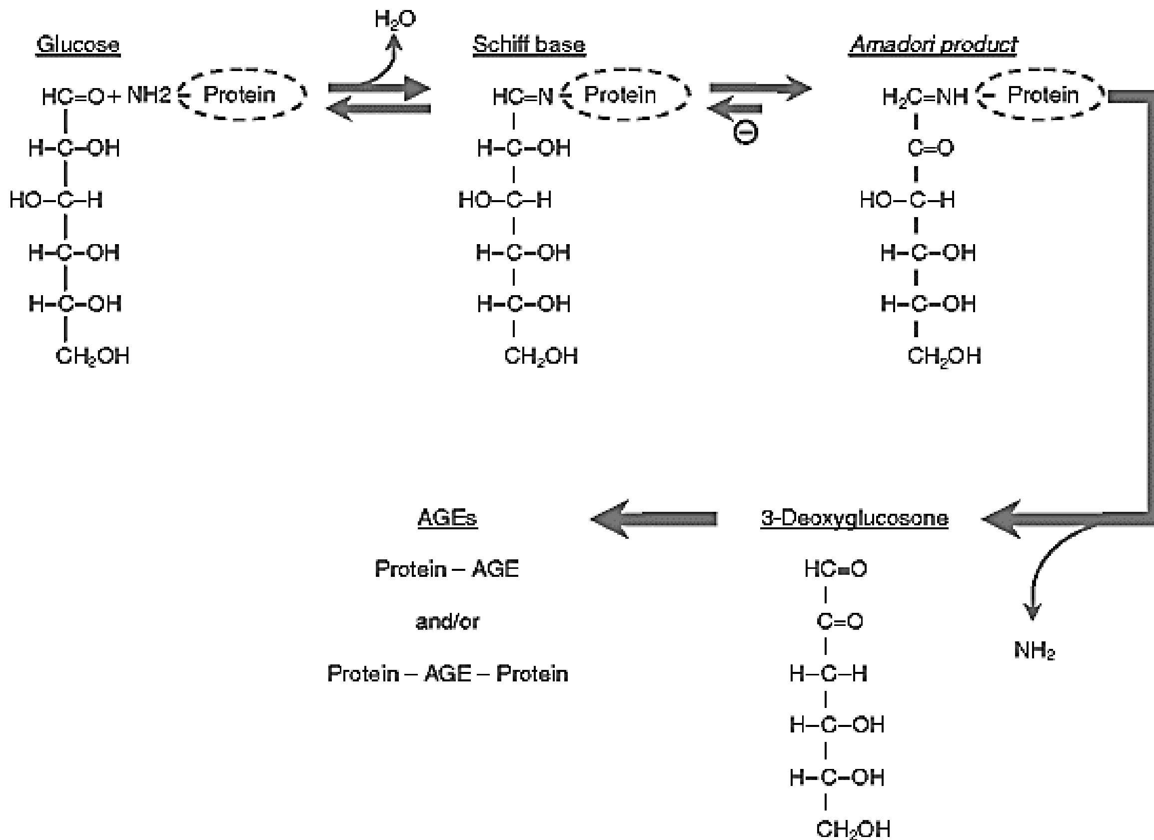


Figure 7: Glycation of a protein and the subsequent formation of AGEs.

I.6.2. Oxidative stress induced by hyperglycemia

OS plays a crucial role in the progression of both types of DM. The metabolic dysregulations observed in diabetes result in mitochondrial dysfunction and an overproduction of free radicals (Piconi *et al.*, 2003). These resulting consequently to the activation of the five pathways of glucotoxicity described above in the pathogenesis of complications (Giacco *et al.*, 2010).

I.6.2.1. Oxidative stress and antioxidant system

A. Oxidative stress and free radicals

OS is generally described as a condition in which cellular antioxidant defenses decrease dramatically and they lose completely their ability to detoxify the free radicals that have been excessively produced (Piconi *et al.*, 2003). Free radicals are reactive unstable chemical species containing one or more unpaired electrons. They are involved physiologically in the signaling process of differentiation and migration (Asmat *et al.*, 2016). However, the excessive levels of free radicals induce damage to cells by passing the unpaired electron resulting in the oxidation

of cell components and molecules such as DNA, proteins, and other macromolecules, which results in a loss of cells function and structure (Bansal *et al.*, 2011; Halliwell *et al.*, 1985). Free radicals can be classified into (Asmat *et al.*, 2016):

1. Reactive oxygen species (ROS).
2. Reactive Nitrogen species (RNS).
3. Reactive chlorine species (RCS).

B. Antioxidant system

The organism has different mechanisms to produce antioxidants, endogenous or exogenous, that will neutralize the elevated number of these free radicals (Pham-Huy et al., 2008). Antioxidants can be categorized into chain-breaking antioxidants or preventive antioxidants, depending on their structure and mechanism of action (Somogyi et al., 2007):

➢ Enzymatic antioxidant

- Superoxide dismutase (SOD): first-line defense against ROS that is involved in cellular damage (Maritim *et al.*, 2003). It catalyzes the conversion of two superoxide radicals to a molecule each of H_2O_2 and molecular O_2 (Heikkila *et al.*, 1976).
- Glutathione peroxidase (GPx): acts as a scavenger of ROS and essentially eliminates the lipid peroxidation products. Molecularly, they ensure the metabolization of H_2O_2 to water using reduced GSH as the hydrogen donor (Maritim *et al.*, 2003).
- Catalase (CAT): it breaks down H_2O_2 into molecular O_2 and water (Kangralkar *et al.*, 2010).

➢ Non- enzymatic antioxidants

These have mainly the role of channeling the single electron to avoid the oxidation of other molecules.

- Glutathione (GSH): it provides the reducing power to maintain other tissue antioxidants in their reduced states, such as Vits C and E (Coleman, 2000).
- α-tocopherol: it is a fat-soluble molecule acting in a lipophilic medium on peroxyl radicals to give Vit E radical and thus prevent the propagation of lipid peroxidation (Lecerf *et al.*, 1994).
- Vit C: it's a water-soluble molecule, it protects water-soluble toxic oxidizing agents such as OH and O_2. It also acts in synergy with Vit E to regenerate this later after the oxidation (Leverve, 2009).

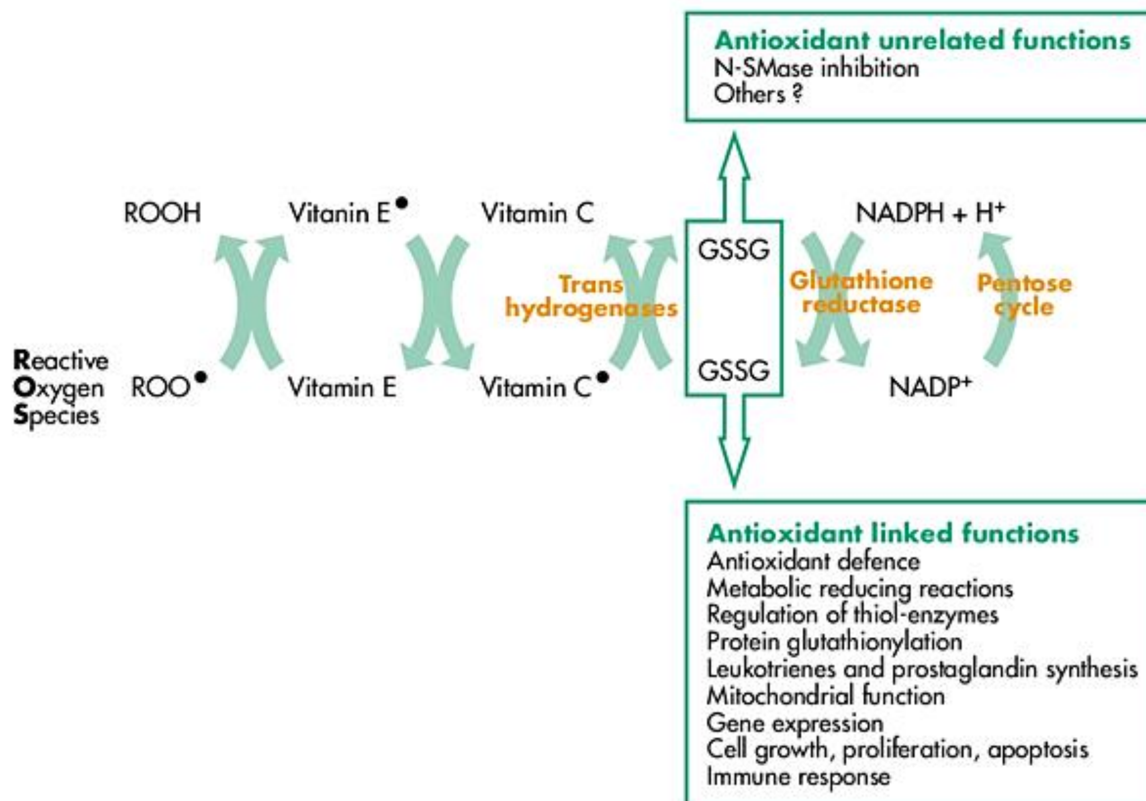


Figure 8: Antioxidant defenses "complementarity between non-enzymatic and enzymatic systems".

I.6.2.2. Oxidative stress in diabetes

The exacerbation of OS in diabetes developed by the glucotoxicity pathways, namely non-enzymatic glycation of proteins, glucose oxidation, and increased lipid peroxidation. In addition, the mitochondria disorders in diabetes play a sensitive role in diabetes progression and they're recognized as a major cause of clinical complications (Sivitz *et al.*, 2010). Hyperglycemia induces pyruvate overproduction, thereby increasing the influx of reducing equivalents (derived from NADH and succinate) into the mitochondrial electron transport chain (Ramalho-Santos *et al.*, 2008). Consequently, an increase in the ATP/ADP ratio and hyperpolarization of the mitochondrial membrane potential are occurs. In parallel, a component of the utilized O_2 in mitochondria is reduced to water, and the remaining O_2 is transformed to oxygen free radical (O^{2-}) which is an important ROS that is converted to other RS such as ONOO, OH, and H_2O_2 (Moussa, 2008). Actually, insulin signaling is modulated by ROS/RNS in two ways. On one side, the ROS and RNS are produced following insulin signals to exert their normal physiological action. On the other side, the ROS and RNS have got negative

regulation on insulin signaling, interpreting them to develop insulin resistance (Erejuwa, 2012). Overall, in hyperglycemic environment, mitochondria undergo rapid fragmentation with a concomitant increase in ROS formation. The mitochondrial morphology alteration observed in hyperglycemic conditions plays a role in the increased production of ROS. Consequently, the regulation of mitochondrial fission/fusion mechanisms emerges as a potential novel approach to manage both acute and chronic ROS production in disorders linked to hyperglycemia (Ramalho-Santos *et al.*, 2008). All of these lead to damage of cellular machinery, increased insulin resistance, and altered the antioxidant enzymes such as CAT, SOD, and GSH–GPx (Maritim *et al.*, 2003) by the glycation of the active site (Coleman, 2000). In addition, insulin is purported to exert regulatory control over the activities of CAT and SOD (Pereira *et al.*, 1995) thus playing a pivotal role in the fluctuations of these enzyme activities observed in diabetes (Coleman, 2000).

I.6.2.3. Biomarkers of glycoxidation

The link between diabetes and OS may be assessed by measuring various biomarkers that include (Giugliano *et al.*, 1995):

1. DNA: the most interesting one is 8-hydroxy-2-deoxyguanosine (8-OHdG) and its free base 8-hydroxyguanine (8-OH-G) in blood cells or urine. Analytical methods to assess these bases are solid phase extraction and HPLC with electrochemical detection, gas chromatography-mass spectrometry (GC-MS), sandwich ELISA, and COMET assay. Various studies have shown that in diabetic patients, serum 8-OHdG levels are increased (Piconi et al., 2003)
2. Lipid peroxidation products: Malondialdehyde (MDA) is the major factor assessed to measure lipid peroxidation after reaction with thiobarbituric acid (TBA) (Asmat et al., 2016a)
3. Glycated proteins: HbA_{1c} which clinically appears to be an essential indicator in diabetes diagnosis (Diabetes-Care, 2019). It provides an assessment of the mean blood glucose concentration over a retrospective period of 2–3 months (Gikas *et al.*, 2009). Also, it may reflect the degree of hyperglycemia, the intensification of AGEs formation, and the extent of OS in the environment (Goldin *et al.*, 2006).
4. Antioxidant enzymes: GPx and GSH in addition to CAT, are the most antioxidant enzymes altered by diabetes and can be evaluated as biomarkers of OS (Asmat *et al.*, 2016).

The variation in these compounds can indicate an alteration in a physiological process and can be used as an early warning against ensuing pathology.

II. Erythrocytes and diabetes

RBCs called erythrocytes, are the most glucose-consuming cells (Wang *et al.*, 2021). In chronic hyperglycemia, erythrocytes undergo morphological, metabolic, and functional changes, consequently influencing hemorheology and microcirculation (Sprague *et al.*, 2006; Zhou *et al.*, 2018). These changes occur in erythrocytes and improved the diabetes progression and the development of diabetes complications (Wang *et al.*, 2021).

II.1. Erythrocytes in non-diabetic patients

The largest abundance of erythrocytes in the blood makes them the most important cells. Their flexibility enables unrestricted traversal through capillaries, facilitating the transport of oxygen (O_2) to tissues and the conveyance of carbon dioxide (CO_2) to the lungs (Wang *et al.*, 2021). The nature and the structure of erythrocytes membrane play an important role in maintaining stability, deformation, aggregation, and adhesion. In addition, it allows them to ensure their function (Benedik *et al.*, 2014). The atypical form of RBCs and their diminutive size afford a substantial surface area-to-volume ratio, facilitating rapid exchange of oxygen O_2 and CO_2 across the cell membrane. Consequently, this characteristic contributes to their remarkable deformability (Wang *et al.*, 2021). In addition to carrying O_2 and CO_2, erythrocytes also have immune functions, such as enhancing phagocytosis, defending against infection, increasing immune adhesion, recognizing and carrying anti-gens, and clearing circulating immune complexes (De Almeida *et al.*, 2012; Jensen, 2009). The mean lifespan of erythrocytes ranges between 110 and 120 days, during which aging erythrocytes are primarily degraded within the reticuloendothelial system of the spleen and liver (Seaman *et al.*, 1977). Several erythrocyte parameter indicators, including hemoglobin concentration, hematocrit, erythrocyte turbidity, mean cell volume (MCV), and mean cell hemoglobin concentration, provide comprehensive insights into the state of erythrocytes. These parameters serve as primary tools for evaluating erythrocyte morphology, structure, function, and production, facilitating the diagnosis of various diseases (Ford, 2013; Kim *et al.*, 2003).

II.2. Erythrocytes in diabetic patients

Many changes were observed in diabetics' erythrocytes, particularly in their morphology and metabolism (Wang *et al.*, 2021). Interestingly, hyperglycemia induces the increase of erythrocytes' perimeter, and the area of erythrocytes decreased with the increasing irregularity in the erythrocyte membrane (Babu *et al.*, 2004). This alteration in cell form was detected by the increase in discocytes (60%) and the decrease in bowl-shaped cells in diabetes (Babu *et al.*, 2004). In addition, Glucose oxidation and protein glycation can induce various modifications in the mechanical and rheological properties of erythrocytes. Specifically, patients with diabetes exhibit a decrease in total protein content, particularly glycoproteins, within the erythrocyte membrane. Concurrently, sialidase activity increases, leading to a reduction in sialic acids on erythrocyte surfaces. Consequently, the superficial negative charge of the cells diminishes, promoting erythrocyte aggregation (Venerando *et al.*, 2002). Moreover, The decreased fluidity and deformability of the erythrocyte membrane, attributed to rising levels of nonenzymatic glycosylation, ROS, and lipid peroxidation, and lead to alterations in the microenvironment of membrane-bound enzymes by modifying phospholipids and fatty acid composition (Higgins, 2015). Therefore, this increases aggregation and induce a high blood viscosity and coagulation. As consequence, the development of microcirculation disorders under these conditions becomes an important cause of diabetic macrovascular and microvascular complications (Wang *et al.*, 2021). On the other hand, nitric oxide (NO), generated by erythrocytes, participates in the process of cell deformation within the microcirculation. Moreover, The decline in NO bioavailability within erythrocytes in diabetes leads to reduced deformability and enhanced adhesion, thereby contributing to microcirculatory disturbances (Bakhtiari *et al.*, 2012; Contreras-Zentella *et al.*, 2019). In parallel, the average life span of erythrocytes in diabetes is shortened by 13% (Qadri *et al.*, 2017). Overall, the rise in hyperglycemia results in osmosis and OS amplification in patients with diabetes. These altered directly the concentration of iron and protein inside and outside the erythrocytes, then activate the eryptosis pathway (Bissinger *et al.*, 2019). Therefore, a low erythrocyte count indicates a reduction in oxygen-carrying cells within the bloodstream. The aggregation of fragmented erythrocytes within microvessels obstructs blood flow, ultimately resulting in tissue hypoxia (Wang *et al.*, 2021).

II.3. Glucose metabolism in diabetic erythrocytes

As blood glucose concentration increases, erythrocytes incur augmented glucose uptake. This is mediated by glucose transporter 1 (GLUT1), a phenomenon characterized by insulin-

independent transmembrane glucose transport (Wang *et al.*, 2021). This accelerates the glucose metabolic pathways inside cells accordingly to the lack of mitochondria. Glycolysis and its products, namely ATP are the main source of erythrocyte energy (Hers *et al.*, 1983). However, The RBCs of diabetic patients undergo various alterations in their cellular mechanisms, including changes in glucose uptake rate, enzyme activity, and production and utilization of intermediate metabolites (Wang *et al.*, 2021). The excess of glucose in erythrocytes will enter the polyol pathway, and it is reduced to sorbitol by AR and then oxidized to fructose by sorbitol dehydrogenase, resulting in the accumulation of sorbitol and fructose (Peterson *et al.*, 1986).

On the other hand, hyperglycemia in DM increased the non-enzymatic glycosylation and causes glycation of membrane proteins and enzymes along with OS leading to a decrease in the activity of Naþ/Kþ-ATPase and other changes in erythrocyte membranes (Viskupicova *et al.*, 2015). This induces the exposure of phosphatidylserine (PS), phospholipids containing amino groups, on the outer surface of erythrocytes, disrupting the asymmetry of membrane phospholipids (Nicolay *et al.*, 2006). In addition to the membrane alterations, the high glucose concentrations and glycoxidation intensified the lipid peroxidation, and loss of activities of GST and GR (Viskupicova *et al.*, 2015). Overall, hemolysis, eryptosis, calcium accumulation, loss of glutathione and CAT activity, and increase in the GSSG/GSH ratio were the most molecular disorders observed inside RBCs (Wang *et al.*, 2021). Generally, these are due to the alteration of glucose metabolism pathways induced by hyperglycemia.

II.4. Oxidative Stress in diabetic erythrocytes

Erythrocytes are vulnerable to OS which targets structural proteins such as cytoskeletons and membrane proteins, as well as functional proteins including enzymes. These oxidative modifications carry considerable implications for erythrocyte function (Pandey *et al.*, 2010). Actually, the autoxidation of glucose was considered the major mechanism for free radical formation in diabetic erythrocytes. Likewise, AGEs are considered pre-deformability oxidants, which can activate several signaling pathways to produce ROS by binding to its receptor. Hyperglycemia also reduces antioxidant capacity by decreasing antioxidant levels of RBCs, such as Vit E, CAT, and SOD (Contreras-Zentella *et al.*, 2019; Maritim *et al.*, 2003). Meanwhile, GSH levels decrease dramatically in diabetic patients, especially those with dyslipidemia (Bissinger *et al.*, 2019). Consequently, the deformability of erythrocytes damaged by OS is greatly reduced, which makes it difficult for erythrocytes to passthrough microvessels and are closely related to diabetic microvascular complications (Wang *et al.*, 2021).

