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Evaluation of the Efficiency of Combined and Separated Antioxidant Supplementation of Vitamin C and E on Semen Parameters in Streptozotocin-Induced Diabetic Male Wistar Rats

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Preface

The current PhD thesis aims to examine the effectiveness of supplementation with vitamin C and E, on the infertility of male diabetes patients, by conducting experiments on male Wistar rats. The study is based on the assessment of semen parameters, blood hormonal levels, and histological investigations of testicular sections. The manuscript is structured as follows:

- The first section describes the theoretical considerations on male infertility (Chapter 1), Oxidative Stress, Mechanisms and Treatment (Chapter 2), and Diabetes Mellitus and Male Infertility (Chapter 3);
- The second part, covers the study design and the protocol carried out according to the preliminary hypothesis and the objectives of the study (chapter 4);
- The third section deals with the results of the study (Chapter 5);
- And the conclusion that draws all the results and prospects for future research.

The wording of this manuscript follows the latest version of ISO-690 (International Organization for Standardization) for bibliographic referencing in the preparation of documents, content, form and structure, published in 2010.

The research work in this thesis involved many people, of which I am indebted, and I want to thank them all for their help and support.

First and foremost, I have to thank "Allah" for providing me blessings, courage, strength, and patience to achieve this modest work.

I express my deepest thanks to my supervisor, Professor KHALED Méghit Boumédiène, researcher lecturer at Djillali Liabès University of Sidi-Bel-Abbes, for the competent help, for his encouragement to accomplish a work begun a long time ago. His critical eye was very valuable for structuring the work and improving the quality of its different sections.

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Special thanks to my parents and my husband for their assistance, understanding and patience.

Abstract

Background and Aims: Male reproductive functions could be affected at multiple levels due to diabetes mellitus. Studies have shown that antioxidant vitamins have direct impact on improving male reproductive capacity. Thus, the aim of the current study was to assess the efficiency of supplementation with vitamin C and E in the case of whether diabetes mellitus could be the cause of infertility or if infertile men suffer from diabetes by inducing diabetes on male Wistar rats.

Material and methods: Adult male Wistar rats were divided into five groups of six animals each: a normoglycemic control and other four groups were given a single dose of streptozotocin (40 mg/kg). These groups were divided into: hyperglycemic control, hyperglycemic +250 mg/kg/day of vitamin C, hyperglycemic +250 mg/kg/day of vitamin E, and hyperglycemic + 250 mg/kg/day of vitamin C + 250 mg/kg/day of vitamin E using gavage during 30 consecutive days. The normoglycemic and the hyperglycemic control groups received vehicle (water and corn oil). The first day after the treatment, the rats were anesthetized and sacrificed to assess body and reproductive organ weights, plasma hormone levels, and semen quality.

Results: Compared to normoglycemic animals, hyperglycemic rats showed a significant decrease in body weight (211 ± 8.7gr; $p<0.05$) and epididymis (1.35 ± 0.10 gr; $p<0.05$). Whereas, the testis weight (3.10 ± 0.14 gr) and sperm count (2.72 ± 4.61 x 10^6) were maintained. Furthermore, a decrease of LH and testosterone levels (0.10 ± 0.00, and 0.25 ± 0.12) respectively, were observed too. There was a significant increase ($p<0.05$) in the final body weight (309 ± 8.77 gr; 268 ± 5.00 gr; 275 ± 3.65 gr), hormonal blood levels of : testosterone (12.53 ± 0.68 ng/mL; 1.46 ± 0.22 ng/mL; 8.61 ± 2.46 ng/mL); LH (0.13 ± 0.01 ng/mL; 0.11 ± 0.00 ng/mL ; 0.13 ± 0.00 ng/mL); and FSH (0.13 ± 0.01 ng/mL ; 0.11 ± 0.00 ng/mL, 0.13 ± 0.00 ng/mL), among hyperglycemic vitamin C, vitamin E, and vitamins C+E supplemented groups, respectively, as compared to the normoglycemic, and thebhyperglycemic control groups. In addition, a significative increase ($p<0.05$) in the epididymis weight (1.69 ± 0.05 gr; 1.61 ± 0.06 gr; 1.69 ± 0.15 gr), and sperm count (9.53 ± 0.73 x10^6; 6.89 ± 0.53 x10^6; 6.75 ± 0.68 x10^6) among hyperglycemic vitamins C, E and C+E, respectively groups, were observed too. Microscopic examination of the vitamins supplemented rats' testes, revealed normal histological structure of most seminiferous tubules with normal spermatogenic series. The mean Johnsen score value was low among diabetic

rats group (5.17 ± 0.30) and was significantly improved in hyperglycemic supplemeted vitamins C (8.17 ± 0.40), E (8.17 ± 0.30), and C+E (8.33 ± 0.49) groups.

Conclusion: Diabetes mellitus induced controversial effects on reproductive capacity. This issue does not affect the efficiency of vitamin C and E supplementation, that could exhibit beneficial effects in preventing the histological changes in the rats testes as well as their serum testosterone levels.

Keywords: Diabetes mellitus, antioxidant vitamins, infertility, semen quality, supplmentation.

Résumé

Objectifs : Les fonctions reproductrices masculines pourraient être affectées à plusieurs niveaux en raison du diabète sucré. Des études ont montré que les vitamines antioxydantes ont un impact direct sur l'amélioration de la capacité reproductrice masculine. Ainsi, l'objectif de ce travail était d'évaluer l'efficacité d'une supplémentation en vitamines C et E dans le cas où le diabète sucré est la cause de l'infertilité ou bien, si les hommes infertiles souffrent de diabète, en induisant le diabète sur les rats Wistar mâles.

Matériel et méthodes : Des rats Wistar mâles adultes ont été divisés en 5 lots de 6 animaux chacun : un témoin normoglycémique et les quatre autres groupes ont reçu une dose unique de streptozotocine (40 mg/kg). Ces groupes étaient divisés en : témoin hyperglycémique, hyperglycémique + 250 mg / kg / jour de vitamine C, hyperglycémique + 250 mg / kg / jour de vitamine E et hyperglycémique + 250 mg / kg / jour de vitamine C + 250 mg / kg / jour de vitamine E, par gavage pendant 30 jours consécutifs. Les lots témoins normoglycémiques et hyperglycémiques ont reçu le véhicule (eau et huile de maïs). A la fin de la période du traitement, les rats ont été anesthésiés et sacrifiés pour évaluer les poids corporels et d'organes reproducteurs. Les taux d'hormones plasmatiques, la qualité du sperme et l'étude des coupes histologiques des testicules, ont aussi été évalués.

Résultats : Comparés aux animaux normoglycémiques, les rats diabétiques ont montré une réduction significative du poids corporel (211± 8,70 gr $p<0,05$) et de l'épididyme (1,35 ± 0,10 gr $p<0,05$). En revanche, le poids des testicules (3,10 ± 0,14 gr) et le nombre de spermatozoïdes (2,72 ± 4,61 x10^6) ont été maintenus. Il a été également observé une réduction des niveaux de LH et de testostérone (0,10 ± 0,00 ng/mL et 0,25 ± 0,12 ng/mL) respectivement. En outre, une augmentation significative ($p<0,05$) du poids corporel final (309 ± 8,77 gr ; 268 ± 5,00 gr ; 275 ± 3,65 gr) ; des niveaux hormonaux du testostérone (12,53 ± 0,68 ng/mL ; 1,46 ± 0,22 ng/mL ; 8,61 ± 2,46 ng/mL), de LH (0,13 ± 0,01 ng/mL ; 0,11 ± 0,00 ng/mL ; 0,13 ± 0,00 ng/mL) et du FSH (0,13 ± 0,01 ng/mL ; 0,11 ± 0,00 ng/mL, 0,13 ± 0,00 ng/mL) a été observée. Le poids de l'épididyme (1,69 ± 0,05 gr ; 1,61 ± 0,06 gr ; 1,69 ± 0,15 gr) et le nombre des spermatozoïdes (9,53 ± 0,73 x10^6 ; 6,89 ± 0,53 x10^6 ; 6,75 ± 0,68 x10^6) ont aussi connu une augmentation significative, dans les lots hyperglycémiques supplémentés de vitamines C, E, C+E respectivement, par rapport aux normoglycémiques et

hyperglycémiques témoins. L'examen microscopique des testicules de rats, ayant reçu des vitamines, a révélé une structure histologique normale de la plupart des tubules séminifères avec des séries spermatogéniques normales. La valeur moyenne du score de Johnsen a été réduite chez les rats hyperglycémiques (5,17 ± 0,30) et elle s'est significativement améliorée dans le groupe des rats hyperglycémiques supplémentés en vitamines C (8,17 ± 0,40), E (8,17 ± 0,30) et C+E (8,33 ± 0,49).

Conclusion : Le diabète sucré induit des effets indésirables sur la capacité de reproduction masculine qui n'affecte pas l'efficacité du traitement avec la vitamine C et E. Ces derniers ont un effet bénéfique dans la prévention des changements histologiques dans les testicules des rats ainsi que leurs niveaux sériques de testostérone.

Mots-clés : Diabète sucré, vitamines antioxydantes, infertilité, qualité du sperme, supplementation.

ملخّـــــص

تتأثر الوظائف التناسلية لدى الذكور على مستويات متعددة بسبب داء السكري .وقد أظهرت الدراسات أن الفيتامينات المضادة للأكسدة لها تأثير مباشر على تحسين القدرة التناسلية للذكور . ولهذا ، كان الهدف من هذه الدراسة تقييم كفاءة العلاج بفيتامين ج و هـ في حالة ما إذا كان مرض السكري هو المتسبب في العقم أو إذا عانى الرجال ، ذوو قدرة إنجابية ضعيفة ، من مرض السكري عن طريق إحداث مرض السكري على ذكور جرذان ويستار.