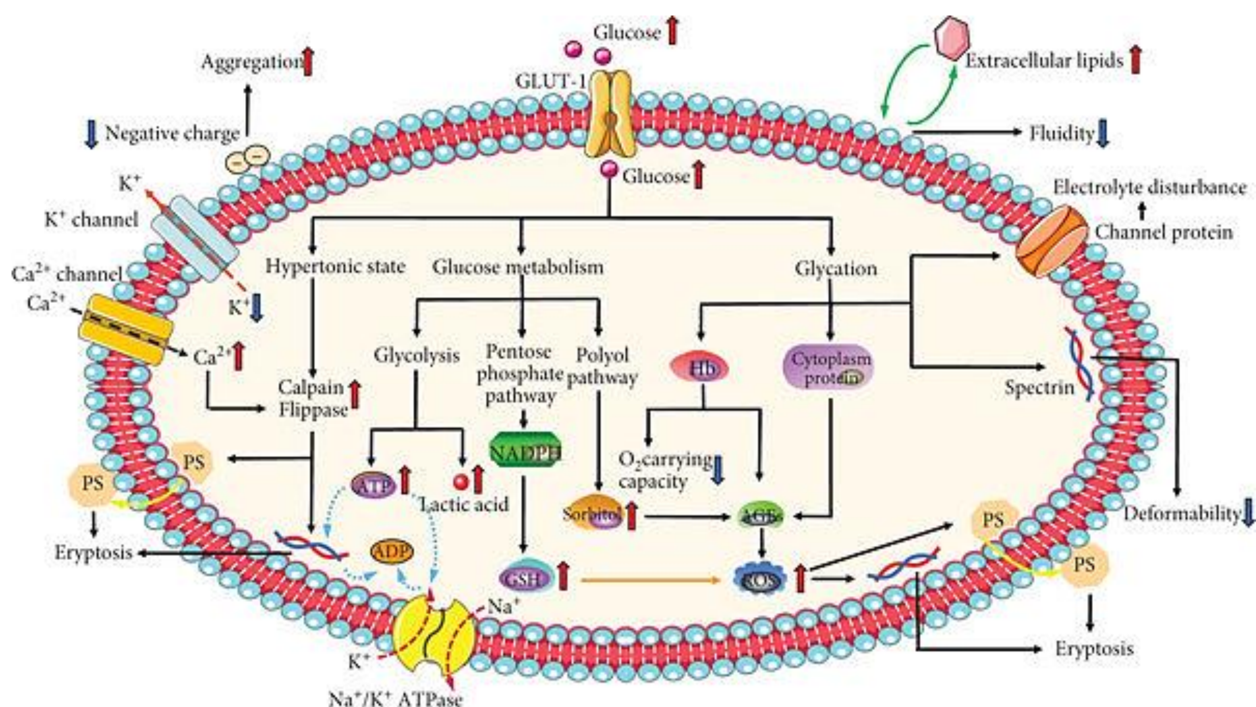


Figure 9: Glucose metabolism disorders in diabetic erythrocytes *(Wang et al., 2021).*

II.5. Hemoglobin alteration in diabetes

Hb is the most abundant protein in RBCs with a main function is oxygen-carrying. Notably, over 90% of the weight of an erythrocyte is Hb that defined as a stable, tetrameric, and hydrophobic ferriprotein. It reversibly binds oxygen and provided that its iron is maintained in the ferrous (Fe^{2+}) state (Stojanović *et al.*, 2012). In the process of binding oxygen, oxyhemoglobin becomes a superoxo-ferrihaem ($Fe^{3+}O^{2-}$) complex, and when tissue release of oxygen occurs, the haem iron is restored to its ferrous state (Coleman, 2000).

II.5.1. Glycated hemoglobin (HbA_{1c})

HbA_{1c} is a minor fraction of Hb in human RBCs and generates by the non-enzymatic binding of glucose to N-terminal amino acids of β-chains of Hb A (Misciagna *et al.*, 2007). HbA_{1c} is formed slowly, continuously, and irreversibly during the 120-day lifespan of the red cells (Bunn *et al.*, 1976). Therefore, the cumulative amount of HbA_{1c} should be directly proportional to a time-averaged concentration of glucose within the erythrocyte and linked to the age of erythrocytes (Bunn *et al.*, 1978). It's important to indicate that HbA_{1c} is described as an Amadori product but it is not an AGE (Quehenberger *et al.*, 2000). However, it may reflect the degree of hyperglycemia, the intensification of AGEs formation, and the extent of OS in the

environment (Goldin *et al.*, 2006). Clinically, HbA_{1c} appears to be an essential indicator in diabetes diagnosis (Diabetes-Care, 2019). It shows the average concentration of blood glucose over the past 2–3 months (Gikas *et al.*, 2009). Consequently, elevated HbA1c concentrations are associated with enhanced affinity for O_2. Therefore, higher HbA_{1c} concentrations lead to difficulties in releasing O_2 to cells and reduced oxygen-transporting function of erythrocytes (Weykamp, 2013). Consequently, many macrovascular and microvascular complications are linked to the rise of HbA_{1c} concentrations (Škrha *et al.*, 2016).

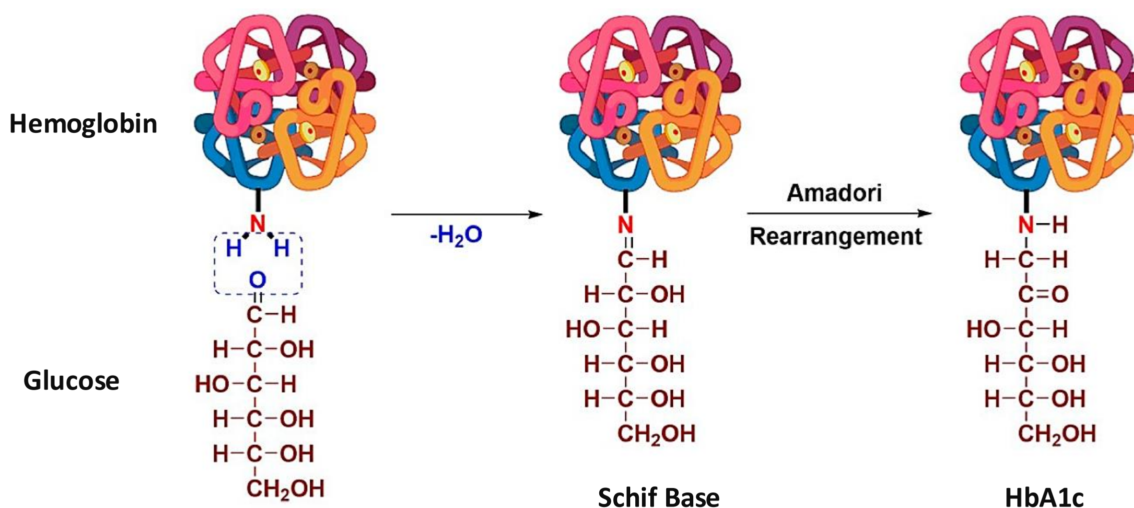


Figure 10: Glycated Hemoglobin formation.

II.5.2. Methemoglobin formation in diabetes

During the process of O_2 carriage, low levels of O_2 are released from Hb as O_2^-, causing it to transition into ferric (Fe^{3+}) metHb, which lacks the capacity to transport O_2 (Misra *et al.*, 1972). Thus, Hb is a comparatively reactive molecule and erythrocytes employ several enzymes as well as CAT and SOD to protect and to maintain Hb in the ferrous (Fe^{2+}) state where it can bind oxygen (Winterbourn, 1990). The formation of small quantities of metHb through the autoxidation of Hb during oxygen carriage occurs constantly in normal erythrocytes (Coleman *et al.*, 1996). Moreover, $NADH^+$-dependent cytochrome b5 metHb reductase (NADH diaphorase) restores efficiently Hb from metHb in a natural manner (Coleman, 2000). This enzyme ensures that usually less than 1% of Hb is oxidized at any one time in healthy individuals (Coleman *et al.*, 1996). However, the erythrocyte's ability to maintain GSH levels and operate other essential enzyme systems namely CAT, decreased importantly in diabetes, and the irreversible metHb formation process will rapidly become manifest. These resulted essentially as a consequence of hyperglycemia that can damage RBCs and make them more

susceptible to oxidation. Actually, it is unclear as to which stages in the metHb process are compromised due to glycation/AGE formation (Coleman, 2000). However, the glycation itself does not change the reactivity of oxyhemoglobin with free radicals and metHb formation. Overall, the metHb amplification process is dependent on the potency of the erythrocyte's antioxidant systems and in general, is less efficient in diabetic cells (Coleman *et al.*, 1998).

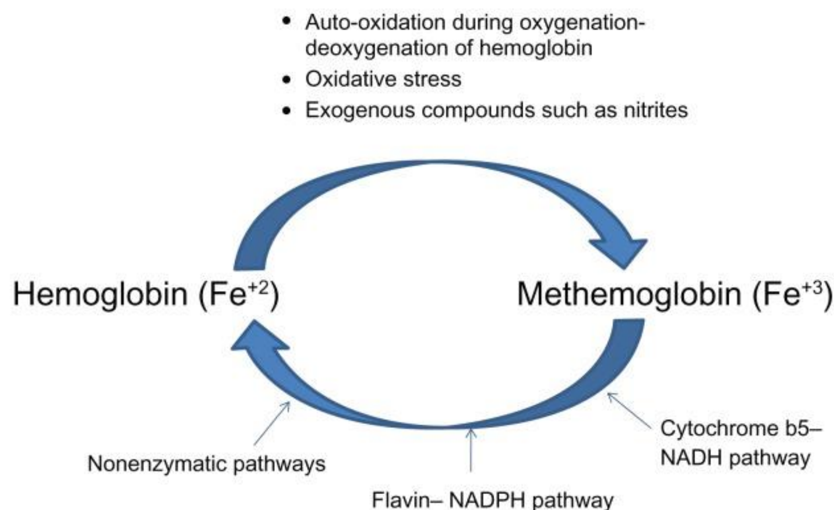


Figure 11: Methemoglobin formation.

II.6. Antioxidant supplementation effect on diabetic erythrocytes

With the aim of minimizing, but not removing the risks of diabetic complications, future therapies might add to the antidiabetic treatment (Table 2), namely anti-AGE agents and antioxidant molecules such as vitamins (Alper *et al.*, 2006; Baynes *et al.*, 1999). Interestingly, the addition of Vit C and E showed an interesting reduction in oxidative-mediated damages in diabetes namely metHb formation *in vitro*, thus they could be used as an alternative medication (Atyabi *et al.*, 2012). By the same token, a daily antioxidant supplement consisting of Vit E (200 IU), Vit C (250 mg) and a-lipoic acid (90 mg) for a period of 6 weeks in diabetic volunteers, improved diabetic plasma antioxidant capacity, attenuated *in vitro* experimental OS of metHb formation and reduced Hb glycation *in vivo* (Coleman *et al.*, 2003). As well, Quercetin (3,3′, 4′,5,7-pentahydroxyflavone), one of the most abundant bioflavonoids commonly present in most edible fruits and vegetables, may provide protection to diabetic patients against some late complications (Rizvi *et al.*, 2009). Overall, both exogenous and endogenous antioxidants can show pro-oxidant effects depending on their concentration and the specific oxidative process involved (Coleman, 2000).

Table 2: Different classes of antidiabetic drugs with their mode of action (Thakur *et al.*, 2010).

Group	Route of administration	Therapeutic action	Drug (s)
Insulin	Subcutaneous	Reduces blood glucose level	Insulin
Sulfonylurea	Oral	Insulin secretagogue	Tolbutamide, Glimeperide
Biguannides	Oral	Reduce hepatic glucose output and increase peripheral glucose uptake	Phenformin
Meglitinides	Oral	Insulin sensitizers and secretogogue	Rosiglitazone
Thiazolidinediones	Oral	Bind to peroxisome proliferator activated receptor gamma help in more production of insulin dependent enzymes	Rapaglamide, Miglitol
Alpha glucosidase inhibitor	Oral	Effect on glucose absorpation from GI tract	Acarbone
Peptide analogue	Oral	Stimulates insulin release	Experimental

III. Male reproductive dysfunction in diabetes mellitus

III.1. Male reproductive system

The male reproductive system is a complex network of organs and hormones that work together to produce, to store, and to release sperm. The male reproductive system is made up of several accessory glands, such as the seminal vesicles, the prostate gland, and bulbourethral gland (Mawhinney *et al.*, 2013). These glands secret fluids that mix with sperm and contribute to the composition of seminal plasma (Cheng *et al.*, 2019). The various biochemical components of seminal plasma were essentially produced in prostate gland and seminal vesicles (Jones *et al.*, 2014). As well, some elements are transferred to the seminal plasma from the endothelium capillaries which surrounded these accessory glands via different mechanisms (Creasy *et al.*, 2013). This is driven by the pressure of the blood flowing through the vessels. The fluid produced is then transported along the vas deferens and mixed with the sperm cells in the epididymis to form semen (Saez *et al.*, 2003). Sperm cells are produced and differentiated during spermatogenesis which is a multi-step process of germ cell division and development that occurs within the testis, more specifically in the seminiferous tubules (Walker, 2010). This process is under strict hormonal control such as follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone (Wilson *et al.*, 1981).While the resulting mature sperm cells present specific metabolic needs, using external hexoses, such as glucose, as their main substrate (Angulo *et al.*, 1998; Miki, 2007)

III.2. Glucose metabolism in sperm cell

The metabolic processes involved in sperm development and function are greatly correlated with glucose uptake and metabolism. For ensuring glucose uptake, the sperm cells presented specialized carriers in their membrane (Dias *et al.*, 2014). These can be divided into two different families of glucose transporters: the Sodium-coupled Glucose Transporters (SGLTs) and the facilitative Glucose Transporters (GLUTs) (Dias *et al.*, 2014). Following the uptake, the hexose metabolism in sperm cells is based on two processes that can be used together or independently: oxidative phosphorylation and glycolysis (Pasupuleti, 2007). All these processes are predominantly under the control of sex steroid hormones (Oliveira *et al.*, 2012; Oliveira *et al.*, 2011), namely FSH and insulin (Boussouar *et al.*, 2004). Actually, the survival and differentiation of germ cells rely on the lactate produced in the glycolytic pathway after the conversion of glucose to pyruvate by the involvement of special enzymes in the process. Interestingly, the germ cells are not capable of metabolizing glucose directly. Wherefore, this is established by metabolic cooperation with the somatic Sertoli cells (SCs) which can produce a high rate of lactate (Boussouar *et al.*, 2004). On the other hand, mature spermatozoa may use glucose in ATP production which would be expected to compromise sperm quality, capacitation, and subsequently fertility (Dias *et al.*, 2014).

III.3. Diabetes and male fertility dysfunction

Diabetes is well-recognized as a cause of male sexual dysfunction. It has been reported that 50% of all diabetic male individuals have some grade of subfertility and/or infertility (Dias *et al.*, 2014). Generally, diabetes has been reported to be associated with retrograde and premature ejaculation, decreased libido, delayed sexual maturation, and compromised semen quality (Liu *et al.*, 1986), such as sperm concentration, motility, and morphology (Bhattacharya *et al.*, 2014). Moreover, diabetic men frequently have small vessel lesions with calcification of scrotal vessels (Bour *et al.*, 1984). Also, it may decrease penile blood pressure and blood flow (Abelson, 1975). Interestingly, vascular complications in diabetes could be responsible for impotence and testicular dysfunction (Defeudis *et al.*, 2022). All of these can be resulted from the alteration of the male reproductive system at different levels. Indeed, three main dysfunctional mechanisms may be postulated to explain the sperm damage observed in diabetic patients: endocrine disorders, diabetic neuropathy, and OS (La Vignera *et al.*, 2012)

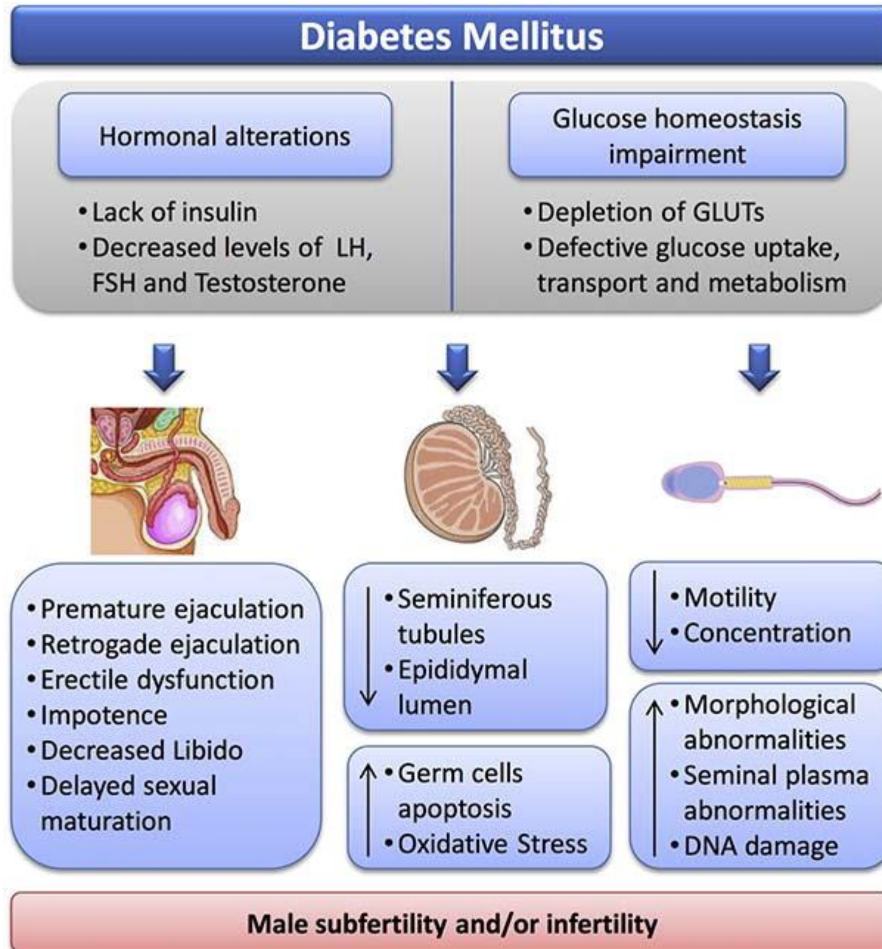


Figure 12: Male reproduction impairment in diabetes *(Dias et al., 2014)*.

The hormonal fluctuation in DM is identified by the decrease of serum testosterone and decreased levels of LH and FSH (Alves *et al.*, 2013; Distiller *et al.*, 1975; Wright *et al.*, 1976). Also, abnormal feedback of the hypothalamus-pituitary axis by sexual steroids was observed, either due to reduced pituitary sensitivity or the inefficient steroid transport into effector cells (Baccetti *et al.*, 2002). These changes are associated with a steroidogenetic defect in Leydig cells and testosterone production that decreases because of the absence of the stimulatory effect of insulin on these cells (Ballester *et al.*, 2004). Interestingly, insulin fluctuations have an important influence on male reproductive health by the alteration of the intracellular metabolism, namely lactate production (Alves *et al.*, 2012) and the differentiation of spermatogonia into primary spermatocytes, via insulin-like growth factor-I (IGF-I) receptor (Nakayama *et al.*, 1999). Moreover, the disruption in sperm metabolism was also related to the defective glucose transport that resulted by the depletion of GLUTs (Handberg *et al.*, 1990). As well, higher levels of nuclear and mitochondrial sperm DNA damage have been consistently

reported in diabetic individuals which all play a role in the impairment of sperm quality in diabetes (Agbaje *et al.*, 2007).

Diabetic neuropathy can cause atonia of seminal vesicles (La Vignera *et al.*, 2012). Thus, the involvement of the pelvic autonomic nervous system by diabetic neuropathy can result in impotence which is associated with bladder dysfunction (Dias *et al.*, 2014). These are related to the frequent neurogenic origin of impotence in diabetic men because erection and bladder function which are controlled by the same nerves and spinal cord centers (Bors, 1960).

III.4. Oxidative stress in diabetic men

OS may play a pathogenic role in DM-related male reproductive function abnormalities (La Vignera *et al.*, 2012). These are identified by the overproduction of ROS which are detected in semen samples of 25% to 40% in infertile men, associated with a decreased efficiency of antioxidant defenses (Ramalho-Santos *et al.*, 2008). It should be noted that spermatozoa are particularly susceptible to OS because of their membranes which contain large quantities of PUFAs, and their cytoplasm contains low concentrations of scavenging enzymes (Ramalho-Santos *et al.*, 2008). However, hyperglycemia-induced overproduction of O_2^- in mitochondria and inhibits significantly G-3-P dehydrogenase activity. Then, this inhibition will activate all the pathways of hyperglycemic damage by diverting upstream glycolytic metabolites to these pathways (Du *et al.*, 2000). Besides hyperglycemia, hormonal factors can also contribute to the generation of OS. Indeed, insulin can stimulate OS directly inducing H_2O_2 production and stimulating the sympathetic nervous system. These lead to the activation of neurotransmitter release and associated enzymatic systems alteration of which induce OS (Wiernsperger, 2003). Additionally, spermatozoa containing defective mitochondria not only produce ATP in less efficiently way which lead to an ultimate energy crisis and a decline of motility and fertility, but also generates more OS. Moreover, the oxidative damage occurred to DNA may accelerate the process of germ cell apoptosis, leading to a decline in sperm counts associated with male infertility (Wei *et al.*, 2000).

OS also plays a role in erectile dysfunction which results primarily from an endothelial dysfunction induced by the loss of biological activity of NO (Musicki *et al.*, 2007). The oxygen free radicals, especially O_2^-, inactivate directly NO and reduce its physiologic impact (De Young *et al.*, 2004). In addition, the high levels of AGE products in the reproductive track namely carboxymethyllysine (CML), induced changes in testicular metabolite levels and spermatogenic gene expression (La Vignera *et al.*, 2012)

III.5. Antioxidant therapy in diabetes with male reproductive disorder

Antioxidant supplementation as a therapy for male reproductive dysfunction in diabetes has been tested and assessed generally on animal models. Actually, a prominent reduction of germ cell apoptosis was found in diabetic rats supplemented with antioxidants, namely Vit E and C, and a-lipoic acid. These vitamins decreased the MDA and caspase-3 activity (Mohasseb *et al.*, 2011). Moreover, Pioglitazone administration reduced significantly sperm shape abnormalities and increased sperm count. Concomitantly, it decreased lipid peroxidation and enhanced GSH and GPx levels in diabetic rats (Rabbani *et al.*, 2010). Glimepiride, known as a third-generation antidiabetic sulphonylurea, decreased sperm shape abnormalities, enhanced sperm counts, and improved antioxidant status in diabetic rats (Rabbani *et al.*, 2009). The evaluation study of hydrated C(60) fullerene (C(60)HyFn) on streptozotocin-diabetic rats demonstrated the bioantioxidant potential that allowed them to overcome testicular dysfunction. Indeed, the treatment with C(60)HyFn restored the normal levels of serum testosterone, testicular reduced glutathione, and a-tocopherol (Bal *et al.*, 2011). These data suggested that antioxidant agents, such as Vit E and C, a-lipoic acid, and pioglitazone, as well as glimepiride reduced germ cell apoptosis, decreased sperm abnormalities, particularly morphologic alterations, and improved conventional sperm parameters (La Vignera *et al.*, 2012).

CHAPITER II: ***Close relationship between high HbA_{1c} levels and methemoglobin generation in human erythrocytes: the enhancement of oxidative stress in the process***

CHAPITER II: Close relationship between high HbA_{1c} levels and methemoglobin generation in human erythrocytes: the enhancement of oxidative stress in the process

ABSTRACT

The present investigation aimed to elucidate the impact of diabetic plasma on human RBCs, specifically focusing on the potential amplification mechanisms of OS relative to metHb production, a putative bioindicator for diabetic disease. An *in vitro* investigation was conducted to explore the impact of poorly controlled diabetes on RBCs. Blood plasma from 24 diabetic patients with varying HbA_{1c} levels was co-incubated with normal RBCs for 0, 24, and 48 h to evaluate cellular turbidity and Hb stability. Additionally, the quantification of both Hb and its oxidized counterpart, metHb, was performed within and outside the RBCs. MDA levels and cellular morphology were concurrently assessed. The results revealed a significant reduction in cell turbidity in the group exposed to diabetic plasma with high HbA_{1c} (0.074 ± 0.010 AU) compared to the control group (0.446 ± 0.019 AU). This suggests a potential impairment of RBCs structure. Furthermore, a significant reduction in intracellular Hb concentration (0.390 ± 0.075 AU) and its stability (0.600 ± 0.001 AU) was observed. Importantly, a substantial increase in metHb levels within RBCs (0.186 ± 0.017 AU) and its supernatant (0.086 ± 0.020 AU) was detected after 48 h. Consequently, RBCs exposed to diabetic plasma with high HbA_{1c} displayed a significant rise in MDA levels. These findings collectively suggest that uncontrol glycemia in DM potentiates metHb generation, thereby serving as a principal contributor to the amplification of OS.

I. Introduction

DM is associated with a high prevalence of microvascular complications and physiological abnormalities (Nordwall *et al.*, 2015). Numerous tissues are affected by chronic hyperglycemia causing their dysfunction including lens fiber cells, vascular endothelial cells, and RBCs (Betz *et al.*, 1983; Davis, 1979; Kinoshita, 1974).

Under physiological conditions, glucose is transported via blood plasma with direct exposure to blood cells, especially RBCs (Manno *et al.*, 2010). Glucose concentration in RBCs is normally close to that of plasma (around 5 mM), which is ensured by passive transport through GLUT1 (Viskupicova *et al.*, 2015). Under hyperglycemic conditions, the concentration of glucose and its metabolites are dramatically elevated in plasma and RBCs (Sharabi *et al.*, 2015). In comparison to normal subjects, it's observed a decrease in RBCs lifespan in diabetic patients (Chandramouli *et al.*, 1975). Various abnormalities in the plasma membrane are also identified as the consequences of an increase in membrane lipid fluidity (Testa *et al.*, 1988), owing to the variation in total cholesterol and phospholipid content (Baldini *et al.*, 1989; Bryszewska *et al.*, 1986). In addition, elevated glucose concentrations are known to modify the hemorheological characteristics of diabetic RBCs. It causes particularly the alteration of membrane phospholipids asymmetry which in turn is involved in the loss of the deformation ability (Wali *et al.*, 1988; Watała, 1988) with the appearance of flat cells or discocytes (Simpson, 1992; Turchetti *et al.*, 1998). This may be the origin of the excessive RBCs aggregation (Satoh *et al.*, 1984; Schmid-Schönbein *et al.*, 1976) and increased tendency of endothelial cells adhesion (Wali *et al.*, 1988). In addition, membrane lipid peroxidation can lead to a reduction of cell survival (Jain *et al.*, 1983) and hypercoagulability (De Lucio Cazaña *et al.*, 1989; Jain, 1985) as shown *in vitro,* in human RBCs and *in vivo* in rats and rabbits (Wali *et al.*, 1987).

Also, hyperglycemia leads to widespread non-enzymatic glycosylation of proteins and accumulation of AGEs, in particular, HbA_{1c} (Gonen *et al.*, 1977). AGEs are considered as possible sources of ROS production in diabetes (Coleman *et al.*, 2000). These free radicals are produced as the consequence of endogenous metabolic reactions such as auto-oxidation of glucose, hexosamine pathway, and AGEs formation (Ighodaro, 2018).

In normal subjects, RBCs evolve several mechanisms to preserve their structural integrity and maintain their Hb in a reduced state (Coleman, 2000). However, the GSH system and

enzymatic antioxidants such as SOD can be reduced and damaged by glycation reactions, aldose reductase activation, and NADPH depletion in diabetic patients with poor glycemic control (Coleman, 2000).

Hb is a reactive molecule that carries O_2 through the organism. In normal RBCs, low levels of O_2 are released as $O^{\cdot 2-}$ from Hb in the process of oxygen carriage which leads it to become the ferric (Fe^{3+}), metHb, in a natural manner, a non-functional molecule unable to transport oxygen (Coleman *et al.*, 1996). In fact, various enzymes as well as CAT and SOD protect the Hb and maintain the level of metHb below 2% (Deshmukh *et al.*, 2013). Moreover, Hb acts as a NO scavenger and deplete NO levels in plasma (Rother *et al.*, 2005). Once Hb is released from its intrinsic environment, it interacts with NO and can be permanently oxidized to metHb (Umbreit, 2007). However, in diabetic RBCs, it is unclear as to which stages in the metHb process that are compromised due to the glycation/AGE formation. The modification of intracellular metabolites and the antioxidant system induced by hyperglycemia may be the contributing factors of metHb amplifications in diabetes. Consequently, the extracellular metHb accelerates the RBCs destruction to further contribute to elevated levels of metHb and Hb degradation products, such as hemin. These products induce endothelial cells inflammation and increase low-density lipoproteins (LDL) oxidation, processes occurring during OS. Further, these products are potentially toxic to various organ systems such as renal, cardiac, pulmonary, and central nervous system (CNS) (Deshmukh *et al.*, 2013; Umbreit, 2007).

Nevertheless, there are only limited studies demonstrating exactly the key factor responsible for various damages that occurred in these cells (Coleman, 2000; Gonen *et al.*, 1977; Schleicher *et al.*, 1981). Previous studies focused more on the evaluation of the harmful effects of diabetes on human RBCs (Bryszewska *et al.*, 1986; Jain *et al.*, 1983; Kung *et al.*, 2009) than OS mechanisms particularly those involved inside RBCs.

Hence, this study was undertaken to explore OS generation and amplification inside RBCs under hyperglycemic conditions. We used RBCs and Hb as *in vitro* models which are invoked in various studies as potent models due to their important role in molecular and physiological mechanisms. Such *in vitro* co-incubations of RBCs with human plasma which have been undertaken in different research investigations (Hjelm *et al.*, 1965; Kempe *et al.*, 2007).

Given the above information, this study aims to investigate the effect of diabetic plasma at different HbA_{1c} levels, on human RBCs in order to highlight the amplification mechanisms

of OS in relation to metHb production, a potential bio-indicator that could be related to diabetes disease.

II. Materials and methods

II.1. Ethical considerations

This research has been done according to Helsinki Declaration, and approved by the independent ethics committee at the Algerian National Agency for the Development of Health Research (ANDRS). All participants provided informed consent prior to enrolment.

II.2. Chemicals

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

II.3. Sample collection

II.4. Preparation of human diabetic plasma

Blood specimens were procured subsequent to an overnight fast from a cohort comprising 24 individuals afflicted with type 2 (non-insulin-dependent) diabetes from the Diabetes Center of Bejaia, Algeria. To minimize the influence of confounding factors and ensure data integrity, individuals presenting with acute medical complications were excluded. These participants exhibited a mean illness duration of 12 ± 6 years. Heparinized tubes (4000 USP units/l of blood) were employed as anticoagulants to prevent coagulation during collection. Plasma separation was achieved through centrifugation at 3000 rpm for 10 min. The isolated plasma fractions were then cryopreserved at -20°C for subsequent analysis (Halder *et al.*, 1998). Categorization of diabetic plasma was executed based on their respective glycated hemoglobin (HbA_{1c}) levels, stratified as follows: low HbA_{1c} ≤ 5% (31 mmol/mol), moderate HbA_{1c} 6%–8% (42–64 mmol/mol), and high HbA_{1c} ≥ 10% (86 mmol/mol; Table 3).

II.4.1. Isolation of human red blood cells

Blood samples were acquired from apparently healthy adult volunteers. To ensure normoglycemia, blood glucose concentrations were verified to be within the physiological range (0.8 g/l). Subsequently, RBCs were isolated via centrifugation at 3000 rpm for 10 min at 4°C and effectively separating them from plasma. The isolated RBCs pellet was then washed twice with a 0.9% NaCl solution to eliminate the residual plasma components. Finally, a standardized 20% hematocrit suspension was prepared using the washed RBCs (isotonic blood).

Table 3: Data of diabetic patients of different HbA_{1c} categories.

Categories	Number of patients	Serum glucose concentration (g/l) (Mean ± SEM)	HbA_{1c} values	Type of diabetes	Duration of illness (year) (Mean ± SEM)	Smoking	Complications
HbA_{1c} L	8	1.31 ± 0.16	≤ 31 mmol/mol) (5%)	Type 2	12 ± 6	No	No
HbA_{1c} M	8	1.37 ± 0.05	≥ 42 mmol (6%) & ≤ 64 mmol / mol (8%)				
HbA_{1c} H	8	2.6 ± 0.14	≥ 86 mmol / mol (10%)				

II.4.2. Isolation of hemoglobin

Normal RBCs underwent controlled hemolysis in distilled water (100 µl isotonic blood with 500 µl distilled water), followed by centrifugation at 3000 rpm for 10 minutes at 4°C. The resultant supernatant, containing intracellular Hb released during hemolysis, was extracted to obtain hemolysate. This hemolysate was subsequently used for the evaluation of hemoglobin stability.

II.5. Experimental design

II.5.1. Effect of diabetic plasma on human red blood cells

A co-incubation experiment explored the impact of diabetic plasma on RBCs function. Isotonic blood was co-incubated with NaCl (Negative control), or diabetic plasma with: Low HbA_{1c} levels (HbA_{1c} L); moderate HbA_{1c} levels (HbA_{1c} M); and high HbA_{1c} levels (HbA_{1c} H), in a 1:9 (v: v) ratio for each group.

Simultaneous assessment of cellular turbidity, Hb, and metHb concentrations was conducted in both the supernatant and intracellular compartments of RBCs across all samples.

II.5.2. Cellular turbidity measurement and morphological study

In brief, following the co-incubation of samples at 37°C for 24 and 48 h, aliquots of 50 µl were taken from each sample. These aliquots were then supplemented with 1000 µl of a 0.9% NaCl solution. Subsequently, cellular turbidity was quantified using spectrophotometric

analysis at 620 nm, serving as a proxy for cellular concentration and thereby indicative of cellular integrity (Takebayashi *et al.*, 2012).

RBC morphology was evaluated under light microscope (10x100 magnification) following a protocol identical to the turbidity test. Suspensions of RBCs, either incubated or not with high-HbA_{1c} diabetic plasma, were examined for potential morphological changes. A Basler A312fc digital camera mounted on a Nikon Eclipse E200 microscope (Nikon, Tokyo, Japan) captured high-resolution images for further analysis.

II.5.3. Measurement of released hemoglobin and methemoglobin generation

Following the quantification of cell turbidity, each aliquot of RBCs suspensions was centrifuged at 3000 rpm for 10 min. Subsequently, the resultant supernatant was carefully harvested to facilitate the assessment of released Hb and metHb, adhering to the methodology described by Drabkin (1946).

II.5.4. Measurement of intracellular hemoglobin and methemoglobin generation

This assay aimed to quantify intact Hb and its oxidation products within RBCs, evaluating the impact of diabetic plasma on cellular Hb levels and oxidation—a pivotal step OS initiation and amplification. Following centrifugation and supernatants removal, distilled water was added to each sample pellet, enabling subsequent spectrophotometric measurement of intracellular Hb and metHb absorbance.

II.5.5. Effect of diabetic plasma on hemoglobin stability

The assessment of Hb stability was carried out according to the protocol described by Mameri *et al.* (2021). Preparation of RBCs suspension was executed in accordance with the described protocol in section II.5.1., followed by cellular lysis utilizing distilled water (100 µl isotonic blood with 500 µl distilled water). Subsequent centrifugation at 3000 rpm for 10 min facilitated the recovery of the supernatant, comprising only of Hb. A volume of 10 µl of Hb was subsequently added to 90 µl of each respective treatment group. The stability of Hb was monitored at 412 nm following 2, 6, and 24 h of co-incubation at 37°C. The observed decline in absorbance served as a quantitative indicator of the extent of Hb alteration induced by the experimental conditions.

II.5.6. Determination of lipid peroxidation levels

MDA, indicative of lipid peroxidation levels, was executed via the determination of thiobarbituric acid-reactive substances (TBARS), following the method established by Stocks *et al.* (1971). Briefly, 50 µl of RBC suspension was co-incubated with 450 µl of each respective treatment group. Following 48 h of co-incubation at 37°C, a mixture comprising 315 µl of trichloroacetic acid (TCA at 30%) and 125 µl of 0.9% NaCl was added to each sample, followed by incubation for 2 h at 0°C. Subsequently, the samples were then centrifuged (3000 rpm for 10 min at 4°C), and the resultant supernatant was subjected to treatment with 158 µl of 1% thiobarbituric acid (TBA) dissolved in 0.05 mol/l of sodium hydroxide (NaOH at 0.05 M), along with 47 µl of ethylenediaminetetraacetic acid (EDTA) at a concentration of 0.1 mol/l. The samples were then subjected to boiling at 95°C for 15 minutes, followed by immediate cooling in an ice bath. The absorbance of the MDA-TBARS complex was subsequently measured at 535 nm. The extent of lipid peroxidation was expressed in absorbance units.

II.6. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 software (San Diego, CA, USA). Data were presented as mean ± standard error of the mean (SEM). Student's t-test compared samples treated with diabetic plasma to the negative control. A p-value of less than 0.05 was considered statistically significant.

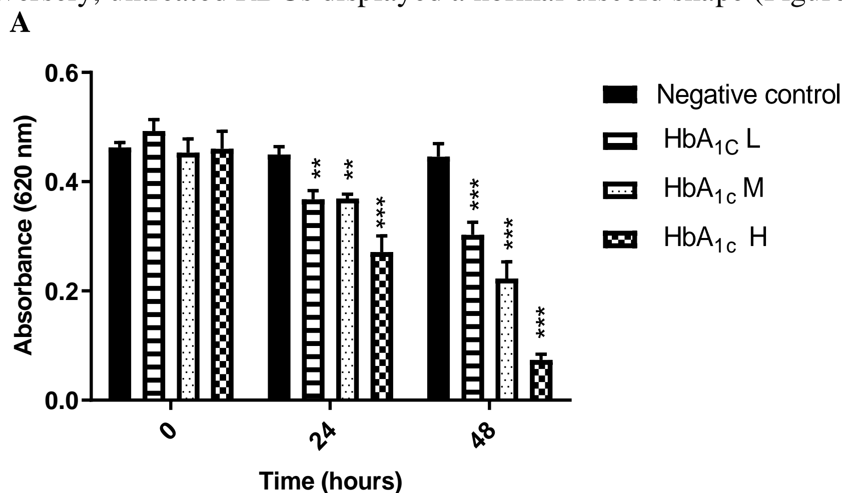
III. Results

III.1. Erythrocyte's turbidity and morphologic analysis

Co-incubation with diabetic plasma for 24 h induced a concentration-dependent cytotoxic effect on RBCs. This is evidenced by a significant decrease in cell turbidity in all diabetic plasma groups (HbA_{1c} L, M, and H) compared to the control (Figure 13.A). Notably, the group treated with high HbA_{1c} plasma (HbA_{1c} H) displayed the most dramatic reduction in cell turbidity (0.271 ± 0.030 AU), indicating extensive cellular damage. This phenomenon continued at the 48-h co-incubation time point, with all diabetic plasma groups exhibiting further cell lysis. The high HbA_{1c} plasma group again showed the most significant decrease in cell turbidity (0.074 ± 0.010 AU), followed by HbA_{1c} M (0.223 ± 0.031 AU) and HbA_{1c} L (0.303 ± 0.023 AU) groups. These findings suggest a correlation between HbA_{1c} levels in diabetic plasma and the degree of cytotoxicity towards RBCs.