قسِّم ذكور جرذان ويستار إلى 5 مجموعات متكونة من 6 حيوانات: السيطرة السليمة ، وأعطيت المجموعات الأربع الأخرى جرعة واحدة من الستربتوزوتوسين (40 مغ /كغ). تم تقسيم هذه المجموعات إلى: السيطرة مصابة بالسكري ، مجموعة السكري + 250 مغ /كغ /يوم من فيتامين ج ، مجموعة السكري + 250 مغ /كغ /يوم من فيتامين هـ ، ومجموعة السكري + 250 مغ /كغ /يوم من فيتامين ج + 250 مع /كغ /يوم من فيتامين هـ.. عن طريق الفم خلال 30 يوما بصفة مستمرة. تم إعطاء مجموعة السيطرة السليمة والسيطرة المصابة بالسكري المركبات المعيارية (الماء وزيت الذرة) .بعد نهاية العلاج ، تم تخدير الجرذان وتضحيتها لقياس أوزان الجسم والأعضاء التناسلية ، ومستويات هرمون التيستوستيرون والـ LH ، والـ FSH ، ونوعية السائل المنوي.

النتائج: أظهرت الجرذان المصابة بداء السكري ، مقارنة مع حيوانات السيطرة ، انخفاض في وزن الجسم (211.33 ± 8.70 غرام) والبربخ (1.35 ± 0.10 غرام) . حيث تم الحفاظ على وزن الخصية (3.10 ± 0.14 غرام) وعدد الحيوانات المنوية ($2.72 \pm 4.61 \times 10^6$) ولوحظ أيضًا انخفاض في مستويات LH ومستويات التستوستيرون (0.10 ± 0.00 ng/mL و 0.25 ± 0.12 ng/mL) على التوالي .بالإضافة إلى ذلك ، كان هناك زيادة معنوية ($p<0.05$) في وزن الجسم النهائي ، ومستويات الهرمونات ، ووزن البربخ ، وعدد الحيوانات المنوية بين مجموعة داء السكري التي تم تزويدها بالفيتامين مقارنة بمجموعة السيطرة السليمة ومجموعة السيطرة المصابة بالسكري.

الخلاصة: خلصنا إلى أن مرض السكري يزيد التأثيرات الضارة على القدرة على الإنجاب عند الذكور. هذا المرض لا يؤثر على فعالية العلاج بفيتامين ج وهـالتي لها تأثير ايجابي في منع التغيرات النسيجية على مستوى الخصيتين وعلى مستويات هرمون التستوستيرون في الدم.

الكلمات المفتاحية: داء السكري ، فيتامينات مضادات للأكسدة ، العقم ، جودة السائل المنوي ، مكملات.

Table of Contents

Chapter 2 : Oxidative Stress, Mechnisms and Treatment

Chapter 3
Diabetes Mellitus and Male Infertility

Part 2
Experimental Study
Chapter 4
Material and Methods

Chapter 5
Results & Discussion

Chapter 6: Conclusion

List of Figures

List of Tables

List of Abbreviations and Acronyms

°C	Degree Celsius
µl	Microlitter
$^{-1}O^{2}$,	Singlet Oxygen
4HNE	4-Hydroxynonenal
AGEs	Advanced Glycated End Products
ALT	Alanine Aminotransferase
AR	Acrosome Reaction
ART	Assisted Reproductive Technologies
ASA	Antisperm Antibodies
ATP	Adenosine triphosphate
BDE	Bond Dissociation Enthalpy
BTB	Blood–Testis Barrier
Ca2 +	Calcium
cAMP	Cyclic Adenosine 3',5'-Monophosphate
CASA	Computer-Assisted Semen Analysis
CF	Cystic Fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
cGy	centigray
CPEMR	Cell Phone-Generated Electromagnetic Radiation
CBAVD	Congenital bilateral absence of the vas deferens
CUAVD	Congenital Unilateral Absence of the Vas Deferens
DAG	Diacylglycerol
DHA	Docosahexaenoic Acid
DM	Diabetes mellitus
DNA	*Deoxyribonucleic acid*
ELISA	Enzyme-Linked Immunosorbent Assay
ERC	Excess Residual Cytoplasm
FSH	Follicle *stimulating hormone*
G6PD	Glucose-6-Phosphate Dehydrogenase
GLUT	Glucose Transporters
GLUT1	Glucose Transporter 1

GLUT2	Glucose Transporter 2
GLUT3	Glucose Transporter 3
GLUT5	Glucose Transporter 5
GLUT8	Glucose Transporter 8
GLUT9a	Glucose Transporter 9a
GLUT9b	Glucose Transporter 9b
GnRH	Gonadotrophine Releasing Hormone
GPx	Glutathione Peroxidase
GR	Glutathione Reductase
gr	Gramme
GSH	Glutathione
GU	Genito-urinary Infections
H_2O_2	Hydrogen Peroxide
HIV	Human Immunodeficiency Virus
HOCL	Hypochloric Acid
HPG	hypothalamic-pituitary–gonadal
IBT	Immunobead Test
ICSI	*Intracytoplasmic Sperm Injection*
IP3	Inosital Triphosphate
IVF	Fertilization In Vitro
KLK3	glycoprotein enzyme
LH	luteinizing hormone
LHRH	luteinizing *hormone*-releasing *hormone*
LOO	Lipid Peroxyl
LOOH	Lipid Peroxide
LPO	Lipid Peroxidation
M	Molar
MAR	Mixed Antiglobulin Reaction
MCTs	Membrane Transporters
MDA	Malondialdehyde
min	Minutes
mL	Milliliter
NCDs	Non Communicable Diseases

ng/dL	nanogramm/dicilitter
nm	Nanometers
NO	Nitric Oxide
O_2^-	Superoxide Ion
O_3	Ozone
OH-	Hydroxyl Ion
OS	Oxidative Stress
PFK	Phosphofructokinase
pH	potential Hydrogen
PIP_2	Phosphatidylinosital-4,5-Biphosphate (
PKA	Protein Kinase A
PLA_2	Phospholipase A2
PSA	Prostate-specific antigen
PTK	Protein Tyrosine Kinase
PTPase	Phosphotyrosine Phosphatase
PUFA	Polyunsaturated Fatty Acids
RAGE	Receptors for Advanced Glycated End Products
RO^2	Peroxyl,
ROS	Reactive Oxygen Species
SC	Sertoli Cell
SGLT	Sodium-Dependent Glucose Transporters
SMTP	Sperm–cervical mucus penetration tests
SOD	Superoxide Dismutase
SPA	Sperm Penetration Assay
STZ	Streptozotocin
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes
TAC	Total Antioxidant Capacity
TEMPO	Tetramethylpiperidine-1-Oxyl3
TZI	Teratozoospermia Index
WHO	World Health Organization
ZP	Zona Pellucida

Introduction

Infertility is an alarming health issue that concerns 48.5 million couples worldwide. In couple infertility, male infertility factors contribute themselves to 20-30% of cases and could then be responsible of 50% of overall cases **(Agarwal *et al.*, 2015)**. Previous research identified infertility causes from most to least common: sperm production issues, blockage of sperm transport, sperm antibodies, sexual issues (ejaculatory and erectile dysfunctions), endocrinology, idiopathic, and other causes **(Parekattil and Agarwal, 2012)**.

Over recent decades, with sophisticated analytical methods, several studies have shown a previously undetectable effect of diabetes on male fertility. In year 2017, the International Diabetes Federation (IDF) reported that 422 million adults were living with diabetes. The increasing diabetes incidence will result in an increased prevalence in men of reproductive age **(IDF, 2017)**.

Diabetes mellitus (DM) constitutes a metabolic disorder of carbohydrates, lipids, and proteins, that induces pathology in various systems and tissues such as eyes, nerves, kidneys, and vessels as well **(Basmatzou, 2016)**. Male reproductive functions could be affected, at multiple levels too, due to DM effects on the endocrine control of spermatogenesis, sperm production, erectile, and ejaculatory functions **(Agbaje *et al.*, 2007)**.

It is well known that glucose autoxidation, with increased oxidative stress, is the consequence of long term uncontrolled DM. This oxidative stress is extremely toxic for cells and may cause direct damages in proteins, lipids, and DNA, or affects normal cellular signaling, and gene regulating **(Fernandes *et al.*, 2011)**, as well as the alteration in antioxidant enzyme levels. In addition, spermatozoa are sensitive to reactive oxygen species (ROS) because of their elevated content of polyunsaturated fatty acids, and their incapacity to repair deoxyribonucleic acid (DNA) damage.

During twenty years, specialists have been foccusing in studying the treatment of infertility at low cost and high effectivness. They resorted to the use of antioxidants. These compounds, which have proven effective treatment of many cases of male infertility, are still

being studied and used to reduce oxidative stress, delaing infertility, caused by diabetes mellitus.

Because of the lack in data in this context, we carried out this study, aiming to assess the efficacy of supplementation with vitamin C and E, on the infertility of male diabetes patients, by conducting experiments on male Wistar rats, after diabetes induction by streptozotosin (STZ) injection, and assessing their sperm parameters, blood hormonal levels, and testis histological sections. We assessed the efficiency of these antioxidants either combined or separated.

Part I: Review of the literature

Chapter 1

Male Infertility, Generalities

Chapter 1

Male Infertility, Generalities

1.1 Sperm Physiology and Pathology

The male reproductive system is a complex and intricate system that produces spermatozoa or sex cells to carry the genetic material of the male. The components of the male reproductive system include both the external and internal sexual organs, as shown on Figure 1.1 and the hypothalamic-pituitary–gonadal (HPG) axis (Figure. 1.2).