Microscopic analysis revealed distinct morphological alterations in RBCs exposed to diabetic plasma, particularly those treated with high HbA_{1c} (HbA_{1c} H). These alterations manifested as changes in cell morphology and the formation of cytoplasmic membrane aggregates. Conversely, untreated RBCs displayed a normal discoid shape (Figure 13. B).

A



B

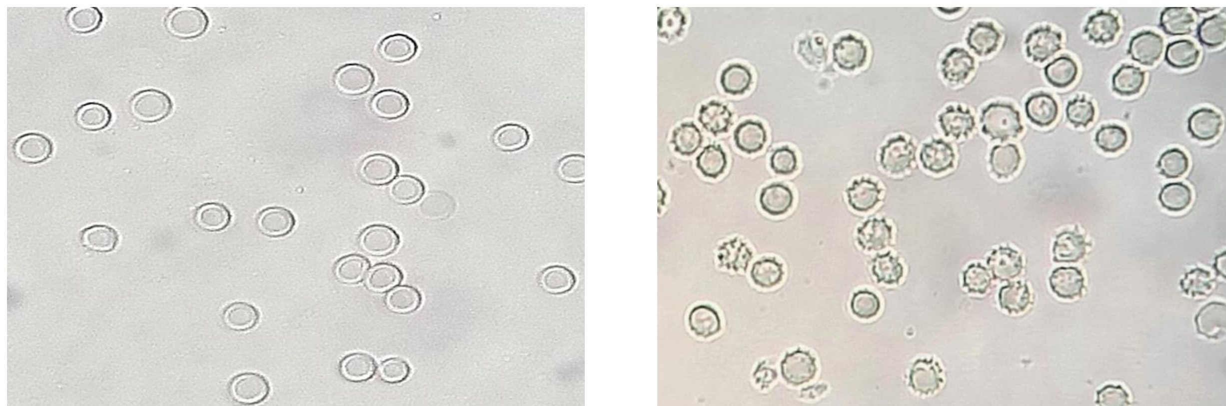


Figure 13: RBCs turbidity and microscopic images morphology. (**A**): RBCs concentration after 0, 24 and 48 h of co-incubation with diabetic plasma at different HbA_{1c} level. Negative control: RBCs treated with 0.9% NaCl, HbA_{1c} L: RBCs treated with diabetic plasma at low HbA_{1c} levels, HbA_{1c} M: RBCs treated with diabetic plasma at moderate HbA_{1c}, and HbA_{1c} H: RBCs treated with diabetic plasma at high HbA_{1c} levels. The data are expressed as means ± S.E.M. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ compared to the negative control group. (**B**) Microscopic images of human RBCs morphology (10x100 magnification). RBCs treated with 0.9% NaCl (left image), RBCs treated with diabetic plasma at high HbA_{1c} level (right image).

III.2. Hemoglobin and methemoglobin concentrations

After 48 h of direct exposure of RBCs with diabetic plasma (HbA_{1c} L, HbA_{1c} M, and HbA_{1c} H), a notable increase in extracellular Hb concentration was observed. Particularly, the

significant Hb release was revealed in the HbA_{1c} H Group (1.074 ± 0.157 AU; Figure 14.A). Concurrently, a dramatic elevation in extracellular metHb concentration was evident in samples co-incubated with diabetic plasma (Figure 14.C).

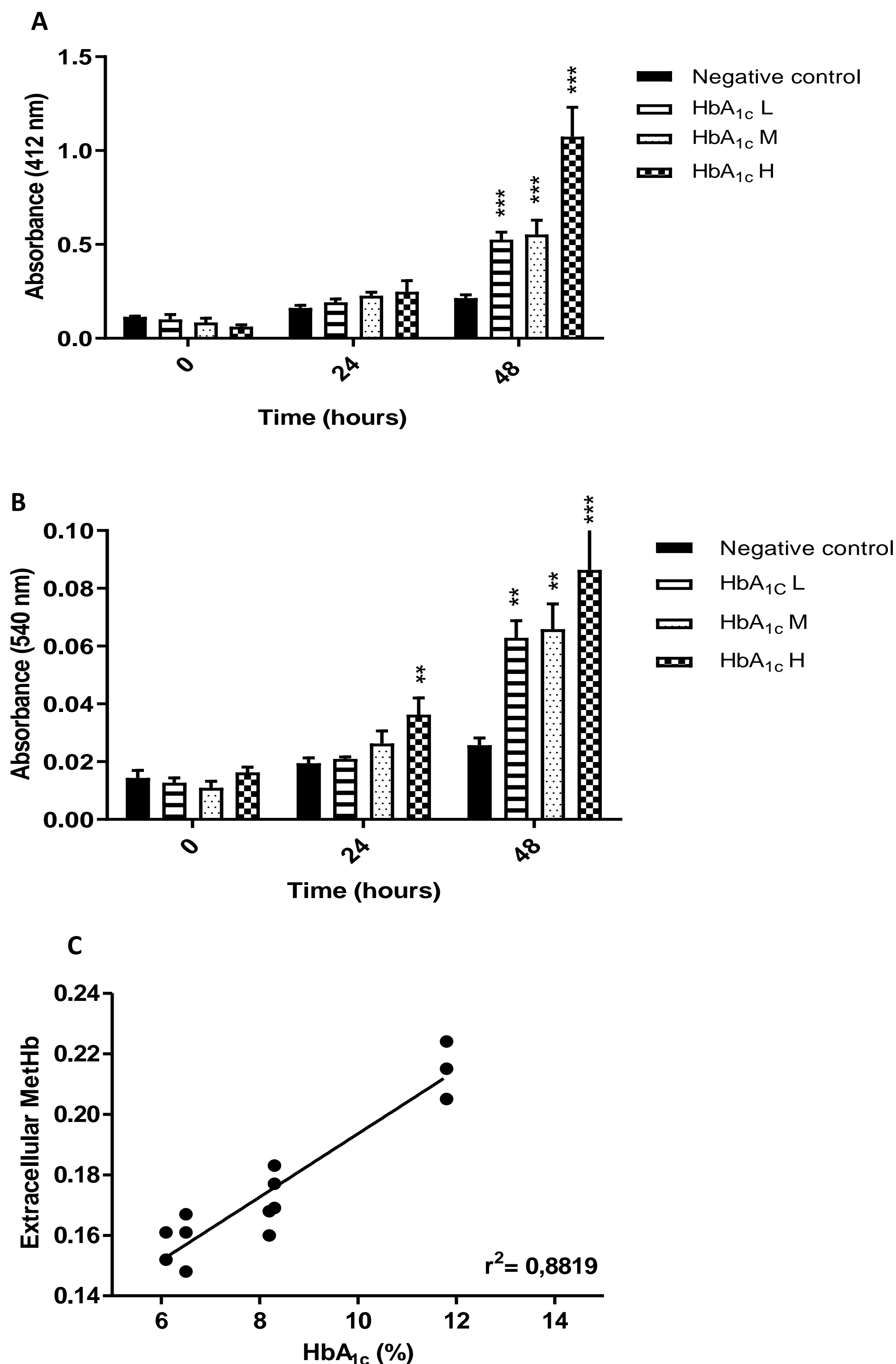
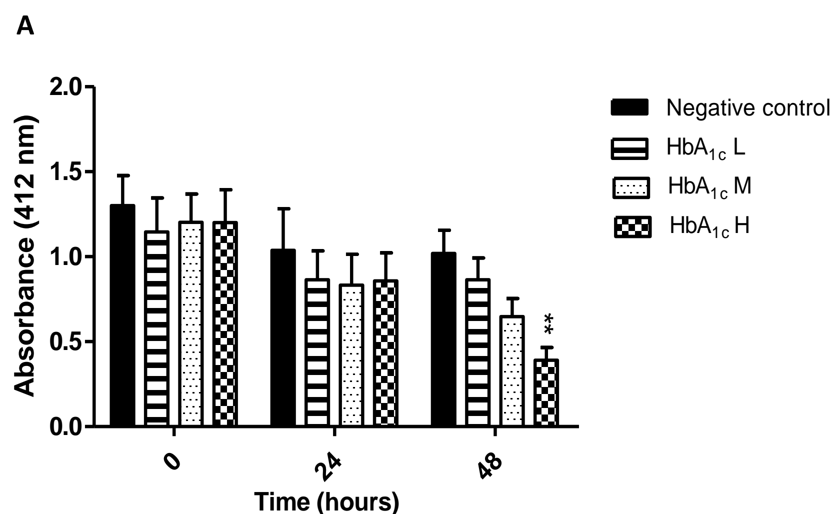


Figure 14: Extracellular hemoglobin and methemoglobin of human RBCs after 0, 24, and 48 h of co-incubation with diabetic plasma at different HbA_{1c} levels. A: extracellular Hb; B: extracellular metHb.

C: correlation between HbA_{1c} values and the intracellular metHb. Negative control: RBCs treated with 0.9% NaCl, HbA_{1c} L: RBCs treated with diabetic plasma at low HbA_{1c} levels, HbA_{1c} M: RBCs treated with diabetic plasma at moderate HbA_{1c} levels, and HbA_{1c} H: RBCs treated with diabetic plasma at high HbA_{1c} levels. The data are expressed as means ± S.E.M. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ compared to the control group.

Figure 15.A illustrates a reduction in intracellular Hb concentrations across all diabetic groups for a duration of up to 48 h. Notably, the most pronounced decrease was observed in HbA_{1c} H group (0.390 ± 0.075 AU). In contrast to Hb, intracellular metHb concentrations displayed a significant increase in all diabetic plasma groups (HbA_{1c} L, M, and H) at the 24 h time point (Figure 15.B). This suggests a potential Hb oxidation process within the RBCs upon exposure to diabetic plasma. Interestingly, Figure 15.B also reveals a decrease in intracellular metHb levels in all diabetic plasma groups after 48 h. This decrease, particularly pronounced in the HbA_{1c} H group (0.055 ± 0.010 AU), suggests a potential release of metHb from the RBCs into the extracellular medium. Furthermore, Figure 15.C highlights a positive correlation between the initial increase in intracellular metHb and the increasing HbA_{1c} levels in the diabetic plasma groups. This suggests a potential association between the severity of the poor glycemic control (as indicated by HbA_{1c}) and the degree of Hb oxidation within the RBCs.



B

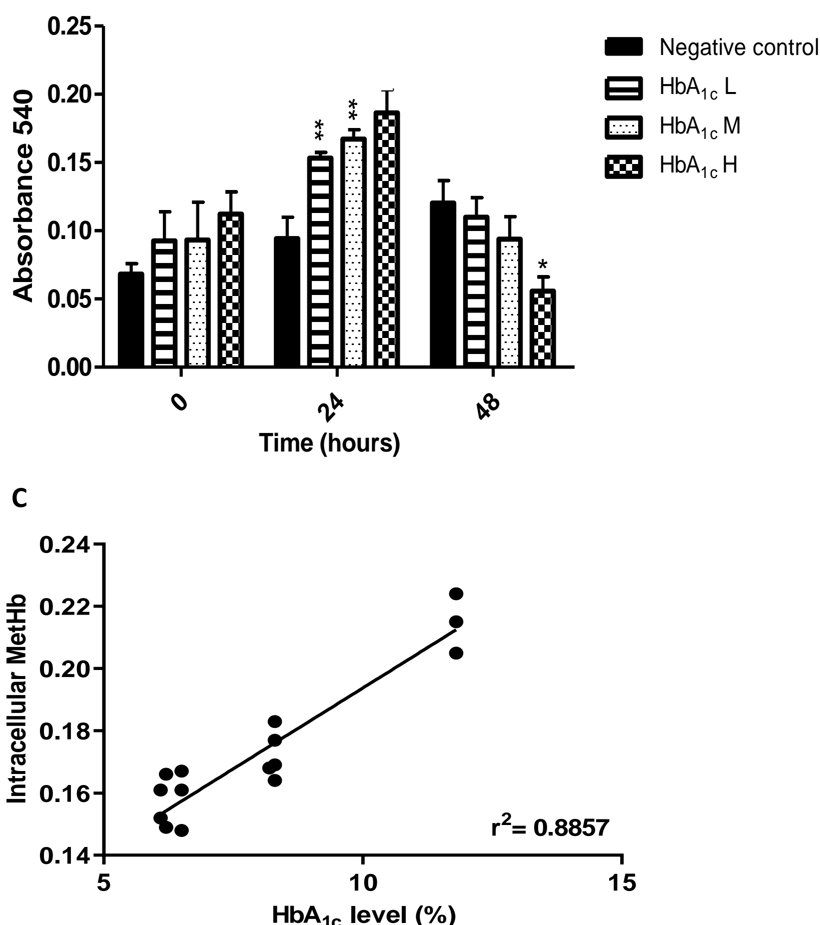


Figure 15: Levels of intracellular hemoglobin and methemoglobin of human RBCs after 0, 24, and 48 h of co-incubation with diabetic plasma at different HbA_{1c} levels. A: intracellular Hb; B: intracellular metHb. C: correlation between HbA_{1c} values and the intracellular metHb after 24 h of co-incubation. Negative control: RBCs treated with 0.9% NaCl, HbA_{1c} L: RBCs treated with diabetic plasma at low HbA_{1c} levels, HbA_{1c} M: RBCs treated with diabetic plasma at moderate HbA_{1c} levels, and HbA_{1c} H: RBCs treated with diabetic plasma at high HbA_{1c} levels. The data are expressed as means ± S.E.M. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ compared to the control group.

III.3. Evaluation of the hemoglobin stability

In the Hb stability assay, initial observations revealed no discernible distinctions across all experimental groups until the 6 h of co-incubation. However, a significant decline was manifested within the HbA_{1c} H Group, denoted by a significant reduction to 0.735 ± 0.080 AU. This finding suggests an early susceptibility to destabilization in RBCs exposed to high HbA_{1c} plasma. Furthermore, Figure 16 demonstrates that at the 24 h of co-incubation, intact Hb levels remained lower in all samples treated with diabetic plasma compared to the negative control (0.898 ± 0.089 AU). Notably, the most critical reduction in Hb levels was observed in the HbA_{1c} H group (0.600 ± 0.001 AU). This substantial decrease in Hb stability highlights the potential

for diabetic plasma, particularly with high HbA_{1c} levels, to accelerate Hb destabilization within RBCs.

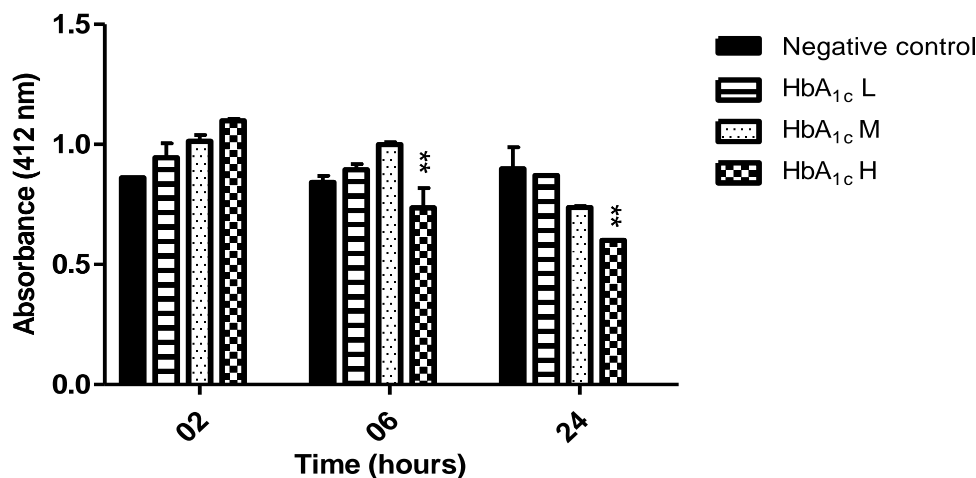


Figure 16: Evaluation of Hb stability after 02, 06 and 24 h of co-incubation with diabetic plasma at different HbA_{1c} levels. Negative control: RBCs treated with 0.9% NaCl, HbA_{1c} L: RBCs treated with diabetic plasma at low HbA_{1c} levels, HbA_{1c} M: RBCs treated with diabetic plasma at moderate HbA_{1c} levels, and HbA_{1c} H: RBCs treated with diabetic plasma at high HbA_{1c} levels. The data are expressed as means ± S.E.M. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ compared with control group.

III.4. Lipid peroxidation

As illustrated in Figure 17, thiobarbituric acid reactivity with RBCs was significantly elevated in RBCs co-incubated with all diabetic plasma groups (HbA_{1c} L, M, and H) compared to the untreated control group (0.082 ± 0.010 AU). This finding suggests a substantial increase in lipid peroxidation within the RBC membranes upon exposure to diabetic plasma. Furthermore, the HbA_{1c} H group displayed the highest level of MDA formation (0.320 ± 0.04 AU) compared to the HbA_{1c} M and HbA_{1c} L groups (0.247 ± 0.080 AU and 0.256 ± 0.090 AU, respectively). This observation suggests a positive correlation between the poor glycemic control (indicated by HbA_{1c} levels) and the degree of lipid peroxidation in the RBC membranes. Briefly, the higher the HbA_{1c} in the diabetic plasma, the greater the level of oxidative damage sustained on RBCs membranes.

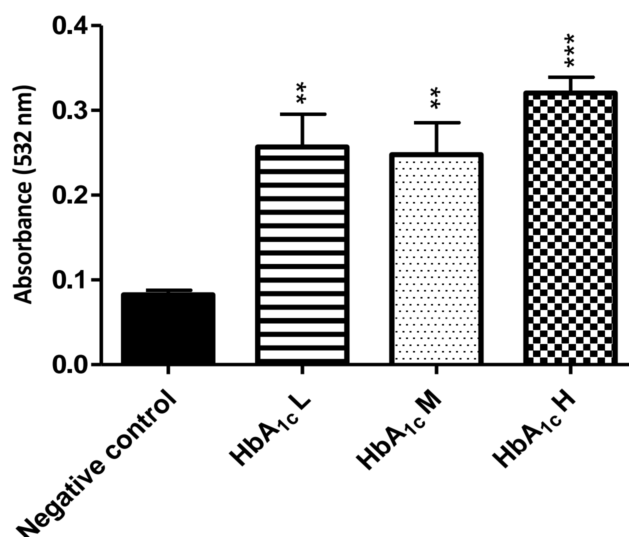


Figure 17: Malondialdehyde levels in human RBCs co-incubated with diabetic plasma at different HbA_{1c} levels. MDA levels in RBCs after 48 h of co-incubation with diabetic plasma at different HbA_{1c} (expressed in absorbance units of TBARS products). Negative control: RBCs treated with 0.9% NaCl, HbA_{1c} L: RBCs treated with diabetic plasma at low HbA_{1c} levels, HbA_{1c} M: RBCs treated with diabetic plasma at moderate HbA_{1c} levels, and HbA_{1c} H: RBCs treated with diabetic plasma at high HbA_{1c} levels. The data are expressed as means ± S.E.M. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ compared to the control group.

IV. Conclusion

In conclusion, our findings indicate a dual impact of diabetic plasma on erythrocyte integrity. This injury manifests as Hb denaturation within RBCs, associated with a concurrent overproduction of metHb and amplification of OS. Notably, the severity of these detrimental effects correlates positively with HbA_{1c} levels and metHb production. Collectively, these results suggest a potential dual role for metHb: (1) As a marker of diabetic status, exhibiting a positive correlation with HbA_{1c}. (2) As a contributor of OS amplification, a known underlying mechanism in various diabetic complications. Clinically, these observations warrant further investigation into the potential of metHb as a biomarker in diabetic patients. Additionally, a strong relationship exists between HbA_{1c} levels and metHb for assessing OS, particularly in patients with poor glycemic control.

CHAPITER III: *High HbA$_{1c}$ levels affect motility parameters and overexpress oxidative stress of human mature spermatozoa*

CHAPITER III: High HbA_{1c} levels affect motility parameters and overexpress oxidative stress of human mature spermatozoa

Abstract

This study aimed to investigate the *in vitro* impact of diabetic plasma on human sperm motility and OS. Plasma samples were collected from 51 male diabetic patients (mean age 62-28 ± 9.28 years) categorized based on HbA_{1c} levels: low (≤5%, 31 mmol/mol), moderate (6-8%, 42-64 mmol/mol), and high (≥10%, 86 mmol/mol). The effects of these plasma samples were then evaluated on eighteen normozoospermic semen samples. Sperm motility parameters were assessed using a computerized Sperm Class Analyzer®. While, OS status was analyzed through a TBARS assay. The results revealed a detrimental impact of diabetic plasma on all sperm motility parameters, with a dose-dependent effect based on HbA_{1c} levels. Notably, the high HbA_{1c} group displayed the most significant adverse effects. Specifically, low straight-line velocity (VSL) was observed in this group, particularly after 20 minutes of co-incubation (8.78 ± 0.47 μm/s). Furthermore, the TBARS assay revealed the highest levels of lipid peroxidation in the high HbA_{1c} group (0.14 ± 0.02 AU), exceeding those observed in sperm treated with H_2O_2 (0.13 ± 0.01 AU). These findings suggest a direct and HbA_{1c}-dependent negative impact of diabetic plasma on spermatozoa, potentially mediated by excessive generation of MDA. In conclusion, this study highlights the detrimental effects of diabetic plasma on sperm motility and the potential role of OS as the underlying mechanism. These observations emphasize the critical importance of clinically managing glycemic control and oxidative stress in diabetic patients to preserve male fertility.

I. Introduction

Glucose metabolism assumes a pivotal position within male reproductive physiology, exerting profound influence over crucial processes such as spermatogenesis, as well as the ultimate quality of gametes, encompassing motility and fertilization competence in mature sperm. (Ding *et al.*, 2015). Nevertheless, DM represents a significant public health concern, demonstrably exerting a detrimental influence on male reproductive function and fertility outcomes. Indeed, chronic hyperglycemia is well documented to contribute to the development of various systemic complications, including male-factor infertility (Ballester *et al.*, 2004). A convergence of experimental and clinical research has focused on elucidating the underlying mechanisms responsible for fertility concerns observed in diabetic patients (Delfino *et al.*, 2007; Hassan *et al.*, 1993; Kriegel *et al.*, 2009; Ramalho-Santos *et al.*, 2008; Scarano *et al.*, 2006).

DM can significantly compromise male fertility through a multifaceted interplay of molecular mechanisms and pathways. These pathways can exert their detrimental effects by either directly disrupting spermatogenesis, or via impairment of penile erection and ejaculation. (Bhattacharya *et al.*, 2014; Sexton *et al.*, 1997). Furthermore, DM disrupts the delicate endocrine control of spermatogenesis and sperm metabolism through a reduction in LH and FSH levels (Ballester *et al.*, 2004; Kriegel *et al.*, 2009). *In vivo* studies, using diabetic animal models, have demonstrated a constellation of adverse effects on male reproductive function. These findings include a reduction in the weight of reproductive organs, particularly the testes, diminished sperm content within the ejaculate, compromised sperm motility, and a concurrent increase in the number of degenerating germ cells at various stages of spermatogenesis (Hassan *et al.*, 1993; Ramalho-Santos *et al.*, 2008; Scarano *et al.*, 2006). Besides, diabetic individuals have demonstrated alterations in human sperm parameters, characterized notably by diminished sperm motility and concentration, alongside an increased prevalence of abnormalities in cell morphology, mitochondria, and plasma membranes (Delfino *et al.*, 2007). In addition, hyperglycemia has been shown to augment damage to sperm nuclear and mitochondrial DNA (Kriegel *et al.*, 2009).

Recently, it has been demonstrated that non-enzymatic glycation is a key contributor, among several biochemical pathways, to the development of pathogenic complications in DM associated with hyperglycemia (Krishnamurti *et al.*, 2001). This mechanism is postulated to significantly elevate the formation of AGEs (Giacco *et al.*, 2010; Giugliano *et al.*, 1996). Interestingly, non-enzymatic glycosylation disrupts the structural and functional integrity of various proteins, including Hb (Brownlee, 1994).

In addition to the increased AGEs formation, hyperglycemia triggers a cascade of additional biochemical mechanisms that culminate in overproduction of free radicals, particularly ROS (Giacco *et al.*, 2010). These mechanisms include: (1) enhanced glycolysis, (2) glucose autoxidation, (3) elevated flux through the hexosamine pathway, and (4) increased activity of the polyol pathway (Ramalho-Santos *et al.*, 2008). However, excessive mitochondrial electron transport chain activity due to hyperglycemia is believed to be the primary source of superoxide anion $O_2^{\cdot -}$ and other ROS. This overload results from hyperpolarization of the mitochondrial membrane potential, accompanied by increased respiration and depletion of NADPH (Ramalho-Santos *et al.*, 2008). Additionally, NADPH oxidase (NOX) enzymes converting NADPH to $NADP^+$, represent a potential source of ROS (Segal *et al.*, 1993). This upsurge in OS within spermatozoa can inflict further damage to mitochondria and mtDNA, ultimately leading to an energy deficit and a decline in sperm motility and fertility (Wei *et al.*, 2000).

Importantly, the interplay between sperm metabolism and DM is closely intertwined with elevated levels of OS. Emerging evidence strongly suggests that the primary factor contributing to reduce fertility in diabetic individuals is the excessive generation of ROS, associated with their detrimental effects on sperm quality (Ramalho-Santos *et al.*, 2008). Sperm cells are exceptionally susceptible to OS induced by ROS, owing to the abundance of PUFAs in their plasma membrane (Aitken *et al.*, 2012). These by-products of aerobic metabolism, increased in hyperglycemic conditions, have the propensity to induce a plethora of modifications in cellular constituents, encompassing proteins, lipids, and DNA, and may even trigger cellular apoptosis (Aitken *et al.*, 2011; Grunewald *et al.*, 2008). Notably, recent emphasis has been placed on assessing levels of lipid peroxidation in the semen of diabetic patients (La Vignera *et al.*, 2012).

However, our understanding of the direct effects of diabetes on sperm cells, devoid of confounding influences such as *in vivo* endocrine disruption, remains limited among diabetic patients. Consequently, *in vitro* models offer a valuable tool to investigate this direct effect, potentially elucidating the multi-level impact of diabetes on fertility within the hypothalamic-pituitary-gonadal axis. Moreover, the precise impact of DM on mature sperm cells at the molecular level remains poorly understood. Notably, there is a notable lack of research and inconsistencies in our understanding of the role of OS mediated by DM in human spermatozoa dysfunction.

In this context, the co-incubation of plasma with sperm cells has emerged as a potent model for assessing the influence of diverse conditions on sperm of human and other animal species. This approach is especially advantageous owing to the similarity of the biochemical compositions of both biological fluids (blood and diabetic plasma) (Benedetti *et al.*, 2012; Rosecrans *et al.*, 1987). Hence, the influence of blood plasma on sperm motility has been extensively explored in a variety of mammalian species, including humans (De Lamirande *et al.*, 1991; Liu *et al.*, 1986), dogs (Verstegen *et al.*, 2005), goats (Saha *et al.*, 2013), hamster (Yanagimachi, 1970), ram (Csiba *et al.*, 2015) and bovine (Nait Mouloud *et al.*, 2017).

Given the contextual background, the current study sought to investigate two main objectives: firstly, to assess the direct impact of diabetic human plasma on sperm motility, and secondly, to discern the underlying mechanisms by analyzing the OS status of mature sperm cells.

II. Materials and methods

II.1. Chemicals

All chemicals were obtained from Sigma-Aldrich Company groups (St. Louis, MO, USA).

II.2. Patients

A consecutive sample of 51 male patients diagnosed with type T2DM and aged 14-88 years were attending the Centre of Diabetes for routine check-ups and participated in this study. These individuals were categorized into three groups (Group II, Group III, and Group IV) based on HbA$_{1c}$ levels. Demographic and clinical characteristics, including age, sex, duration of diabetes, smoking status, and presence of complications, were collected for all participants (Table 4).

<table>
<tr><th>Groups</th><th>No of patients</th><th>Serum glucose concentration (g/l)
(Mean ± SEM)</th><th>HbA$_{1c}$ values</th><th>Age (year)
(Mean ±SEM)</th><th>Duration of illness (year)
(Mean ± SEM)</th><th>Smoking</th><th>Compli cations</th><th>Pharmacotherapy</th></tr>
<tr><td>Group I</td><td>17</td><td>1.32 ± 0.16</td><td>≤ 31 mmol/mol) (5%)</td><td rowspan="3">62.28± 9.28</td><td rowspan="3">16.50 ± 6.98</td><td rowspan="3">No</td><td rowspan="3">No</td><td rowspan="3">Oral Antidiabetic Drugs + Insulin</td></tr>
<tr><td>Group II</td><td>17</td><td>1.37 ± 0.05</td><td>> 42 mmol (6%) & ≤ 64 mmol / mol (8%)</td></tr>
<tr><td>Group III</td><td>17</td><td>2.5 ± 0.14</td><td>≥ 86 mmol / mol (10%)</td></tr>
</table>

Table 4: The different groups of diabetic men according to their mean HbA1c level.

Group I served as negative control and received a Tris extender solution unsupplemented with plasma.

II.3. Ethical considerations

This research has been done according to Helsinki Declaration, and approved by the independent ethics committee at the Algerian National Agency for the Development of Health Research (ANDRS). All participants provided informed consent prior to enrolment.

II.3.1. Preparation of diabetic Plasma

Venous blood samples were obtained from diabetic human donors via venipuncture into heparin tubes (4000 USP units/mL). Following blood collection, plasma was isolated from cellular components by centrifugation at 3000 rpm for 10 min. The isolated plasma was then stored at -20°C until further analysis (Halder *et al.*, 1998).

II.3.2. Selection criteria of semen samples

Semen samples in this study were obtained from normozoospermic donors as defined by the most recent WHO criteria (World Health Organization, 2010). Following an abstinence period of 3-5 days, healthy donors provided a total of 18 ejaculate samples via masturbation into sterile containers. After liquefaction at room temperature for 20 min, each aliquot was assessed for sperm concentration, motility, and pH according to the latest WHO standard guidelines (Lu *et al.*, 2010).

II.3.3. Semen preparation and treatment

Semen samples were centrifuged at room temperature for 2000 g for 7 min (Vignera *et al.*, 2012). Following centrifugation, the seminal plasma was aspirated, and the sperm pellet was resuspended in a Tris-based extender solution with a volume equal to the discarded seminal plasma. The extender solution composition included Tris (30.8 g/L), citric acid (17 g/L), and fructose (12.5 g/L). To assess sperm motility, sperm suspensions were diluted in a Tris buffer to achieve a final concentration of 60 million spermatozoa after the addition of diabetic plasma.

The diluted sperm suspension was aliquoted into four groups. Three aliquots were supplemented with diabetic plasma with different HbA_{1c} levels at a final concentration of 20% (Liu *et al.*, 1986). Additionally, a control aliquot was maintained with Tris extender solution

without plasma supplementation. Subsequently, all diluted samples were co-incubated at room temperature prior to analysis.

II.3.4. Motility assessment

Sperm kinematic parameters were assessed at 10 min intervals for each sample until complete stopping of sperm motility using a Sperm Class Analyzer® (SCA) version 5.4 system (Microptic S.L., Barcelona, Spain). This system (Computer-Assisted Sperm Analysis) incorporates a Basler A312fc digital camera mounted on a Nikon Eclipse E200 microscope (Nikon, Tokyo, Japan) for automated sperm motility analysis.

For each sample, a 10 µL aliquot was loaded onto a Makler chamber (Sefi-Medical Instruments Ltd., Italy) with a 10 µm depth and pre-warmed to 37°C for 30 seconds to ensure optimal physiological conditions. Sperm motility was then evaluated using a 10x phase-contrast objective. The following kinematic parameters were measured:

- Straight-line velocity (VSL): represents the average speed of a sperm's movement in a straight line from its starting to ending position.
- Curvilinear velocity (VCL): represents the average velocity calculated based on the specific path followed by the cell from point to point.
- Average path velocity (VAP): represents the average velocity along the smoothed, non-linear sperm trajectory.
- Progressive rapid motility (PR): defined as the percentage of spermatozoa exhibiting a VCL exceeding 25 µm/s and demonstrating straight-line motility.

II.3.5. Measurement of malondialdehyde

Following a period of 60 min of co-incubation with the various treatments, the sperm concentration in the final pellet was standardized to a range of 10 to 20 million spermatozoa per ml for subsequent lipid peroxidation analysis (Rao *et al.*, 1989).

The MDA concentration was quantified independently in both spermatozoa and each of the dilution treatments to serve as a convenient indicator of lipid peroxidation, utilizing TBA-assay according to Buege and Aust (Buege *et al.*, 1978) with some modifications. Following a 1 h co-incubation of spermatozoa with the diverse treatments (Tris, diabetic plasma at different HbA_{1c} levels, and H_2O_2 at 100 µM, as a positive control), all samples were centrifuged at 800 g for 10 min to isolate the spermatozoa from the treatment medias. Then, distilled water was introduced to the pellets, and the cells were subjected to sonication (20hz/ 40 S). Additionally,

315 µl of TCA (30%) and 160 µl of TBA (1%) were added to both the sonicated sperm (pellets) and the supernatant of each treatment media. The samples were then boiled for 15 min at 95 °C and subsequently cooled in an ice bath. Finally, the absorbance was measured at 535 nm.

II.3.6. Statistical analysis

Data were collected and expressed as mean ± SEM and analyzed by GraphPad Prism 5.0 (San Diego, California, USA). Student's t-test was used to compare the samples treated with diabetic plasma to the negative control. $p < 0.05$ was considered statistically significant.

III. Results

III.1. Kinematic parameters

Figure 18 illustrates the effects of diabetic plasma on sperm kinematic parameters, including VCL, VSL, and VAP, which were measured in all samples after co-incubation periods of 10, 20, 30, 40, 50, 60, 70, 80, and 90 min. A decrease in both VSL and VAP was observed in all treatment groups after only 10 min of co-incubation. However, the decline in VCL occurred with a slight delay, becoming statistically significant only after 20 min of co-incubation. At this stage, VCL values in the HbA_{1c} L, HbA_{1c} M, and HbA_{1c} H groups were 26.89 ± 0.59 µm/s, 26.45 ± 0.71 µm/s, and 15.83 ± 1.55 µm/s, respectively. Compared to the control group, the co-incubation with diabetic plasma resulted in a significant and detrimental effect on sperm motility starting at 20 min. Notably, the HbA_{1c} H group displayed the most severe impairment, exhibiting complete cessation of motility by 80 min of co-incubation (Figure 13). Sperm treated with diabetic plasma having lower HbA_{1c} levels (HbA_{1c} L and HbA_{1c} M) exhibited intermediate motility decline. A significant difference between these groups was observed from 30 min onwards for VSL and VAP, with the HbA_{1c} L group, demonstrating the highest values (10.72 ± 0.20 µm/s for VSL and 14.87 ± 0.20 µm/s for VAP)..

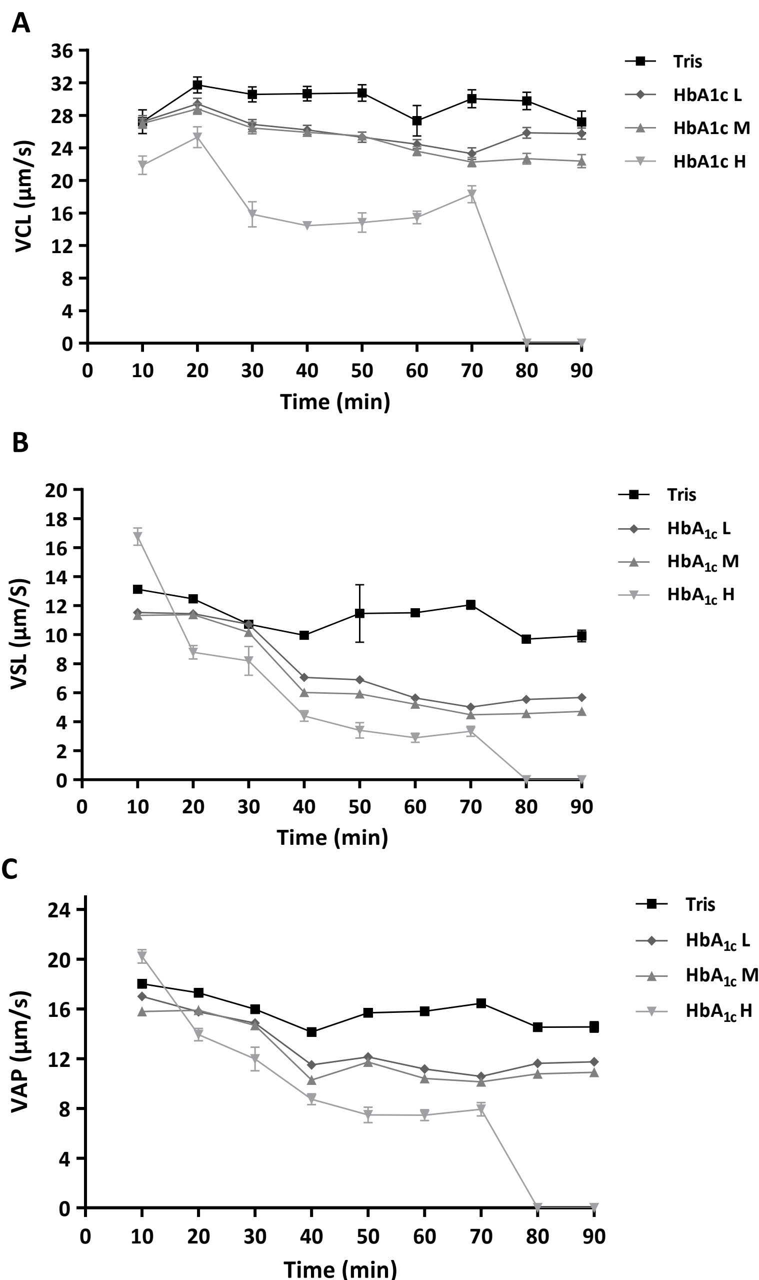


Figure 18: Kinematic parameters of human sperm co-incubated with diabetic plasma at different HbA_{1c} levels.(**A**) curvilinear velocity (VCL), (**B**) straight linear velocity (VSL) and (**C**) average path velocity (VAP), after 10, 20, 30, 40, 50, 60, 70, 80 and 90 min of co-incubation of human sperm in the different tested samples: control group (Tris) and groups treated with plasma of diabetic males at different concentration of HbA_{1c}. HbA_{1c} L: low glycated hemoglobin, HbA_{1c} M: moderate glycated hemoglobin, HbA_{1c} H: high glycated hemoglobin.