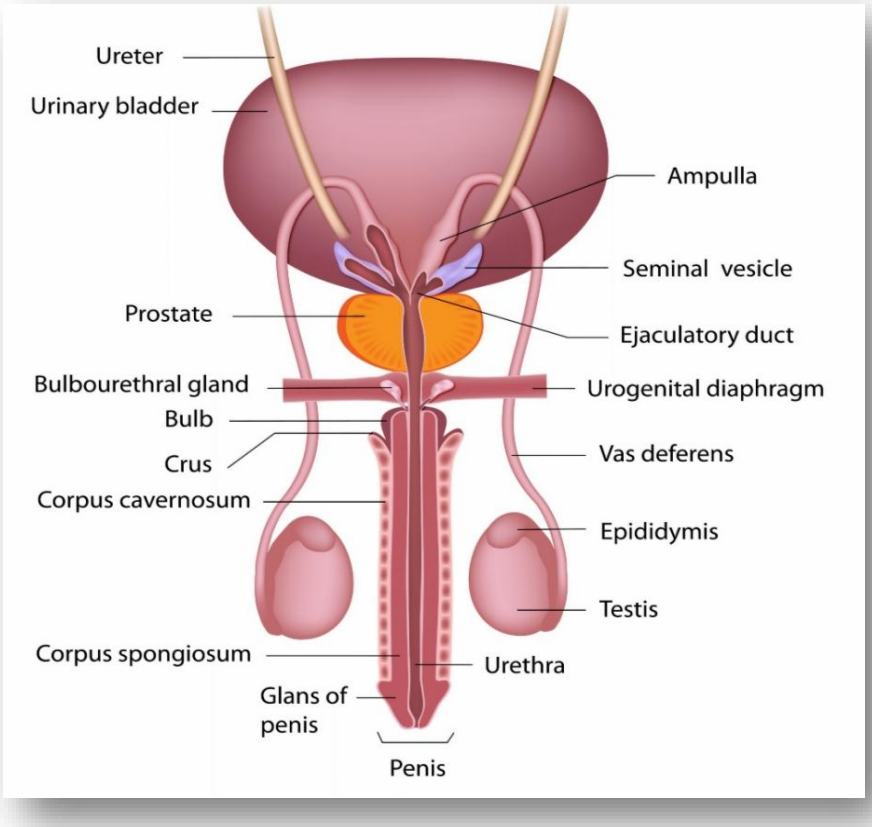

Figure 1.1: Male genital system (**AHC, 2017**)

The male reproductive system formed during the early embryonic development stages, becomes fertile during puberty and maintains the masculinity of adult male. The external genitalia include the scrotum, testes, and penis whereas the internal genitalia include the epididymis, seminal ducts, spermatic cords, seminal vesicles, ejaculatory ducts, bulbourethral or Cowper's glands, and the prostate gland (Figure 1.1). The testes produce the male gametes (spermatozoa). The excurrent duct system matures, stores, and transports the gametes to the penis for expulsion, and the accessory glands produce and modify the contents of the semen **(Durairajanayagam *et al.*, 2015)**.

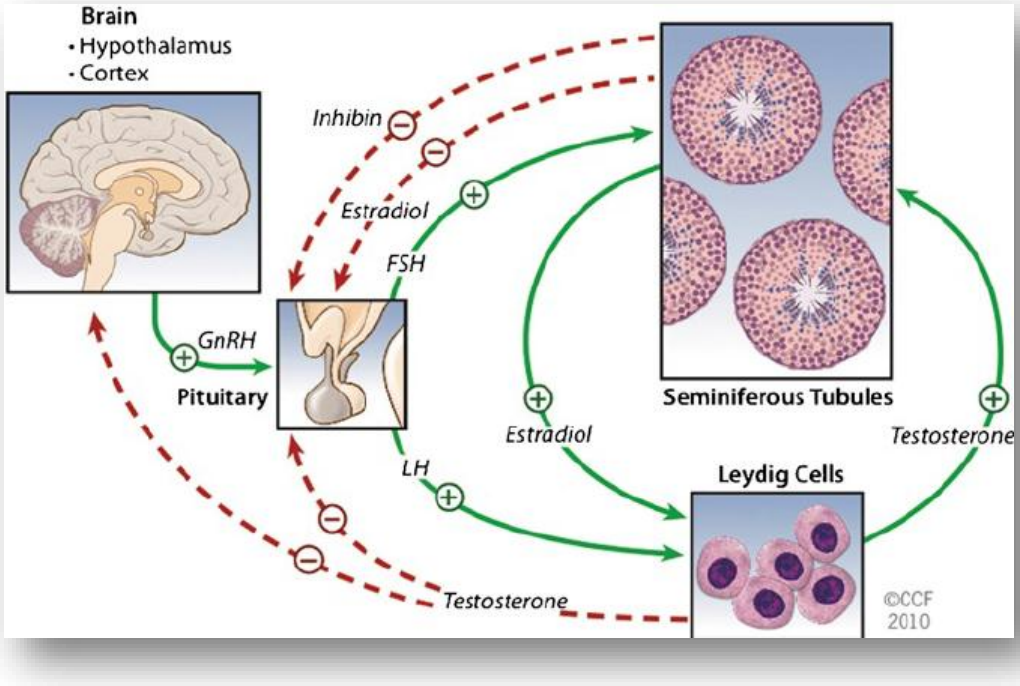

Figure 1.2: Hypothalamic-pituitary–gonadal (HPG) axis (**Mohanty and Singh, 2017**)

1.1.1 Spermatogenesis

Spermatogenesis is an extremely intricate process of cell differentiation, starting with germ cell (spermatogonia) development and culminating in the production of highly specialized spermatozoa. This process produces the genetic material required for species replication. Spermatogenesis occurs in the lumen of the seminiferous tubules. It was classically

believed that human spermatogenesis takes about 64 days in the testis (from spermatogonium to spermatid) with an additional 10–14 days in the epididymis for maturation of spermatozoa. Thus, the entire process takes about 70 ± 4 days to complete. However, a more recent report, published by **Durairajanayagam *et al.*, (2015),** has suggested that the entire process from production to ejaculation of spermatozoa is completed within a shorter period: an average of 64 ± 8 days (with a range of 42–76 days). Spermatogenesis begins at puberty and occurs continually throughout the entire male adult life span in contrast to oogenesis, which is finite in women. The baseline number of precursor cells in the testes is regulated by Follicle Stimulating Hormone (FSH) **(Durairajanayagam *et al.*, 2015)**. Early in embryonic development, the gonocytes, which precede the spermatogonial germ cells formation, undergo active mitotic replication. Spermatogenesis involves a series of cellular events that start in the basal compartment and finishe in the apical compartment. The basal and the luminal compartments are kept separate by tight junctions. In the seminiferous tubules, the developing cells are arranged in a highly ordered sequence from the basement membrane toward the lumen (Figure 1.3) **(Neto *et al.*, 2016)**.

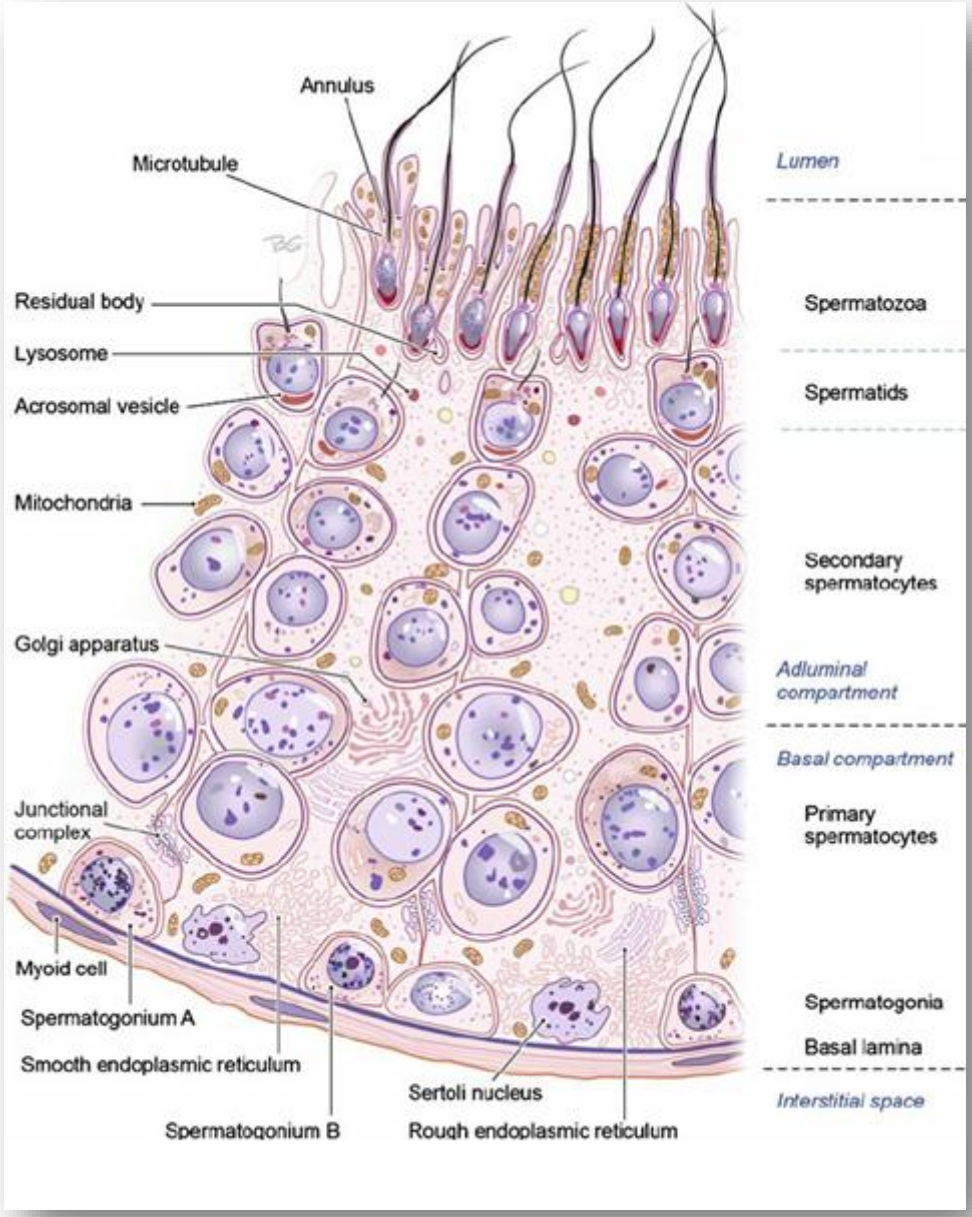

Figure 1.3: Seminiferous tubule. A cross section of the germinal epithelium in the seminiferous tubule. The germinal epithelium is divided by the Sertoli cell into two compartments, i.e., the basal and ad luminal compartments. Fully formed spermatozoa are released into the lumen **(Durairajanayagam *et al.*, 2015)**

Spermatogenesis is a multistep process involving three major phases (mitosis, meiosis, and spermiogenesis), as well as other cellular events such as cells migration, differentiation, and apoptosis (Table 1.1). These events are highly regulated and the understanding of the molecular mechanisms that regulate the three spermatogenesis phases has been a major focus of study for decades (Figure 1.4) **(Oliveira and Alves, 2015)**.