Figure 19 illustrates a notable decline in PR spermatozoa across all groups treated with diabetic plasma. PR spermatozoa are characterized by substantial forward movement and are considered crucial for fertilization success. Notably, co-incubation with HbA_{1c} H plasma resulted in a dramatic decline in PR, with values reaching 0.93 ± 0.60% after only 30 min. Up

to the 30-min mark, HbA_{1c} L group revealed a higher percentage of progressively motile spermatozoa (3.62 ± 1.70%) compared to the HbA_{1c} M group (2.24 ± 0.67%). However, following 30 min of co-incubation, all diabetic plasma groups (HbA_{1c} L, HbA_{1c} M, and HbA_{1c} H) displayed values approaching zero, while the control group maintained the highest PR values.

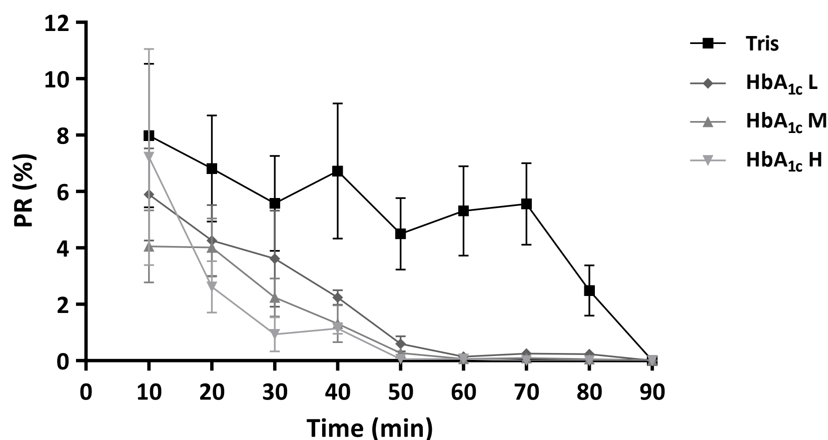


Figure 19: Progressive rapid motility of human sperm co-incubated with diabetic plasma at different HbA_{1c} levels. The % of PR were obtained after 10, 20, 30, 40, 50, 60, 70, 80, and 90 min of co-incubation of human sperm in different tested samples: control group (Tris) and groups treated with plasma of male diabetic at different concentration of HbA_{1c}. HbA_{1c} L: low glycated hemoglobin, HbA_{1c} M: moderate glycated hemoglobin, HbA_{1c} H: high glycated hemoglobin).

III.2. Lipid peroxidation

Figure 20 demonstrated the evaluation of lipid peroxidation levels in human sperm following 1 h co-incubation with diabetic plasma at different HbA_{1c} levels. Measurements were performed in both the sperm pellet (cells) and the supernatant (treatment media) of each sample. The data revealed significant differences in mean MDA levels across all groups, with the same tendency observed in both the pellet and supernatant fractions. Compared to the control group (Tris extender), MDA formation increased significantly in sperm treated with diabetic plasma and H_2O_2 (positive control). The HbA_{1c} H group displayed the highest MDA level (0.14 ± 0.01 AU), followed by the HbA_{1c} M group (0.12 ± 0.01 AU) and the HbA_{1c} L group (0.08 ± 0.01 AU). Notably, these findings suggest an HbA_{1c}-dependent effect on OS, with MDA values progressively decreasing from the highest to the lowest HbA_{1c} group (HbA_{1c} H > HbA_{1c} M > HbA_{1c} L). Interestingly, the HbA_{1c} H group even surpassed the H_2O_2-treated samples in terms of OS levels (0.13 ± 0.01 AU).

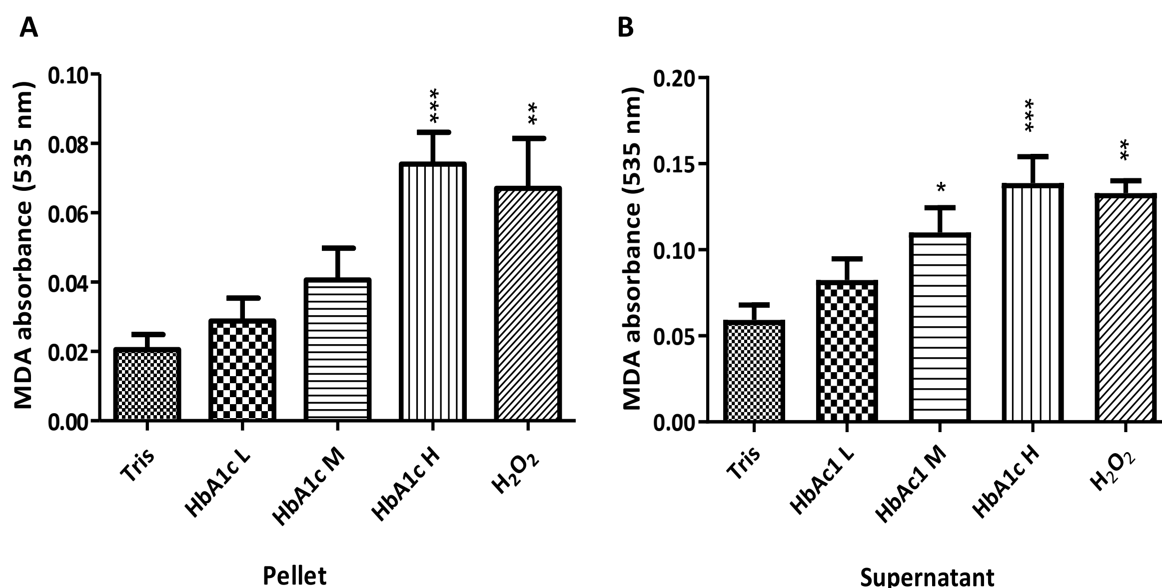


Figure 20: Malondialdehyde levels after co-incubation during 60 min with human diabetic plasma at different concentration of HbA_{1c}. (**A**) MDA levels measured in supernatant, (**B**) MDA levels measured in pellet. Tris: negative control, H_2O_2: positive control, HbA_{1c} L: low glycated hemoglobin, HbA_{1c} M: moderate glycated hemoglobin, HbA_{1c} H: high glycated hemoglobin. The data are expressed as means ± S.E.M. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ compared with control group.

IV. Conclusion

The present study suggests a direct detrimental effect of diabetic plasma on mature spermatozoa, potentially contributing to fertility issues in diabetic patients. This effect is independent of other reported factors, such as altered sexual hormone levels and impaired spermatogenesis. Our findings implicate OS as a key underlying mechanism responsible for sperm dysfunction. The observed correlation between increasing HbA_{1c} levels and declining sperm quality further emphasizes the importance of glycemic control in diabetic men. Maintaining optimal glycemic levels may offer a strategy to mitigate OS and potentially preserve fertility. Furthermore, the study suggested the potential benefits of exploring protective effects offered by antioxidant therapies, particularly utilizing antioxidant compounds known for their significant impacts on fertility outcomes.

CHAPTER IV: ***Combined Vitamin C and Cyclodextrin- Vitamin E reduces oxidative stress and improves the motility of human sperm exposed to diabetic plasma at high HbA_{1c} levels***

CHAPTER IV: Combined Vitamin C and Cyclodextrin- Vitamin E reduces oxidative stress and improves the motility of human sperm exposed to diabetic plasma at high HbA_{1c}

ABSTRACT

The aim of the current study was to evaluate the protective effect of vitamin C (Vit C) and vitamin E loaded in cyclodextrin (CD-Vit E) alone or in association on human sperm incubated with blood plasma of patients with uncontrolled diabetes (high HbA_{1c} levels). Semen samples with normozoospermic (N= 16) were exposed to high HbA_{1c} levels in plasma from diabetic individuals (≥10%), considered as the control (sperm + plasma). In the treated groups (sperm + plasma + vitamins), Vit C and CD-Vit E were added to the sperm samples. The sperm quality was determined by the assessment of sperm motility using Computer Assisted Semen Analysis (CASA), and the oxidative status by MDA production using a Thiobarbituric Acid Reactive Substance (TBARS) assay. The sperm kinematic parameters (VCL: 10.587 ± 0.204 µm/s, VSL: 4.902 ± 0.121 µm/s, and VAP: 6.981 ± 0.142 µm/s) and gametes progressive movements (moderate progressive: 13.299 ± 1.791% and rapid progressive: 3.583 ± 0.579%) showed a dramatic decline after 30 min following the incubation with diabetic plasma. However, sperm pre-treated with Vit C (0.1 mg/ml) or CD-Vit E (0.25 mg/ml) alone and in association, reported an effective improvement of all sperm parameters. The association of the two vitamins showed the highest values in VCL, VSL and VAP (21.181 ± 0.289 µm/s, 10.364 ± 0.182 µm/s, and 13.636 ± 0.128µm/s, respectively), and enhanced moderate and rapid progressive gametes (27.862 ± 1.708% and 11.214± 1.877%, respectively) with a significant decrease in immobile cells (30.539 ±3.722 %). Also, the co-treatment with Vit C and CD-Vit E reduced significantly MDA levels (0.059 ± 0.011 AU) compared to the non-protected group (0.910 ± 0.022 AU). The present results indicate that the association of Vit C and CD-Vit E exhibited a potent protective effect on human sperm by reducing the adverse side effects of hyperglycemia-induced OS.

I. Introduction

Oxygen is an essential element, but its metabolic byproducts, particularly ROS, can trigger a cascade of cellular dysfunction. Particularly, at their elevated rate, ROS cause systemic dysfunctions of the male reproductive system (De Lamirande *et al.*, 1997). In this respect, different pathologies can be involved in the overproduction of ROS including DM (Ramalho-Santos *et al.*, 2008).

DM is a chronic disease characterized by a multi-faceted metabolic disorder and represents a major global health concern due to its serious complications, namely male sexual dysfunction (Agbaje *et al.*, 2007). Elevated free radical levels in DM coincide with a concomitant decrease in antioxidant defense mechanisms, ultimately resulting in cellular organelle dysfunction (proteins, lipids, and DNA) (Ramalho-Santos *et al.*, 2008).

During normal physiological processes, human sperm generate controlled levels of ROS, acting as signaling molecules crucial for capacitation, the acrosome reaction, and ultimately, fertilization (Griveau *et al.*, 1994). However, excessive ROS are strongly associated with the loss of sperm function by declining motility, viability, and cause sub-fertility and even male infertility (Tremellen, 2008). Indeed, the absence of DNA repair mechanisms in sperm cells, the high levels of polyunsaturated fatty acids in the membrane, and the low levels of cytoplasmic antioxidant enzymes are the mean causes for the sensitivity of mature spermatozoa against ROS attacks (Agarwal *et al.*, 2008; Prakash *et al.*, 2014). However, seminal plasma is well endowed with an antioxidant system that protects cell metabolism from damages caused by OS (Sikka, 2004). This system of cellular defenses is divided into primary antioxidant enzymes, including superoxide dismutase (SOD), Catalase (CAT) and glutathione peroxidase (GPX), and secondary non-enzymatic antioxidants such as vitamin C (ascorbic acid), vitamin E (α-tocopherol), pyruvate, glutathione, and carnitine (Saleh *et al.*, 2002). Nevertheless, a dramatic decrease in antioxidants and their activity is observed during DM.

Actually, an ongoing research is developed about the potential benefits of antioxidant supplements in diabetic patients (Golbidi *et al.*, 2011). In this respect, different antioxidants, especially vitamins, were tested individually and in combination at different doses on male infertility (Agarwal *et al.*, 2004; Mazloom *et al.*, 2011). These small molecules such as vitamin E (Vit E) and vitamin C (Vit C) have a powerful effect on free radicals scavenging (Jones *et al.*, 1995).

Vit C is well known to have a critical role in protecting spermatogenesis and in improving sperm integrity and fertility against OS in many species including humans (Greco *et al.*, 2005) and animals (Shrilatha, 2007). Therefore, it plays a major role in increasing testosterone levels, prevents agglutination of sperm, improves its quality (Thiele *et al.*, 1995), and protects human spermatozoa against endogenous oxidative DNA damage (Fraga *et al.*, 1991).

Also, Vit E, a lipid-soluble molecule, acts mainly within cell membranes (Ford *et al.*, 1998). Supplementation with appropriate doses of this antioxidant in infertile men causes a slight increase in its seminal plasma concentration (Moilanen *et al.*, 1995; Moilanen *et al.*, 1993). Several *in vitro* studies have reported its effectiveness in protecting motility, the morphology of sperm, and in reducing its MDA concentrations (Suleiman *et al.*, 1996). When this vitamin is preloaded in cyclodextrins, this increases its solubility and enhances its protective activity on different sperm motility parameters, membrane functionality, and lipid peroxidation (Benhenia *et al.*, 2016).

When associated, these two vitamins act synergically to protect membrane lipids from damage, prevent ROS accumulation, and therefore preserve cell's function (Packer *et al.*, 1979). Several experimental and clinical trials have highlighted therapeutic strategies using antioxidant supplementation with the aim of reducing ROS damages (Agarwal *et al.*, 2004). However, there is no well-defined therapeutically protocol using vitamins in diabetes-induced OS in human reproductive medicine. Particularly, there are no sufficient data exploring the protective effect of the combined Vit C and CD-Vit E against OS induced by diabetic plasma on human spermatozoa.

In this regard, this study was carried out to investigate the protective effect of Vit C and CD-Vit E, alone or in association on mature spermatozoa exposed to diabetic plasma with high HbA_{1c} levels.

II. Material and methods

II.1. Chemicals

All chemicals were obtained from Sigma-Aldrich Company groups (St. Louis, MO, USA).

II.2. Collection of biological samples

II.2.1. Selection of semen samples

Human sperm was collected from a total of 16 healthy men with normozoospermic parameters following a recommended minimum of 3 days and a maximum of 5 days of sexual abstinence. The samples were brought directly to the laboratory and liquefied completely at 37°C. The handling of samples was in compliance with the recommendations of World Health Organization (WHO).

II.2.2. Selection of diabetic plasma

Blood samples were obtained from 20 diabetic patients and collected in heparin tubes (4,000 USP units/L of blood). Glycated hemoglobin was then measured for each sample, and only those with high levels ($HbA_{1c} \geq 10\%$; 86 mmol/mol) were selected in this study. Following centrifugation at 865 g for 10 minutes, plasma was collected and stored at -20°C until further analysis (Halder *et al.*, 1998).

II.3. Ethical considerations

This research has been done according to Helsinki Declaration and approved by the independent ethics committee at the Algerian National Agency for the Development of Health Research (ANDRS). All participants provided informed consent prior to enrolment.

II.4. General experimental design

The aim of the study was to evaluate the protective effect of Vit C and CD-Vit E alone or in association on human sperm quality incubated with blood plasma of patients with uncontrolled diabetes (high HbA_{1c} level). The control groups (sperm + Tris or plasma) and the treated samples groups (sperm + plasma + vitamins) were assessed for sperm motility and oxidative status.

II.4.1. Preparation of media

Vitamin C was prepared daily. The required quantity (0.1 mg/ml) was diluted in Tris extender and stored in a dark container.

Permethyl-cyclodextrins-vitamin E (CD-Vit E) complexes were prepared with a co-evaporation method with a molar ratio of 2:1 (141.03 mg: 100. mg). The cyclodextrin with vitamin E was dissolved in 75 ml of ethanol. The resultant mixture was maintained under stirring for 24 h at room temperature and shielded from light. The solvent was then evaporated under vacuum at 40°C by rotary evaporation and the residue was kept in a desiccator until used (Benhenia *et al.*, 2016). The CD-Vit E complexes were daily solubilized in Tris buffer to obtain the finally concentration (0.25 mg/ml).

II.4.2. Antioxidant treatments and diabetic plasma co-incubation

Semen samples selected were divided into five equal aliquots, then centrifuged at 2000 g for 7 min at room temperature (La Vignera *et al.*, 2012). The sperm pellet was resuspended in a volume of the original seminal plasma equivalent to the discarded supernatant in:

- Tris extender lonely.
- Tris with vitamin C at 0.1 mg/ml (Vit C).
- Tris with Permethyl-cyclodextrins-vitamin E at 0.25 mg/ml (CD-Vit E).
- Tris with Permethyl-cyclodextrins-vitamin E at 0.25 mg/ml + Vitamin C at 0.1 mg/ml (Vit C+ CD-Vit E).

After 20 min of incubation at 37°C of washed spermatozoa with the different media described above, 80% of each aliquot underwent co-incubation with diabetic plasma at a final concentration of 20% (v/v). While the fifth group was considered as a negative control (treated only with Tris with no addition of diabetic plasma and no treatment).

II.4.3. Motility analysis by computer-assisted sperm analysis (CASA)

Sperm motility was assessed using the Sperm Class Analyzer® (SCA) Version 5.4 (Microptic S.L., Barcelona, Spain). This system integrates a Basler A312fc digital camera mounted on a Nikon Eclipse E200 microscope (Nikon, Tokyo, Japan). A 10 µm-depth Makler counting chamber (Sefi-Medical Instruments Ltd., Biosigma S.r.l., Italy) was loaded with 10 µl of each sperm solution and pre-warmed for 30 seconds at 37°C. Sperm motility was evaluated

under a phase-contrast microscope at 10x magnification by a single investigator throughout the study.

The sperm samples were analyzed by measuring: VAP, VCL, and VSL, progressive and immotile spermatozoa. Values were recorded for each sperm group after 0, 30, 60, 90, and 120 min of incubation.

Spermatozoa were classified as mobile with progressive movement or immobile. This analysis was performed as described by Perobelli *et al.* (2010).

II.4.4. Estimation of lipid peroxidation

Lipid peroxidation was estimated calorimetrically by measuring thiobarbituric acid reactive substances TBARS, using the methods of Buege *et al.* (1978) with some modifications. In brief, spermatozoa of each group were separated from the treatment media (Tris, diabetic plasma at high HbA_{1c} level with/ without antioxidant treatment) after 30 min of the incubation, by centrifugation at 800 *g* for 10 min. Following centrifugation, the sperm pellets were resuspended in distilled water and subjected to sonication for cell lysis. Subsequently, 315 µL of TCA and 160 µL of TBA were added to both the sonicated sperm (pellets) and the respective incubation media supernatants. Samples were then boiled for 15 min, followed by rapid cooling in an ice bath. Finally, the absorbance of both the supernatant and pellet fractions was measured at 535 nm.

II.5. Statistical Analysis

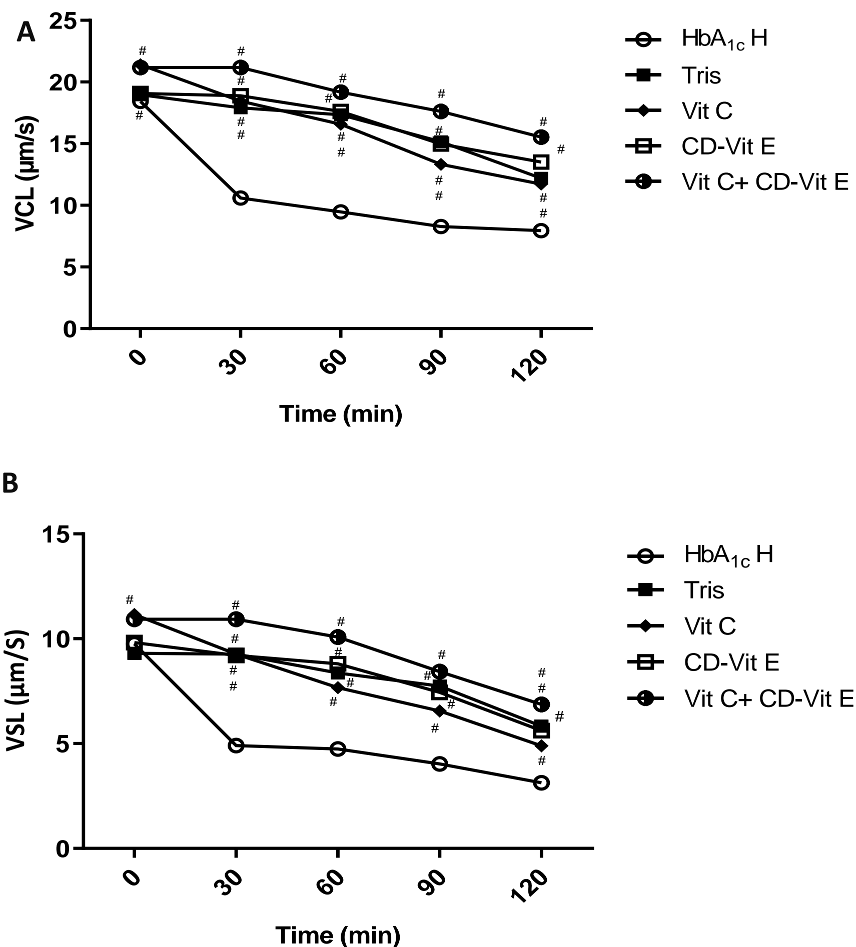
Statistical analyses were carried out using Statview 4.02 software (Abacus Concepts Inc., Berkeley, CA, USA). One-way and two-way ANOVA analyses were used to compare the differences concerning lipid peroxidation and Kinematic parameters, respectively, followed by Bonferroni *post hoc* test. #p: Comparison with diabetic plasma group (HbA_{1c}). The results are expressed as mean ± SD of the different experiments (# $p < 0.05$).

III. RESULTS

III.1. Sperm kinematic parameters

Figure 25 shows a significant decline of all sperm velocities (VCL, VSL, and VAP) in the HbA_{1c} H group (10.587 ± 0.204 µm/s, 4.902 ± 0.121 µm/s, and 6.981 ± 0.142 µm/s, respectively) following the 30 min of the incubation with diabetic plasma. Instead, the pre-

treatment with Vit C and CD-Vit E expressed the same improvement tendency as the negative control, with a significant difference between all groups remarkably after 30 min of the treatment. In fact, the highest values of VCL, VSL, and VAP were revealed in Vit C and CD-Vit E association (21.181 ± 0.289 µm/s, 10.364 ± 0.182 µm/s, and 13.636 ± 0.128µm/s, respectively), followed by CD-Vit E alone (18.885 ± 0.239 µm/s, 9.211 ± 0.150 µm/s, and 12.403 ± 0.167µm/s, respectively), then by Vit C alone (18.445± 0.207 µm/s, 9.272 ± 0.121 µm/s, and 12.443 ± 0.138µm/s, respectively).



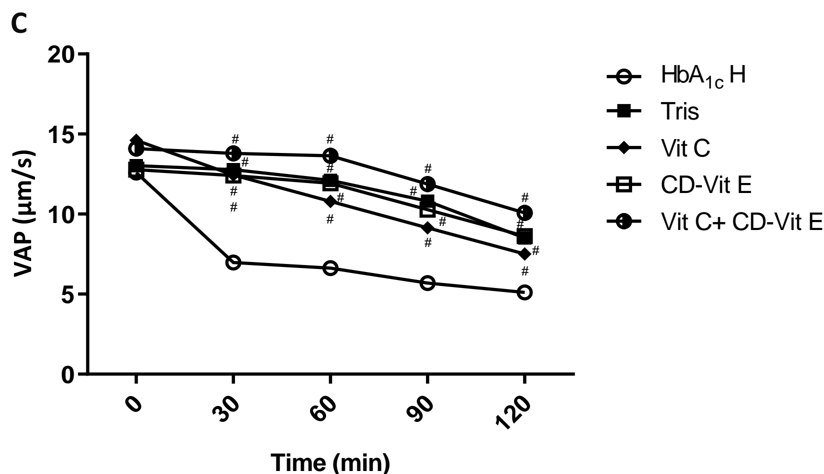


Figure 21: Effect of CD-Vit E and Vit C pre-treatment, alone or in association, on human spermatozoa velocities following the *in vitro* co-incubation with diabetic plasma with high HbA_{1c} levels. (a): curvilinear velocity (VCL), (b): straight linear velocity (VSL), and (c): average path velocity (VAP). HbA_{1c} H: Sperm cells co-incubated in diabetic plasma with high HbA_{1c} levels without any treatment; Tris: sperm cells suspended in Tris extender lonely; Vit C: sperm cells co-incubated with diabetic plasma after a pre-treatment with vitamin C; CD-Vit E: sperm cells co-incubated with diabetic plasma after a pre-treatment with vitamin E loaded in cyclodextrin; and Vit C+ CD-Vit E: sperm cells co-incubated with diabetic plasma after a co-treatment with vitamin E loaded in cyclodextrin and Vit C. #p: Comparison with diabetic plasma group (HbA_{1c}). The results are expressed as mean ± SD of the different experiments (# $p < 0.05$).

III.2. Progressive motility and percentage of immobile cells

The progressive motility of spermatozoa following co-incubation with diabetic plasma exhibiting high HbA_{1c} levels (HbA_{1c} H group) showed a significant decrease (13.299 ± 1.791% and 3.583 ± 0.579%, for moderate progressive “MP” and rapid progressive “RP” respectively), especially after 30 min of incubation. In parallel, the percentage of immobilized expressed an excessive augmentation (66.636 ± 3.541%) just after 30 min of the contact with diabetic plasma. Adversely, all these parameters were totally preserved in the pre-treatment with the association of Vit C and CD-Vit E. Interestingly, this co-treatment allowed simultaneously the best improvement in progressive motility (MP: 27.862 ± 1.708% and RP:11.214± 1.877%) and prevents cell immobility (30.539 ±3.722 %), better even than CD-Vit E (26.818 ± 2.207%; 10.016 ± 1.591%, and 40.444 ± 2.675 % for MP, RP, and immobile, respectively) and Vit C (22.919 ± 0.509%; 7.737 ± 0.865%, and 42.301 ± 1.509 % for MP, RP, and immobile, respectively). Indeed, the synergic action of these two antioxidants revealed a powerful protective effect on sperm motility and progressivity.

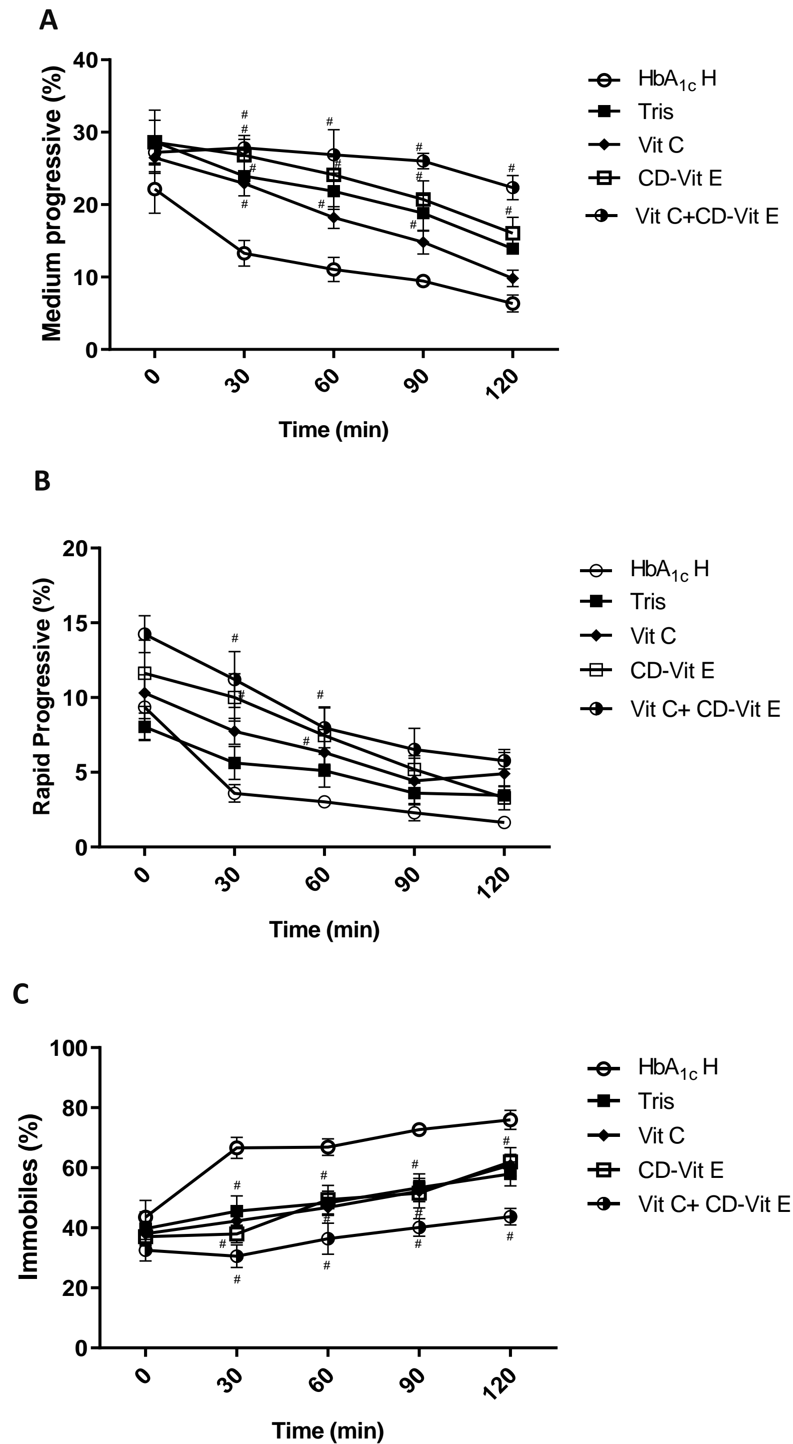


Figure 22: Effect of CD-Vit E and Vit C, separately and in combination, on Rapid progressive (a), Moderate progressive (b), and Immobile (c) spermatozoa co-incubated with diabetic plasma at high HbA_{1c} levels. HbA_{1c} H: Sperm cells co-incubated in diabetic plasma with high HbA_{1c} levels without any treatment; Tris: sperm cells suspended in Tris extender; Vit C: sperm cells co-incubated with diabetic plasma after a pre-treatment with vitamin C; CD-Vit E: sperm cells co-incubated with diabetic plasma after a pre-treatment with vitamin E loaded in cyclodextrin; and Vit C+ CD-Vit E: sperm cells co-incubated with diabetic plasma after a co-treatment with vitamin E loaded in cyclodextrin and Vit C. #p: Comparison with diabetic plasma group (HbA_{1c}). The results are expressed as mean ± SD of the different experiments (# $p < 0.05$).

III.3. MDA levels assessment

The degree of lipid peroxidation in all groups (controls and treated groups) is determined by the levels of MDA in the pellet and supernatant of each condition (Fig 23). Compared to the negative control (Tris group), the diabetic plasma enhanced significantly the quantities of MDA produced (0.910 ± 0.022 AU) during the incubation. However, this was totally reduced after the treatment with CD-Vit E and Vit C alone and in association. Notably, the lowest MDA levels were observed in the association treatment (0.059 ± 0.011 AU), followed by CD-Vit E (0.165 ± 0.38), and finally by Vit C treatment (0.353 ± 0.026 AU). The same trend was observed in pellet results.

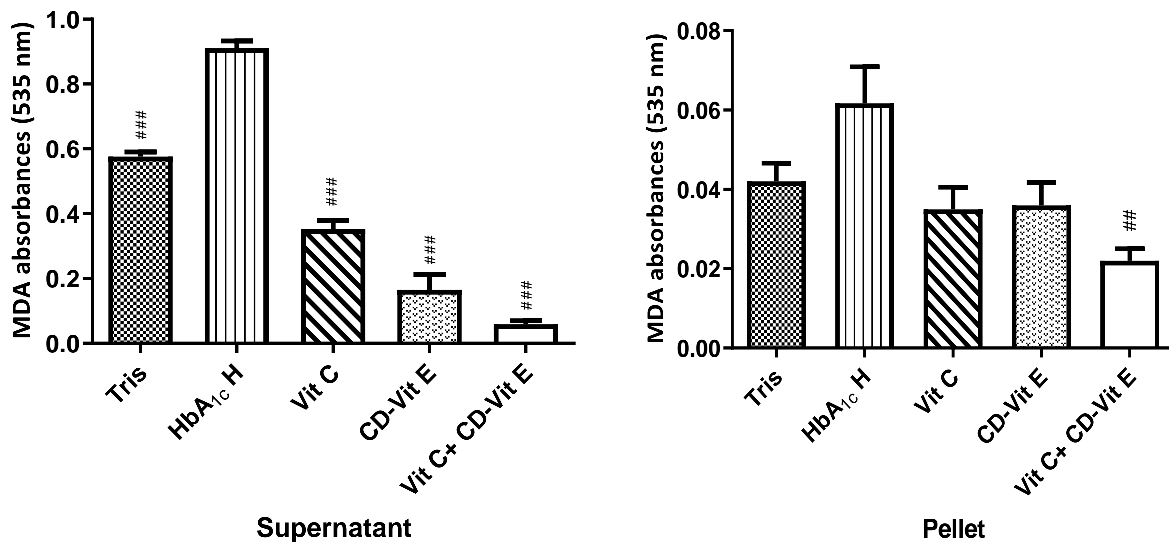


Figure 23: Malondialdehyde (MDA) levels in sperm cells exposed to diabetic plasma after a pre-treatment with CD-Vit E and Vit C, alone or in association. #p: Comparison with diabetic plasma group (HbA_{1c}). The results are expressed as mean ± SD of the different experiments (# $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$).

IV. Conclusion

The production of free radicals and OS under uncontrolled hyperglycemia appears to play a crucial role as a pathogenic factor in the alteration of mature spermatozoa and ultimately the reduction in male fertility. The co-treatment with Vit C and CD-Vit E plays a positive role in the protection of human spermatozoa in diabetic individuals. This protection may be mediated through the mitigation of damage to the mitochondrial respiratory chain and its antioxidant properties. Indeed, this combination treatment seems to protect strongly against the loss of sperm motility caused by lipid peroxidation. This suggested that Vit C and CD-Vit E association

may be suitable and useful approach to prevent damages caused by chronic and uncontrolled hyperglycemia on mature spermatozoa.

General discussion and Conclusion

General discussion and Conclusion

Diabetes is a chronic metabolic disorder of the endocrine system characterized by hyperglycemia (American-Diabetes-Association, 2009). Diabetic patients are eventually face to much more cellular and tissue injuries and suffer from different degrees of complications (Brownlee, 2001). Further, it leads to premature organ occurrence, vital systems failure, and significantly reducing lifespan (Coleman *et al.*, 2001). Previous researches have largely investigated the role of high glucose levels as the only and direct trigger for these complications (Brownlee, 2001). However, glucose levels may remain irregular for considerable periods throughout the diabetic's life, but they can live long periods, specifically exceeding 20 years, without the emergence of serious complications (Coleman *et al.*, 2001). Therefore, many researchers are recently delving into cellular levels to thoroughly investigate the underlying mechanisms responsible for these disorders (Coleman *et al.*, 2001). Great evidence suggested that OS enhancement may ultimately contribute to diabetic complications (Coleman, 2000).

Indeed, the most cell type subjected to significant carbonyl stresses throughout its lifespan, is RBCs (Coleman, 2000). The absence of a nucleus and the inability to express new enzymes limit their adaptability to alter biochemical states (Coleman, 2000). In diabetes, RBCs are the most glucose-consuming cells, leading to inevitable alterations in their morphology, metabolism, and function (Wang *et al.*, 2021). Due to their crucial role and their impact on microcirculation, the damage of RBCs may lead to repercussions on various other physiological systems (Grossin *et al.*, 2009; Wang *et al.*, 2021; Zhou *et al.*, 2018). In fact, various biochemical components that increased in diabetic RBCs and plasma, such as glucose and its derivative metabolites (AGEs, free radicals, and lipid peroxidation products) may alter other peripheral cells and tissues, as they lead to premature organ and systems failure (Çimen, 2008; Del Prato, 2009; Sivitz, 2001; Yamagishi *et al.*, 2015) namely male reproductive system.

This thesis was designed with the overall two main aims: 1) revealing the molecular mechanism by which diabetic plasma causes molecular alterations and cellular damage inside RBCs and in mature spermatozoa; 2) investigation of the protective potential of combined Vit C and CD-Vit E against the damage occurred on mature sperm cells. Initially, we co-incubated, in *vitro*, the normal human erythrocytes with diabetic plasma at different HbA_{1c} levels. Then, this study employed a multifaceted approach utilizing both established diabetic biomarkers (cell turbidity and MDA levels) and recently explored parameters (intracellular Hb and metHb production). On the other hand, to understand how these alterations in RBCs and blood of diabetic patients may affect other cells namely sperm cells. The human mature spermatozoa

were cultivated with diabetic plasma at different HbA_{1c} levels. Indeed, *in vivo,* various biochemical components of blood plasma were essentially transferred from the endothelium capillaries which surrounded the accessory glands of the male reproductive system, to seminal plasma (Creasy *et al.*, 2013). Hence, we analyzed and assessed the gametes' motility (kinematic parameters and progressive motility) and their corresponding OS status (MDA assessment). The second part of our study aimed to monitor the protective potential of Vit C and CD-Vit E, both individually and in combination against cells-damage occurring in high HbA_{1c} conditions. In this regard, we added a pre-treatment with Vit C and CD-Vit E to mature spermatozoa before their incubation in diabetic plasma. Then, the cytoprotective effect was assessed following the same protocol and analyzing the same parameters of our first approach.