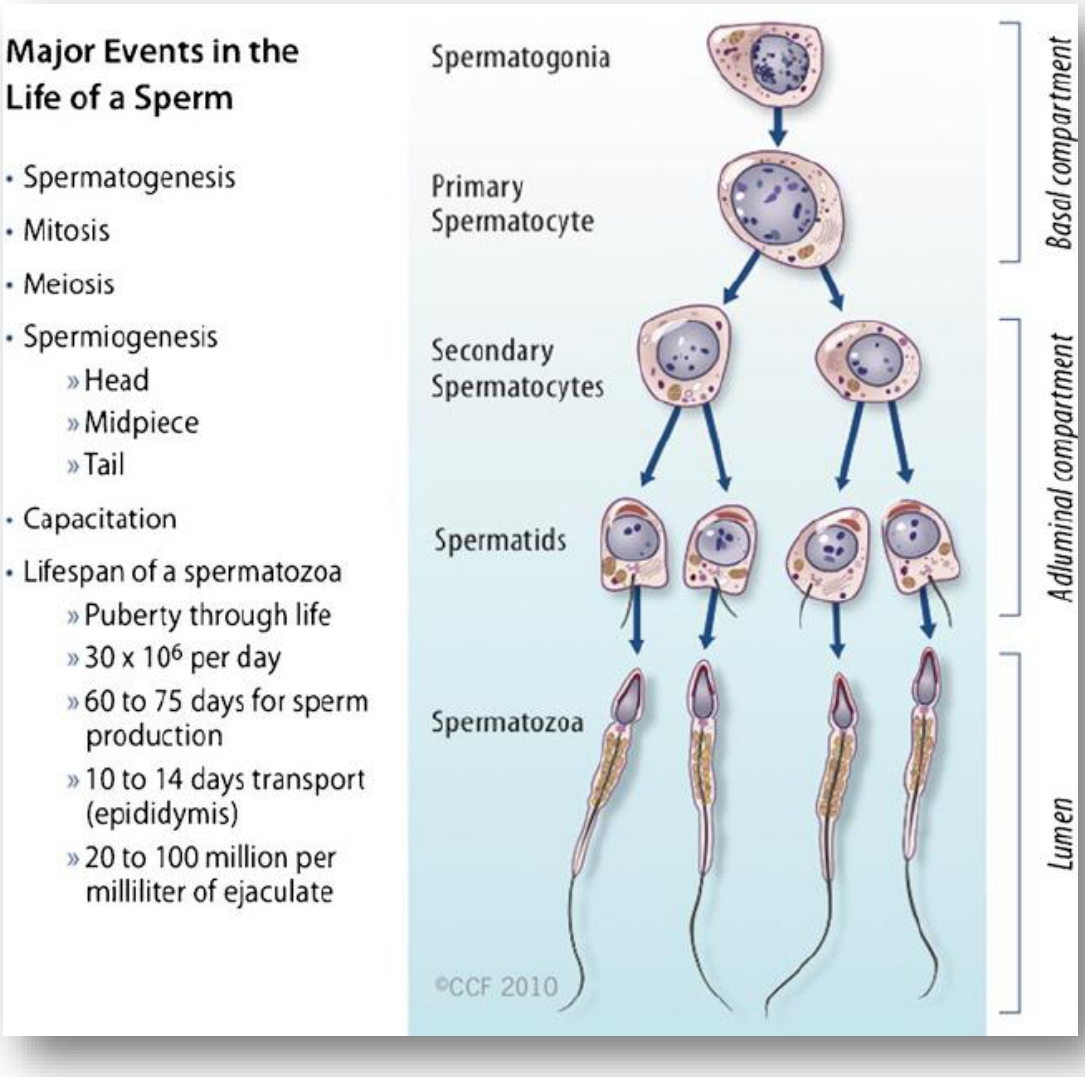

Figure 1.4: Spermatogenesis. Major events in the life of a sperm involving spermatogenesis, spermiogenesis, and spermiation **(Durairajanayagam *et al.*, 2015)**

Table 1.1: Terminology in spermatogenesis **(Oliveira and Alves, 2015)**

Process	Description
Spermatogoniogenesis	Process of producing spermatogonia through multiple mitoses to amass a large population of stem cells, most of which undergo meiosis to produce spermatozoa
Spermatogenesis	Process of differentiation of a spermatogonium into a spermatid Purpose: to produce (via mitosis and meiosis) the necessary genetic material for species replication
Spermatocytogenesis	Process of producing spermatocytes that occurs in the basal compartment of the seminiferous tubules
Spermiogenesis	A complex metamorphosis that transforms round spermatids (from the final division of meiosis) into a complex structure spermatozoon
Spermiation	Process whereby a mature spermatid frees itself from the Sertoli cell and enters the tubular lumen

1.1.2 Sperm Structure

A normal human spermatozoon is 55 to 70 μm in length and has three main structural regions: the head, midpiece, and tail (flagellum). The principal function of the sperm head is the contribution of its haploid set of chromosomes to the oocyte at fertilization, whereas the midpiece and tail provide the motility essential for the spermatozoon to reach the fertilization site (Figure 1.5).

The sperm head contains the cell's nuclear DNA, when the chromatin is heavily condensed, and its protamines are highly cross-linked so that the sperm nucleus is stabilized and effectively inactivated upon fertilization. This makes the head of the spermatozoon inflexible, which assists in penetration of the oocyte's zona pellucida during fertilization. The anterior half to two-thirds of the sperm head is covered by the acrosome, the membrane-bound structure that originated from the Golgi complex during spermiogenesis **(Patton and Battaglia, 2005)**.

The acrosome contains a number of hydrolytic enzymes, such as hyaluronidase and acrosin, which are required for fertilization. During fertilization, the acrosomal membrane fuses with the oocyte plasma membrane oocyte at numerous sites. This is followed by the acrosome reaction, an event characterized by acrosomal enzyme released from the head tip.

Among the common abnormalities of the sperm head are defective shape or size and the presence of numerous vacuoles (> 20 %) within the head surface. Shape defects include large, small, tapering, pyriform, amorphous, double heads, and various other combinations

The tail measures 40–50 μm in length (nearly ten times the length of the head) and provides motility for the cell. The sperm cell's entire motility apparatus is contained in the tail, propelling the sperm body via waves generated in the neck region that pass along distally in a whiplash manner. The tail can be divided into: the midpiece (anterior portion); principal piece; and endpiece (posterior portion) **(Durairajanayagam *et al.*, 2015)**.

The sperm midpiece contains the mitochondria that generate energy via oxidative phosphorylation; the centriole, used by the fertilized oocyte in its first cell division; and the beginnings of axoneme, the motility apparatus. The mitochondria are arranged helically around the proximal part of the axoneme, and they supply the adenosine triphosphate (ATP) necessary for flagellar motility **(Patton and Battaglia, 2005)**.

Ideally, the midpiece supports the head at exactly the center position. It should be slender as well (maximum width of 1 μm), yet thicker than the rest of the tail and between 7.0 and 8.0 μm in length. The tail diameter should be between 0.4 and 0.5 μm, measuring about 50 μm in length. The tail should have a well-defined endpiece, without any coiling or abnormal bending (over 90°). Throughout its length, the flagellum shows specific modifications. A central axoneme consists of nine microtubular doublets circularly arranged to form a cylinder around a central pair of single microtubules (Figure 1.6). In the middle piece, the axoneme is surrounded by an outer cylinder of nine outer dense fibers arranged as nine rod-like structures that run parallel to the axoneme; they play a role in protecting sperm against shearing forces, defining waveform and generating motility **(Carrell, 2010)**. Motility plays a key role in sperm transport through the cervix; the sperm cells need to maintain motility despite being suspended in fluid secreted by the female reproductive organs. Moreover, motility is required

to avoid phagocytosis by polymorphonucleocytes found in female body fluid (**Durairajanayagam *et al.*, 2015**).

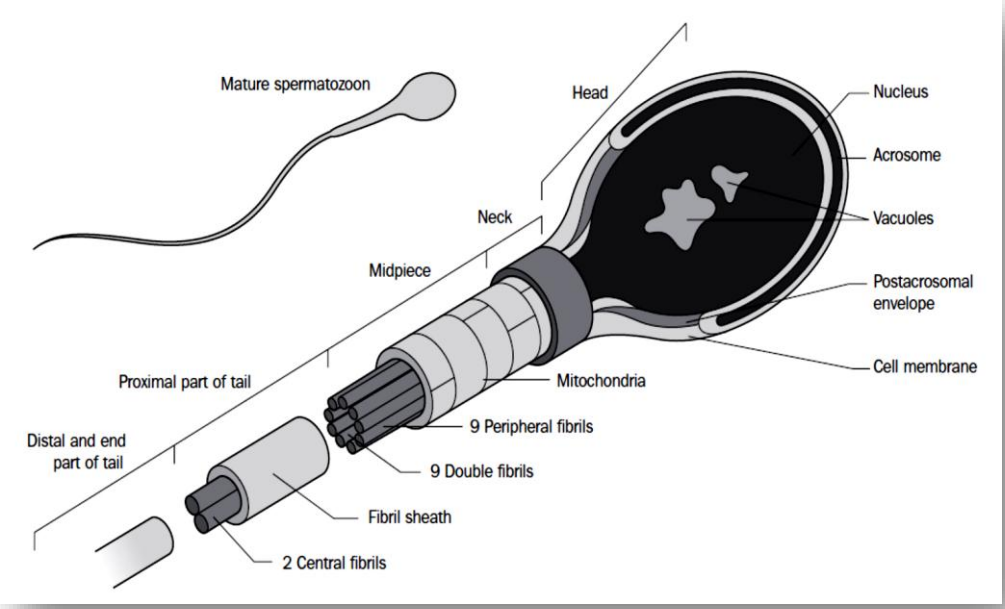

Figure 1.5: Light and electron microscopic diagrams of human spermatozoon **(Oehninger and Kruger, 2007)**

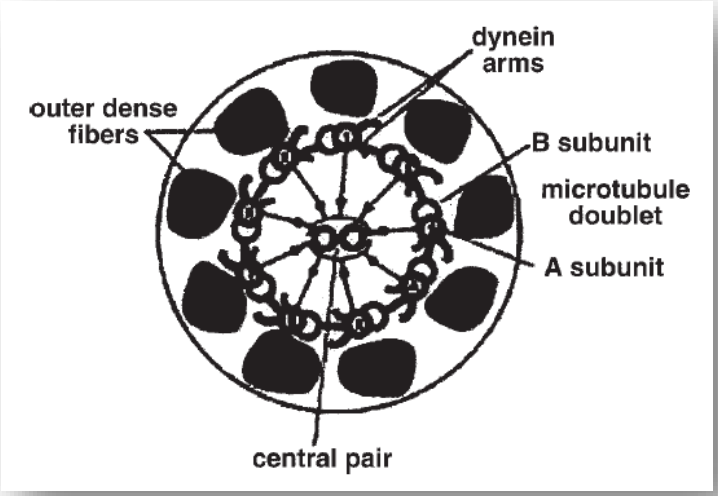

Figure 1.6: Diagram of a cross-section of the sperm axoneme **(Patton and Battaglia, 2005)**

1.1.3 Sperm Function and Physiology

After production in the testes, immature spermatozoa are moved through the corpus and caput regions of the epididyma and are then stored in the proximal section of the cauda epididymis. The spermatozoa move a total of 6 m through the reproductive tract before leaving the urethra. The average transit time in the epididymis is estimated at 12 days. Sperm motion is driven by hydrostatic pressure that is created by the combination of fluids secreted by the seminiferous tubules and tubular peristalsis. Movement through the proximal epididymis is mediated by peripheral smooth muscle contractions, and movement throughout the epididymal head is mediated by contraction of the tunica albuginea. Fluidic rhythmic movements of the cilia, lining the walls of the ducts, and the cyclic contractions of contractile cells along the wall of the epididymal duct further propel seminal constituents. Epididymal duct contraction is believed to be regulated by cholinergic, adrenergic factors, and vasopressin **(Mohanty and Singh, 2017)**.

The spermatozoa mature during epididymal transit and storage and acquire functional competence. The most obvious maturational change in spermatozoa is the motility acquisition when in contact with seminal plasma or physiological culture media - a process referred to as "activation." Other changes occuring during epididymal maturation of spermatozoa are alterations of the plasma membrane, chromatin condensation and stabilization, and possibly some final modifications to the shape of the acrosome **(Patton and Battaglia, 2005)**. These changes are induced by the fluid medium, secretions of the epithelium of the seminiferous tubules and epididymal lumen that the cells are exposed to. This epididymal fluid contains many substances such as sodium, glutamate, albumin, bicarbonate, transferrin, immobulin, inositol, potassium, L-carnitine, sialic acid, lactate, metalloproteins, proenkephalin, taurine, clusterin, glycerophosphorylcholine, and chloride. These substances are in part responsible for epididymal cell metabolism, activation of sperm motility, and regulation of fluid retention of sperm and epididymal cells **(Mohanty and Singh, 2017)**.

At ejaculation, sperm are transported from their storage site and are mixed with prostatic fluid and seminal vesicle fluid before passage along the penile urethra. The first fraction of the ejaculate contains most of the spermatozoa, suspended in epididymal and prostatic fluid, whereas subsequent fractions contain both prostatic and vesicular fluid **(Patton and Battaglia, 2005)**.

It is commonly stated that, the seminal plasma provides a nutritive and protective medium for the spermatozoa during their journey through the female reproductive tract. The main constituents of semen are listed below along with their functions:

- Water: fluid mechanism of transport for sperm;
- Buffers: protect sperm in acidic vaginal environment,
- Nutrients: fructose, carnitine, vitamin C, and citric acid provide nourishment for spermatozoa;
- Mucus: acts as lubricant for sexual intercourse;
- Spermatozoa: oocyte fertilization;
- Enzymes: semen clotting in vagina and further liquefaction of clot. Prostate-specific antigen (PSA) is a glycoprotein enzyme encoded by the KLK3 gene produced by the prostate for semen liquefaction and dissolving of the cervical mucus for sperm penetration;
- Prostaglandins: stimulate smooth muscle contraction and transport through both reproductive tracts and improve sperm motility;
- Immunity particles: lysozyme, immunoglobulin, and leukocytes act as antibacterial agents and wash out the urethra; zinc acts as antioxidant;
- Other cells: genitourinary tract epithelial cells and immature germ cells **(Mohanty and Singh, 2017).**

During intercourse, the spermatozoa are deposited into the vagina, near the cervical os, and must swim through the cervical mucus, accross the uterus, enter the oviduct, and reach the oocyte in its ampullary portion for fertilization to occur **(Patton and Battaglia, 2005)**.

As with epididymal maturation, capacitation is required too before fertilization can occur. Capacitation takes place after ejaculation into the female reproductive tract. During capacitation, spermatozoa undergo a biochemical changes sequence that ultimately enable them to fertilize an ovum. The sperm plasmalemma is reorganized to support the subsequent acrosome reaction; seminal plasma factors are removed and modifications are made to the sperm membrane, sterols, lipids, glycoproteins, outer acrosomal membrane, and surface charge. The concentration of intracellular free Ca^{2+} increases as well. In particular, it is the removal of cholesterol from the surface membrane that allows for the acrosome reaction to occur **(Durairajanayagam *et al.*, 2015)**. In addition, D-mannose binding lectins are involved

too in the binding of human sperm to the zona pellucida. Thus, all these series of changes are necessary to transform the stem cells into fully mature, functional spermatozoa equipped to fertilize an egg (Figure 1.7) **(Zini and Agarwal, 2011)**.

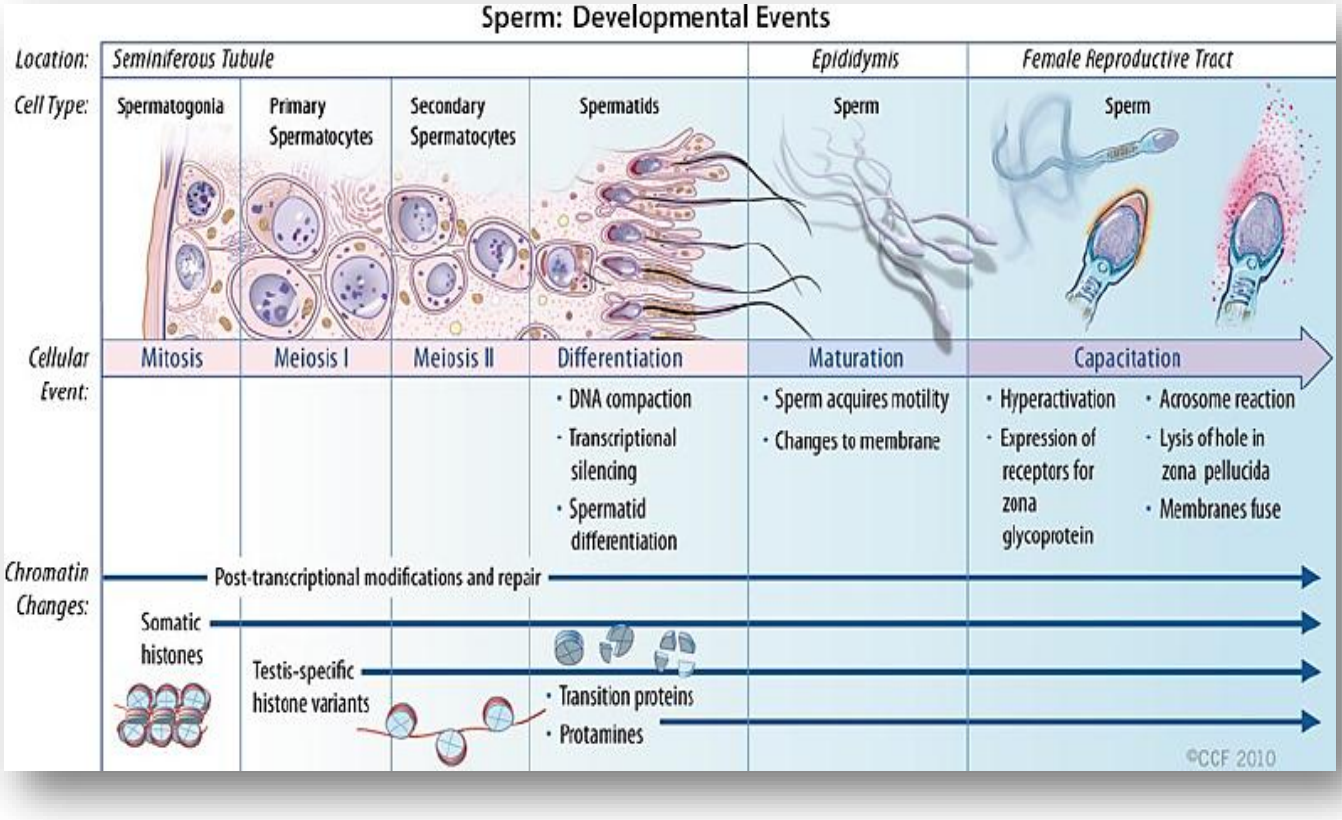

Figure 1.7: Diagrammatic representation of sperm developmental events. Changes that occur during the development of a germ cell into a spermatozoon leading to its release and subsequent maturation and storage in the epididymis, prior to its journey into the female reproductive tract **(Zini and Agarwal, 2011)**

1.1.4 Pathophysiology of Sperm and Infertility

The complexity of sperm structure and function means that it can be difficult to determine the pathophysiological reasons a man's infertility. If the developmental or maturational processes are disturbed, this could result in issues, such:

- Low sperm concentration (from inefficient spermatogenesis);
- Poor sperm motility (caused by midpiece or axonemal abnormalities);
- Abnormal sperm morphology (owing to errors in spermiogenesis).