Our results demonstrated that incubation of RBCs with diabetic plasma triggers a chain of cellular injuries, shortening their lifespan via HbA_{1c}-dependent hemolysis. Studies link this to increased RBCs fragility, suggesting compromised flexibility and reduced ability to handle circulation stresses. This enhanced hemolysis may contribute to chronic anemia, potentially impacting oxygen delivery throughout the body (Kung *et al.*, 2009; Lippi *et al.*, 2012; Osuntokl *et al.*, 2007). In order to reveal the potential role of Hb and metHb in mediating the cytotoxic effects observed, we quantified their intracellular and extracellular levels. This investigation aims to explore the possibility that Hb and metHb alteration contributes to RBC injury. The results revealed a high level of denaturation and oxidation of intracellular Hb to metHb in close relationship to HbA_{1c} levels. These were contributes to the formation of Heinz bodies resulting from metHb production and the fastening of denatured Hb to the cell cytoskeleton and consequently to expose senescence antigenic sites (Chaves *et al.*, 2008; Grossman *et al.*, 1992). These reports were clearly demonstrated in the microscopic analysis which illustrated the RBCs morphology alteration. These oxidative damages induced in diabetes were essentially conducted by the non-enzymatic glycation of protein and the auto-oxidation of glucose which generated OS in their processes (Ighodaro, 2018). Consequently, the overproduced free radicals reacted with the transition metals (iron and copper) that are highly abundant in RBCs which are known with their environments saturated with O_2 (Wang *et al.*, 2021). This resulted in the irreversible metHb production with the prevention of the enzymes that reduced the oxidation form of Hb. In addition, The high abundance of PUFAs within the RBCs plasma membrane makes them susceptibles to free radical attack (Çimen, 2008).Therefore, we opted to evaluate the lipid peroxidation in RBCs exposed to diabetic plasma. Our result revealed a significant increase in MDA concentrations in total correlation with HbA_{1c} levels. In accordance with our finding, Jain et al. observed a positive correlation between lipid peroxidation levels and glycated

Hb in diabetic RBCs (Jain *et al.*, 1989). These findings provide the oxidative potential of diabetic plasma and highlighted the underlying molecular mechanisms associated with membrane damage, hemolysis, and the release of Hb and metHb into the extracellular environment. Under homeostatic condition, the antioxidant defense system within RBCs effectively reduces metHb back to its ferrous form (ferrohemoglobin) and efficiently neutralizes free radicals, thereby preventing the cellular injuries observed in this study (Mansouri *et al.*, 1993). However, the increase generation of HbA_{1c} in diabetic plasma acts as pro-oxidant stimuli, potentially exacerbating metHb formation, especially in states characterized by diminished antioxidant capacity. Actually, the antioxidant mechanisms within RBCs were mitigated in effectiveness by glycation, polyol pathway, and pentose phosphate pathway (Wang *et al.*, 2021). In parallel, diabetic plasma with high HbA_{1c} presents a severe depletion in the antioxidant system (Annadurai *et al.*, 2014). This leads to shortages in reducing power necessary for optimal enzyme function, as well as the high needs for reducing power caused by compensatory cellular responses to oxidative and carbonyl stresses present in diabetes (Coleman, 2000). Clinically, these may be manifested by the development of anemia (Kabamba, 2016) and RBCs aggregation, which are an independent risk factors in the development of vascular complications in diabetic patients (Pretorius et al., 2015).

On the other hand, the co-incubation of human mature spermatozoa with diabetic plasma affected importantly sperm motility and the kinematic parameters (VCL, VSL, and VAP) in correlation with HbA_{1c} level. Similarly, RP motility, a key parameter related to fertility success, was decreased significantly with the increase of HbA_{1c}. The impairment in sperm motility relied on the excessively elevated glucose levels, which trigger all glucotoxicity pathways inside spermatozoa. These are directly modulated by high level of glucose or as a result of protein glycation, leading in increased free radicals production (Mooradian *et al.*, 1999). As well, the improve in ROS levels and the deficits in antioxidant system are closely linked to the increase of AGEs formation namely HbA_{1c}, in diabetic plasma (Annadurai *et al.*, 2014). The observed alterations in mature sperm motility following the cells exposure to diabetic plasma, as evidenced by OS investigations, strongly suggest that these factors may be the key contributors to the impaired motility. Consistent with our findings, Delfino *et al.* (2007) demonstrated a notable decline in the kinetic properties of spermatozoa among diabetic patients, particularly in progressive motility. This decline was linked to the alterations in membrane integrity, resulting in exacerbation of OS within sperm cells. Notably, the abundant presence of PUFAs in spermatozoa membranes, which confer cellular fluidity crucial for sperm motility, interact with ROS, thereby increase lipid peroxidation processes (Aitken *et al.*, 2012). Consequently, this

explains the significant elevation of MDA levels in the extracellular medium, correlating with HbA_{1c} levels. Clinically, increased free radicals and other toxic components of diabetic blood plasma that are transported into seminal plasma by different ways (Benedetti *et al.*, 2012), altered sperm membrane and amplified lipid peroxidation that exerted adverse effects on sperm quality. Our data aligns with findings by La Vignera *et al.* (2012) who reported a detrimental effect of poor glycemic control on sperm quality, particularly with respect to lipid peroxidation levels in diabetic patients. We observed a significant inverse correlation between lipid peroxidation and sperm motility, both of which are intricately linked to the levels of glycemic control. Independently of other factors previously reported, such as sexual hormones and impairments in spermatogenesis, it was the first time to elaborate on the adverse effect of high HbA_{1c} levels on mature spermatozoa. HbA_{1c}, indicates the average of blood glucose concentration over the preceding 2–3 months (Gikas *et al.*, 2009). A period that is approximately close to human spermatogenesis process which takes about 72 days (Muciaccia *et al.*, 2013). Unfortunately, the diabetes-mediated oxidative damages were irreversible and unstoppable due to the impairment of the antioxidant system, notably the reduction in the activities of GPx, SOD, and other scavengers such as Vit E and C, leads to a depletion in the GSH pool (Ramalho-Santos *et al.*, 2008).

Secondly, as antioxidant therapy becomes a rational step in the prevention of oxidant and carbonyl stresses in the face of intermittent hyperglycemia, we have evaluated the cytoprotective effect of both Vit C and CD-Vit E alone and in combination on spermatozoa exposed with diabetic plasma with elevated concentration of HbA_{1c}.

Vit E presents poor solubility limiting consequently its action against ROS due to its lipophilic character. While, when it was preloaded in cyclodextrins, it enhanced the vitamin solubility without affecting its antioxidant activity. In addition, it appears to have the best effect on sperm motility, membrane functionality, and lipid peroxidation prevention (Benhenia *et al.*, 2016). Indeed, the beneficial effect of CD-Vit E, as a complex, was exclusively tested in our study especially, on human cells in hyperglycemic conditions. Moreover, Vit C and Vit E act synergistically when the initiating radicals are generated within the lipid core to neutralize ROS (Murray *et al.*, 2006). These may partly explain the metabolic role and support the interest in using the combination of these two vitamins against spermatozoa damage induced by diabetes-mediated OS.

Given this evidence, the asked question is whether this antioxidant supplementation has the same potential improvement in hyperglycemic men who have developed an impaired reproductive system as a result of HbA_{1c} elevation and OS exacerbation. So, the pre-treatment

with Vit C and CD-Vit E, individually and in combination on human sperm cells that were exposed to diabetic plasma at high HbA_{1c} levels, was performed. Our data showed an important improvement in almost all sperm motility alerted with high HbA_{1c} levels, after a pretreatment with the two selected antioxidants. The addition of Vit C and CD-Vit E respectively at 0.1 and 0.25 mg/ml, individually and in combination, preserved all sperm kinematics parameters (VCL, VSL, and VAP). Similarly, the findings revealed a significant amelioration of progressive motility (rapid and moderate progressivity), which is fully related to fertility success. In parallel, the percentage of immotile cells was exceptionally lower than those in the diabetic group (HbA_{1c} H). These were due to the high potency of these two vitamins and their synergistic action for scavenging ROS (Zini *et al.*, 2014). Indeed, Vit C is known to contribute up to 65% of the total antioxidant capacity of seminal plasma found intracellularly and extracellularly (Agarwal *et al.*, 2004). It contributes to recycling other antioxidants, protecting against DNA damage, and preventing lipid peroxidation in semen cells (Buettner, 1993). This latter was confirmed by the MDA measurement test, which demonstrated a reduced percentage of MDA with Vit C treatment at a concentration of 0.1 mg/ml. Interestingly, this reflects the beneficial impact of the right dose of Vit C on semen quality namely in diabetes. The excessive intake of ascorbic acid has been reported to cause reproductive failure in male patients (Paul *et al.*, 1978). Moreover, ascorbic acid deficiency causes a reduction in reproductive performance (Chinoy *et al.*, 1986). This is developed by decreased antioxidant enzyme activities, and increased levels of H_2O_2 and lipid peroxidation in spermatozoa (Potts *et al.*, 1999). Comparatively, the protective potential of CD-Vit E on sperm motility was more efficacy than the Vit C treatment. This indicates that CD-Vit E has more potent effects in the reduction of adverse side effects of OS induced by diabetes on mature spermatozoa. Indeed, Vit E is one of the most important antioxidant molecules, involved mainly in the cell membranes. This chain-breaking antioxidant is thought to interrupt the chain reactions with lipid peroxidation and scavenges free radicals generated during the univalent reduction of molecular O_2 and stabilizes cell membranes integrity (Agarwal *et al.*, 2011). In this regard, the authors suggested that α-tocopherol could be involved in association with antioxidant enzymes, to maintain the functional capacity of sperm when exposed to oxidative attack (Therond *et al.*, 1996). As well, Moslemi *et al.* (2011) showed that supplementation with Vit E can enhance significantly sperm motility and improve pregnancy occurrence. Likewise, Suleiman *et al.* (1996) reported that the treatment of asthenospermic patients with oral Vit E improved sperm motility and decreased significantly MDA concentration in spermatozoa. The same data was seen in our study by the MDA assessment test, showing a reduced concentration of these by-products in the CD-Vit E treated

group. Actually, the sperm motility improvement by the treatment with CD-Vit E was directly correlated with the reduction in lipid peroxidation and ultimately to the protection of spermatozoa (Suleiman *et al.*, 1996). Consequently, this improved sperm motility and membrane functionality and prevented lipid peroxidation (López-Nicolás *et al.*, 2014). Similarly, Benhenia *et al.* (2016) showed in their findings a significant improvement of CD-Vit E, on the animal semen quality.

Interestingly, the combination of Vit C and CD-Vit E has the most effective improvement on all sperm motility, presenting the lowest percentage of immotile cells and MDA concentration compared to the other treated groups. This potent combined effect was due mainly due to the synergistic actions of those vitamins in the suppression of ROS and their damage on cells. Generally, Vit C scavenges oxygen radicals in the aqueous phase, whereas Vit E scavenges oxygen radicals within the membranes (Traber *et al.*, 2011). In cells, when a molecule of Vit E neutralizes a free radical, it loses its antioxidant ability which is subsequently restored and regenerated by the interaction of Vit C with GSH (Niki, 1987). Several *in vitro* studies have reported the beneficial effects of Vit E and C on the inhibition of lipid peroxidation of plasma sperm membrane, sperm DNA integrity, and on the capacity of fusion with oocyte in animal species (Geva *et al.*, 1996; Irvine, 1996; Parinaud *et al.*, 1997). As well, Donnelly *et al.* (1999) reported a decrease in H_2O_2-induced ROS production and DNA damage after the utilization of both Vit C (300 and 600 mmol/l) and Vit E (40 and 60 mmol/l) to normozoospermic and asthenozoospermic samples.

In summary, our findings demonstrated that the injuries of RBCs in uncontrolled hyperglycemia initiated from the intracellular environment and was further exacerbated by overproduction of metHb in close relationship with the elevated HbA_{1c} levels. This was mainly associated with the intensive formation of ROS which is related the OS exacerbation, the underlying mechanism of the different damages in RBCs and other peripheral cells. Similarly, mature sperm cells presented an important alteration in their quality and motility related to the rise of HbA_{1c}. These suggest that elevations in HbA_{1c} and metHb values may indicate the onset of micro-vascular and male reproductive problems. Vit C and CD-Vit E association seems to protect strongly the mature spermatozoa by the improvement of its motility and quality against lipid peroxidation and OS in poor hyperglycemic conditions. Overall, these suggest that the use of antioxidant supplements complementary to antiglycation treatment may add some protective potential in the future clinical management of diabetes. Especially since antioxidants lack major side effects and are relatively inexpensive. However, it may not be possible to reverse

completely the diabetic complications or enhance separately male reproduction systems. Future studies will concentrate on concentration/dose-related and more refinement in antioxidant dosage/benefit ratios. It is also apparent that tissues require antioxidant capacity throughout their lipophilic as well as aqueous phases, necessitating the activity of molecules that differ widely in physiochemical and structural parameter, but that carry out essentially the same function.

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Appendix

Table 1: Study participants with diabetes and their medical data. T2: Type 2 DM; O.A.D: Oral Antidiabetic Drugs; Insu: Insulin.

Number Of Patients	Sex	Type of DM	Glucose g/L	HbA1c values (%)	Age (year)	Durations of illness (year)	Smoking	Complications	Pharmacology
1	Male	T2	1	5.55	51	9	No	No	O.A.D
2	Male	T2	1.29	8	72	14	No	No	O.A.D
3	Male	T2	2.20	12.2	47	7	No	No	O.A.D
4	Male	T2	0.98	5.56	49	9	No	No	O.A.D
5	Male	T2	1.30	8.3	46	15	No	No	O.A.D
6	Male	T2	2.60	11.4	77	16	No	No	O.A.D
7	Male	T2	1	5.09	7	/	No	No	Insu
8	Male	T2	1.42	8	64	19	No	No	O.A.D
9	Male	T2	2.75	12.3	58	7	No	No	O.A.D
10	Male	T2	0.99	5.5	64	10	No	No	O.A.D
11	Male	T2	1.40	8.4	56	11	No	No	O.A.D
12	Male	T2	2.81	13	49	9	No	No	O.A.D
13	Male	T2	1.10	5.75	55	7	No	No	O.A.D
14	Male	T2	1.23	5.99	75	17	No	No	O.A.D + Insu
15	Male	T2	1.20	5.8	40	8	No	No	O.A.D
16	Male	T2	1.39	8.2	71	11	No	No	O.A.D
17	Male	T2	1.48	8.1	77	10	No	No	O.A.D
18	Male	T2	2.66	15.6	85	18	No	No	O.A.D + Insu
19	Male	T2	2	14.5	47	9	No	No	O.A.D + Insu
20	Male	T2	2.13	15	15	/	No	No	Insu
21	Male	T2	1.26	8.5	51	4	No	No	O.A.D
22	Male	T2	1	7.8	60	7	No	No	O.A.D
23	Male	T2	1.56	4.78	52	6	No	No	/
24	Male	T2	2.13	10.7	65	10	No	No	O.A.D
25	Male	T2	1.6	8.2	65	10	No	No	O.A.D
26	Male	T2	0.90	4.87	63	13	No	No	O.A.D + Insu
27	Male	T2	1.24	8	57	14	No	No	O.A.D
28	Male	T2	1.49	8.3	46	15	No	No	O.A.D
29	Male	T2	2.22	10.7	65	10	No	No	O.A.D
30	Male	T2	1.67	8	72	14	No	No	O.A.D
31	Male	T2	1	5.9	65	18	No	No	O.A.D
32	Male	T2	1.22	7.4	52	18	No	No	O.A.D
33	Male	T2	2.56	11.6	49	12	No	No	O.A.D + Insu
34	Male	T2	1.13	5.6	47	9	No	No	O.A.D
35	Male	T2	1.39	7.8	51	23	No	No	O.A.D + Insu
36	Male	T2	1.50	6.2	71	6	No	No	O.A.D
37	Male	T2	1.92	12	70	7	No	No	O.A.D
38	Male	T2	1.43	7.6	63	17	No	No	O.A.D
39	Male	T2	1.10	6.3	69	18	No	No	O.A.D + Insu
40	Male	T2	1.34	8.2	72	12	No	No	O.A.D + Insu
41	Male	T2	1.29	8.1	60	11	No	No	O.A.D + Insu
42	Male	T2	2	10.9	69	10	No	No	O.A.D + Insu
43	Male	T2	2.32	12.2	47	7	No	No	O.A.D
44	Male	T2	1.15	5.8	41	8	No	No	O.A.D
45	Male	T2	3	20.5	67	12	No	No	O.A.D
46	Male	T2	1.99	10.4	71	15	No	No	O.A.D + Insu
47	Male	T2	0.96	5.64	76	5	No	No	O.A.D
48	Male	T2	2.50	13.5	69	15	No	No	O.A.D + Insu

49	Male	T2	1.46	7.8	54	10	No	No	O.A.D
50	Male	T2	2.49	13.94	48	9	No	No	O.A.D
51	Male	T2	1.43	8.2	66	11	No	No	O.A.D + Insu
52	Male	T2	2.11	11.8	42	6	No	No	O.A.D
53	Male	T2	1.89	10.7	64	9	No	No	O.A.D
54	Male	T2	1.31	8	63	18	No	No	O.A.D + Insu
55	Male	T2	1.20	5.16	74	9	No	No	O.A.D + Insu
56	Male	T2	2.31	12	69	6	No	No	O.A.D
57	Male	T2	1.39	7.6	62	12	No	No	O.A.D + Insu
58	Male	T2	1.40	8.9	65	14	No	No	O.A.D + Insu
59	Male	T2	2.42	12.2	46	6	No	No	O.A.D
60	Male	T2	1	5.8	58	10	No	No	O.A.D + Insu
61	Male	T2	1.19	6	67	9	No	No	O.A.D
62	Male	T2	0.93	5.9	70	7	No	No	O.A.D
63	Male	T2	1.99	10.08	50	7	No	No	O.A.D
64	Male	T2	2.45	12.2	31	5	No	No	O.A.D
65	Male	T2	1.14	5.19	67	10	No	No	O.A.D + Insu
66	Male	T2	2.36	13.2	56	6	No	No	O.A.D
67	Male	T2	1.31	8	57	7	No	No	O.A.D + Insu
68	Male	T2	2.49	12	70	7	No	No	O.A.D
69	Male	T2	1.15	5.30	44	7	No	No	O.A.D
70	Male	T2	2.43	11.40	80	16	No	No	O.A.D + Insu
71	Male	T2	3.11	16.3	56	9	No	No	O.A.D
72	Male	T2	1.41	8.5	61	9	No	No	O.A.D
73	Male	T2	2.30	12	52	6	No	No	O.A.D
74	Male	T2	2.99	14.6	49	9	No	No	O.A.D
75	Male	T2	1.22	5.38	65	10	No	No	O.A.D + Insu
76	Male	T2	3.42	19.7	53	7	No	No	O.A.D
78	Male	T2	2.56	13.4	56	9	No	No	O.A.D
79	Male	T2	0.85	5.6	67	5	No	No	O.A.D
80	Male	T2	1.41	10.5	52	5	No	No	O.A.D
81	Male	T2	1.89	12	53	5	No	No	O.A.D
82	Male	T2	1.27	6	54	8	No	No	O.A.D
83	Male	T2	1.11	5.42	67	11	No	No	O.A.D + Insu
84	Male	T2	2.57	14.1	79	16	No	No	O.A.D + Insu
85	Male	T2	1.32	8.1	48	9	No	No	O.A.D
86	Male	T2	2.45	13.9	63	9	No	No	O.A.D
87	Male	T2	1.23	4.92	32	4	No	No	O.A.D
88	Male	T2	2	12.2	37	4	No	No	O.A.D
89	Male	T2	1.30	5.87	67	12	No	No	O.A.D + Insu
90	Male	T2	1.90	11.3	57	8	No	No	O.A.D + Insu
91	Male	T2	2.50	15.4	52	8	No	No	O.A.D
92	Male	T2	1.13	5.6	52	9	No	No	O.A.D
93	Male	T2	2.51	12.4	47	7	No	No	O.A.D
94	Male	T2	2	12.6	85	12	No	No	O.A.D
95	Male	T2	1.32	5.25	39	3	No	No	O.A.D