This list is just illustrative, it could be extended to take in every step of each process, from the activation of spermatogonium to fertilization, and even further, because embryo development is affected by the quality of the nuclear DNA of fertilizing spermatozoon **(Patton and Battaglia, 2005)**.

1.2 Male Infertility

1.2.1 Definition

Infertility is defined as failure to conceive after 12 or more months of regular unprotected intercourse. A comprehensive male infertility assessment should be pursued sooner than a year if there is a previous infertility history, a known risk factor, advanced female age or a specific request by the couple **(Sabanegh, 2011)**.

It has been recorded that 48.5 million couples that have unprotected intercourse suffer from infertility worldwide. However, this statistic does not clearly define infertility by geographic region. In general, 50% of infertility cases are due to a solely female factor, pure male factor accounts for 20-30% of the problem, and the remaining 20-30% is due to a combination of both male and female factors.

At least 30 million men worldwide are infertile with the highest rates in Africa and Eastern Europe as illustrated on Figure 1.8 **(Agarwal *et al.*, 2015)**.

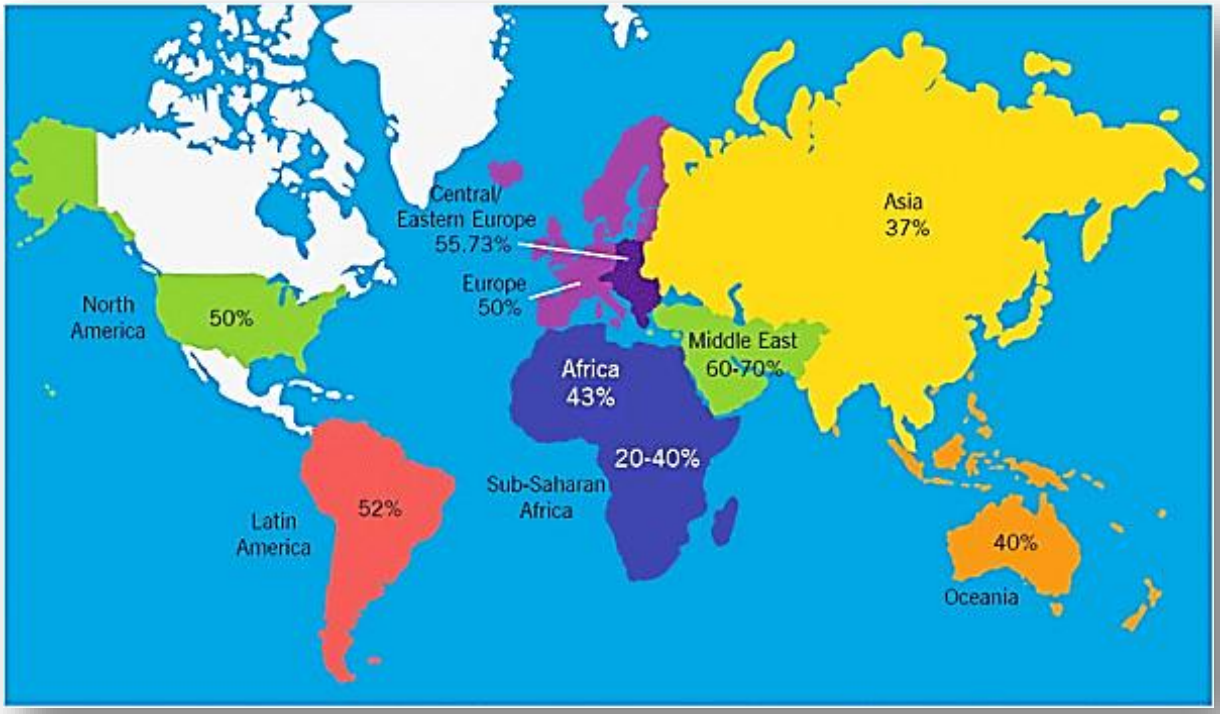

Figure 1.8: World map containing percentages of infertility cases per region that are due to male factor **(Agarwal *et al.*, 2015)**

The figure above demonstrates rates of infertility cases in each region studied (North America, Latin America, Africa, Europe, Central/Eastern Europe, Middle East, Asia, and Oceania) due to male factor involvement **(Agarwal *et al.*, 2015).**

1.2.2 Etiology

1.2.2.1 Pre-testicular

a. Hypo gonadotropic hypogonadism

Hypo gonadotropic hypogonadism affects fertility at multiple levels. Sperm production is deleteriously affected via a lack of testosterone and a lack of a stimulatory effect on the Sertoli/germ cell complex. Sexual function is negatively impacted with effects seen at the level of erectile function, ejaculatory function, and sexual desire too. There are several hypo gonadotropic hypogonadism etiologies. The most common are elevated prolactin,

medications, illicit drugs, and pituitary damage. Kallmann syndrome is another, albeit rare, cause of hypo gonadotropic hypogonadism **(Parekattil and Agarwal, 2012)**.

b. Elevated prolactin

Elevated prolactin may cause hypogonadism by suppressing the release of GnRH. Symptoms of hypogonadism, especially erectile dysfunction and loss of libido, are the most common presenting symptoms in males with hyper-prolactinemia, though galactorrhea and gynecomastia may also be evident. Elevated prolactin may be secondary to various etiologies. The most common is a prolactinoma, **(Carrell, 2010)**, renal failure, hypothyroidism and cirrhosis. Prolactin levels may be elevated too in certain systemic diseases such as systemic lupus erythematous, rheumatoid arthritis, celiac disease, and systemic sclerosis. Many drugs elevate prolactin levels, especially those which block the effects of dopamine, such as antipsychotics **(Plessis, 2014)**.

c. Pharmacologic

Various medications may cause hypo gonadotropic hypogonadism. Estrogens and progestin may cause a decrease in testosterone levels via negative feedback to the hypothalamic-pituitary-gonadal axis. Marijuana is known to decrease testosterone levels by working on the endocannabinoid receptors present at multiple levels of the hypothalamic-pituitary axis. Both ethanol and cannabinoids suppress GnRH secretion at the level of the hypothalamus. Endocannabinoid receptors have also been found in the pituitary and may affect the hypothalamic-pituitary axis at that level as well. LHRH agonists and antagonists are used for the treatment of prostate cancer, precocious puberty, and gender reassignment surgeries **(Carrell, 2010)**. In the male, both induce deep hypogonadism. LHRH antagonists directly and intuitively decrease LH and FSH levels. LHRH agonists produce a tonically stimulated state, which, unlike the physiologic circadian rhythmicity of normal LHRH stimulation, acts to decrease LH and FSH secretion. Narcotics may also produce profound hypogonadism. Nearly 40% of men using methadone were found to have total testosterone levels less than 230 ng/dL **(Esteves and Agarwal, 2011)**.

d. Kallmann Syndrome

Kallmann Syndrome affects between one in 8,000–10,000 males. It is a spectrum of disease in which the primary manifestations are anosmia and hypo gonadotropic hypogonadism that leads to an absence of puberty **(Plessis, 2014)**. Multiple genetic disorders can lead to Kallmann Syndrome. These most disorders commonly manifest through the same mechanism whereby GnRH secreting neurons fail to migrate to the hypothalamus. Lack of these neurons in the hypothalamus results in a lack of GnRH secretion and thus hypogonadism **(Parekattil and Agarwal, 2012)**.

e. Hyper gonadotropic hypogonadism

One of the most common causes of hyper gonadotropic hypogonadism is Klinefelter Syndrome (Klinefelter's). Klinefelter's affects male fertility by altering spermatogenesis both directly and indirectly by altering the hormonal midium. Interestingly, sex hormone levels are normal until puberty. During puberty, they do rise to low-normal levels, however, by adulthood, serum testosterone levels are typically below normal. Histologic studies demonstrate gradual degeneration of the testes during development with hyperplasia of poorly functioning Leydig cells **(Carrell, 2010).**

1.2.2.2 Testicular etiologies

a. Varicocele

A varicocele consists of a pampiniform plexus dilation likely caused by the absence or incompetence of the venous valves at the level of the internal spermatic vein. Varicoceles have long been associated with infertility. It affects multiple semen parameters; total sperm count, sperm motility, and sperm morphology that are all negatively affected. There are several theories about the underlying pathophysiology of a varicocele, with heat, renal metabolites, and hormonal abnormalities all playing a role. However, most agree that disruption of the countercurrent heat exchange mechanism in the testis, causing hyperthermia, is the most likely mechanism. The mechanism by which heat causes decreased sperm counts is poorly understood. However, one hypothesis is that high temperatures could increase the metabolic rate of testicular and epididymal sperm. Furthermore it could increase the oxidative damage

amount to both the structure and the DNA of the spermatocytes and spermatids **(Parekattil and Agarwal, 2012)**.

b. Cryptorchidism

Cryptorchidism is well known to affect fertility too, with bilateral cryptorchidism having more severe effects than unilateral, and with higher testes having worse function than lower testes. The pathophysiology is complex, with heat likely playing a partial but significant role. Other factors are implicated too, including the underlying genetics, hormonal status, and environmental exposures, that originally lead to the cryptorchidism **(Ono and Sofikitis, 1997)**.

c. Testicular cancer

It has been proved that testicular cancer is strongly associated with infertility. There are multiple ways in which testicular cancer is related to and can contribute to reduce fertility. Both testicular cancer and impaired spermatogenesis may be related in their etiology of embryologic testicular dysgenesis. The testicular dysgenesis syndrome is a spectrum of disease that may involve cryptorchidism, hypospadias, decreased spermatogenesis, and testis cancer. In this syndrome, it is thought that all of these share an origin of abnormal fetal testis development. As a result of this abnormal development, any number of these symptoms may be present in a boy **(Menon *et al.*, 2008)**. Testicular tumors may directly contribute to infertility too by secreting hormones, which can down regulate sperm production in the contralateral testis. This is uncommon and has been seen with Leydig and Sertoli cell tumors as well as seminomas. Tumors may directly disrupt spermatogenesis by mass effect or by the effects of the inflammatory reaction to the tumor **(Esteves and Agarwal, 2011)**. Hence, cancer treatments may decrease fertility **(Kacem *et al.*, 2014)**.

d. Ionizing radiation

Excellent data on the effects of ionizing radiation is available from two similar studies, which are unlikely to be repeated. Researchers in these studies prospectively irradiated the testes of prisoners with single or multiple doses of radiation up to 600 cGy. Sperm counts were followed, and serial testicular biopsies were performed. These studies showed that sperm counts declined when testes were irradiated and that decline was dose dependant. At low doses

of ~7.5 cGy, a mild decline of sperm counts was observed, and this decline increased to severe oligospermia by 30–40 cGy and azoospermia by 78 cGy. The time to recovery was seen to be dose-dependent too, with those receiving 20 cGy starting to develop a recovery of sperm counts by 6 months, those with 100 cGy at 7 months, 200 cGy at 11 months, and 600 cGy at 24 months. The percentage of men achieving a complete recovery even in time declined with increasing radiation doses **(Rowley *et al.*, 1974)**.

e. Chemotherapy

Chemotherapy typically targets rapidly dividing cells and thus has severe effects on the germinal epithelium. As such, the expected outcome of acute chemotherapy is a decline in spermatogenesis. BEP: Bleomycin, etoposide, and cisplatin or carboplatin, constitutes the most commonly used chemotherapy regimen for testicular germ cell tumors. The decrease in fertility seen post-BEP chemotherapy is likely the result of a direct reduction of spermatogenesis and not as a result of any change in the hormonal midium. Indeed, testosterone levels are not seen to be significantly reduced at 12 months' post-chemotherapy, and FSH levels are appropriately elevated. FSH levels decline as spermatogenesis returns over the following 2–4 years. It should be noticed, however, that return of spermatogenesis is not guaranteed **(Parekattil and Agarwal, 2012)**.

f. Genetic azoospermia/Oligospermia

It is estimated that 2–8% of infertile men have an underlying genetic abnormality, with this number rising to 15% in azoospermic men. Although the majority of male infertility does not have an identifiable genetic cause, two potential etiologies are Y chromosome micro deletions and karyotypic abnormalities. The two most common karyotypic abnormalities are Klinefelter's (47, XXY) and chromosomal translocations. Robertsonian translocations represent a third significant genetic cause of infertility. They occur in 0.8% of infertile men, and this number rises to 1.6% in oligospermic individuals **(Visser and Repping, 2009)**.

g. Environmental factors

Hyperthermia is considered to be a major contributor in the pathogenesis of infertility in men with varicocele and cryptorchidism. Many lifestyle factors have the potential to increase scrotal temperatures too, including underwear type, heated car seats, and occupational heat

exposure **(Jung and Schuppe, 2007)**. Recently, cell phones have been implicated as possibly playing a role in decreasing male fertility. The mechanism by which cell phones affect semen parameters has not yet been elucidated, but one hypothesis is that cell phone-generated electromagnetic radiation (CPEMR) alters mitochondrial function and acts to increase reactive oxygen species (ROS) **(Parekattil and Agarwal, 2012)**. Tobacco consumption has been implicated in the pathogenesis of numerous cancers and medical diseases. While this consumption significantly impacts female fertility comparing to male for whom the effect is less clear. Semen parameters, including sperm density, motility, and morphology, have all been shown to be worsened with tobacco use **(Künzle *et al.*, 2003; Collodel *et al.*, 2009)**. However, a significant reduction in fertility has not yet been proven.

h. DNA damage

There are numerous etiologies of sperm DNA damage. Radiation, toxins, genital tract inflammation, varicocele, advanced paternal age, and testicular hyperthermia, all induce significant DNA damage **(Belloc *et al.*, 2009; Hammiche *et al.*, 2010)**.

1.2.2.3 Post-testicular

a. Absence of the vas deferens

Congenital Bilateral Absence of the Vas Deferens (CBAVD) consitutes a condition strongly related to cystic fibrosis (CF) and has even been considered as a diagnostic criterion for CF.

Congenital unilateral absence of the vas deferens (CUAVD) is a different entity altogether **(Donohue, 1989)**. While there is still a significant rate of CFTR mutations in men with CUAVD, especially when the obstructive azoospermia is present **(Lissens *et al.*, 1996)**. The bulk of CUAVD is the result of an embryologic Wolffian duct aberrancy **(Shapiro *et al.*, 2003)**. Since CUAVD not associated with a CFTR mutation is usually a unilateral and isolated phenomenon, fertility is often preserved **(Parekattil and Agarwal, 2012)**.

b. Young's Syndrome

Young's Syndrome is a rare disorder, which presents clinically as obstructive azoospermia and chronic sinopulmonary infections **(Handelsman *et al.*, 1984)**. Thus, it could be difficult to differentiate clinically from cystic fibrosis variants and primary ciliary dyskinesia. Indeed, definitive diagnosis of Young's Syndrome requires negative CFTR genetic testing as well as investigation of ciliary ultrastructure to rule out primary ciliary dyskinesia. Normal spermatogenesis is seen, and the obstructive azoospermia is due to intensified secretions in the vas deferens.

The etiology of Young's Syndrome is unclear with childhood mercury exposure having been postulated to play a role in the past **(Goeminne and Dupont, 2010)**.

c. EjDO/Seminal vesicle dysfunction

Ejaculatory duct obstruction is a common etiology of male infertility, occurring in 1–5% of men presenting with infertility **(Smith *et al.*, 2008)**. There are many causes of ejaculatory duct obstruction, including cystic fibrosis spectrum disease, Wolffian or Muellerian origin cysts, calcifications, tuberculosis and other genital urinary infections, calculi, and urinary tract instrumentation **(Carson, 1984; Paick *et al.*, 2001)**.

d. Vasectomy and vasectomy reversal

Vasectomy is a procedure that is intended to produce infertility, and it is successful in over 90% of cases **(Labrecque *et al.*, 2002)**. Some of the key determinants of success are related to aspects of surgical technique. The manner of ligating the ends, non- ligation versus clipping versus suture ligation, length of vas removed, as well as whether to fold vas ends are all controversial **(Hallan and May, 1988; Adams and Wald, 2009)**. Two maneuvers which do seem to provide significant benefits are luminal cauterization and fascial interposition **(Cook *et al.*, 2007; Sokal and Labrecque, 2009)**. Reversal vasectomy may be performed in an attempt to return fertility to the sterilized man. The outcomes of reversal vasectomy are dependent on a number of factors. Surgical technique is a one, with the use of a microscope significantly improving pregnancy rates over loupe-assisted vasovasostomy **(Jee and Hong, 2010)**. Time elapsed since fertility also plays a significant role with a 97% patency rate and

76% pregnancy rate being achieved if surgery is performed at less than 3 years since vasectomy **(Belker *et al.*, 1991)**.

e. Nerve injury

Nervous injury, affecting ejaculation, may occur at many levels and have a diverse etiology ranging from spinal cord injury to neural damage during retroperitoneal or pelvic surgery to neuropathy from systemic diseases. Ejaculatory dysfunction is present in 90% of spinal cord injury patients. The type and severity of ejaculatory dysfunction are dependent on the level and extent of the injury **(Parekattil and Agarwal, 2012)**.

f. Medications

Medications could affect ejaculation process by altering adrenergic signaling. This is most clearly seen with alpha-1 antagonists. Recent studies have shown that the ejaculatory dysfunction induced by alpha-1 antagonists is actually a failure of emission **(Hisasue *et al.*, 2006; Kobayashi *et al.*, 2008)**.

Antipsychotics have long been associated with sexual dysfunction, including ejaculatory dysfunction. Antipsychotics have effects on many different neurotransmitters including dopamine, norepinephrine, acetylcholine, and serotonin. Predictably, altered ejaculatory function with antipsychotics use correlates with anti-adrenergic actions of the antipsychotics **(Smith *et al.*, 2002)**.

g. Prostate resection

Surgery of the prostate is well known to cause retrograde ejaculation. Transurethral resection of the prostate as well as the laser photovaporization and enucleation all have a high likelihood of inducing retrograde ejaculation since removal of the proximal prostatic urethra severely diminishes the resistance to back flow of semen **(Parekattil and Agarwal, 2012)**.

h. Coital

Abnormal coital practices may play a role in infertility when they interfere with semen deposition in the vagina or affect their timing with the female reproductive cycle. Similarly,

erectile dysfunction and penile abnormalities such as hypospadias and chordee may interfere with semen deposition and thus may play a role in infertility.

Lubricants are commonly used by infertile couples, and many vaginal lubricants have been shown to negatively affect fertility. Many synthetic lubricants not only affect sperm motility but have also been shown to increase the DNA fragmentation index **(Agarwal *et al,* 2008a)**.

1.2.3 Male Infertility Diagnosis

1.2.3.1 Assessment of subfertile male

The diagnosis and treatment options for male infertility have recently undergone significant change as advancements in technology and understanding in the fields of molecular biology, genetics, and laboratory medicine. Further, advancements in assisted reproductive technologies (ART) have rendered previously subfertile and infertile couples with various options for pregnancy. Such changes in the understanding and treatment of fertility and infertility have necessitated a much more detailed assessment of the couple presenting with infertility. At the basis of this assessment is a sophisticated and methodological evaluation of male factor infertility including laboratory tests of urine, serum, and semen, as well as radiological and genetic studies **(Parekattil and Agarwal, 2012)**. Conception requires a balanced coordination between the endocrinologic and reproductive systems of both male and female partners **(AUS, 2010)**. The workup and diagnosis of infertility is unique in medicine in that it involves multiple organ systems of two individuals. Pathology is often difficult to isolate given this complexity. Isolated male factor has been shown to be causative in 20% of infertility cases and is a contributing factor in conjunction with female factor pathology in an additional 30% of cases **(Slama *et al.,* 2002)**.

On the other hand, since evaluation of the male partner in an infertile couple is simple, fast, inexpensive and usually non-invasive, it may be performed as soon as the infertile couple seeks medical assistance, or whenever the male partner decides to evaluate his fertility status **(Sharlip *et al.,* 2002)**. Evaluation of the male partner should be carried out following basic medical guidelines, which are: the patient's history, physical examination, as well as all the laboratory and imaging resources available at the time (Figure 1.9). While the patient's

history and physical examination are of fundamental importance to all patients, imaging and laboratory techniques should be used when necessary. Several patients will require only two separate standard semen analyses, while others will need to go through many tests in order to elucidate the infertility cause. Each patient must be individually evaluated **(Oehninger and Kruger, 2007)**.

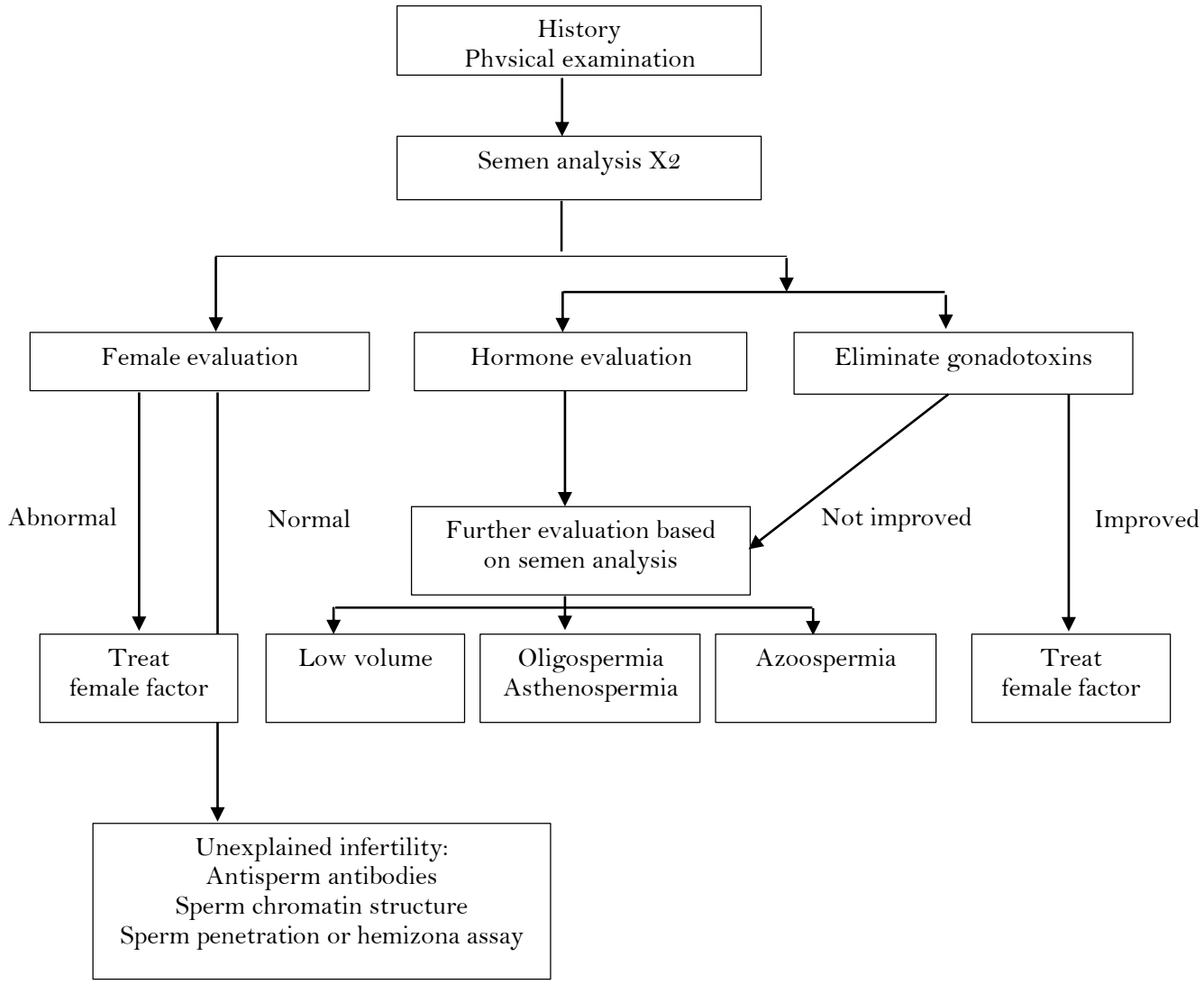

Figure 1.9: General Algorithm for diagnostic evaluation of male infertility **(Turek, 2005)**

1.2.3.2 Basic semen analysis

The semen analysis is the cornerstone of the assessment of the male partner for infertility, and evaluates not only spermatozoa, seminal plasma and non-sperm cells are assessed too.

Human spermatozoa show marked heterogeneity, and two semen samples should be evaluated, collected at least 7 days between specimens, and 3 months after any febrile illness. Samples are collected after a period of abstinence of greater than 48 h, but less than 7 days. Specimens should be analyzed within 1 h of collection, as semen liquefies at room temperature within 60 min **(Samplaski *et al.*, 2010)**. The semen analysis is evaluated using standardized reference ranges. Most commonly the World Health Organization (WHO) values, which were published as manuals in 1980, 1987, 1992, 1999, and 2009, providing standard reference values for semen characteristics (Table 1.2) **(Cooper *et al.*, 2009)**.

Table 1.2: 2009 World Health Organization standard reference values for semen characteristics **(Cooper *et al.*, 2009)**

Volume	1.5 mL or more
Ph	7.2 or more
Sperm concentration	15 million or more spermatozoa/mL
Total sperm count	39 million or more spermatozoa/ejaculate
Progressive motility	39% or more
Total (progressive + non-progressive) motility	40% or more
Morphology	4.0% or more normal forms
Vitality	58% or more live

a. Specimen handling

Semen samples present a possible biohazard since they may contain harmful viruses, e.g. human immunodeficiency virus (HIV), hepatitis B and herpes. Therefore, semen samples should always be handled with care, as if infected, and the wearing of protective gear is advised (gloves, masks, and spectacles) **(Oehninger and Kruger, 2007)**.

The way in which the sample is produced is controlled. Several patients may produce a sample by coitus interruptus or by using a spermicidal condom if the sample is produced at home. Coitus interruptus has the disadvantage that the first part of the sample may be lost. An indication that the sample may have been produced by coitus interruptus will be the presence of vaginal epithelium cells **(Alexander, 1982)**.

When the patient collects the sample at the laboratory, a relationship can be built with the patient, information is easy to obtain and the patient's enquiries can be answered. When a sample is brought to the laboratory and left on a counter without any information, the results of such a semen analysis cannot be evaluated or interpreted because the method of production, days of abstinence and time of ejaculation are not known. Semen samples should thus be produced by masturbation into a clean plastic container that is sterile-packed at shipment, or otherwise should be separately sterilized at the laboratory. The patient is instructed to urinate and then to wash his hands with soap and water and the glans of the penis with water alone, before producing the sample **(Oehninger and Kruger, 2007)**.

The patient should be asked about the precise period of abstinence, as well as a short medical history. Questions regarding his medical history should include information on the occurrence of any previous infections or illnesses, especially in the past 3 months, if it is his first visit, or since his previous semen analysis when a repeated analysis is being performed. Medical history, any recent medication or anesthesia during the laest 3 months, any previous history of operations of the urogenital tract, especially involving the bladder, an orchidopexy, orchiectomy, varicocelectomy or testicular biopsy, or whether severe injuries of the testicles or orchitis, should be considered in assessment or questionning. A note should be made about smoking and drinking habits too **(Menkveld and Kruger, 1996)**.

b. Physical parameters

Parameters describing the appearance of the sample include color, liquefaction, and viscosity, while coagulation and odor can also be added to this category. pH is included in this group too. All these parameters are simple to assess and are primarily determined by visual examination.

- **Coagulation:** this parameter is an important aspect of semen analysis that is ignored by several investigators, essentially because numerous semen samples are still produced at home instead of at the laboratory. Human semen is ejaculated in a liquefied state, but is quickly transformed into a semisolid state or coagulum, probably under the influence of the enzyme protein kinase secreted by the seminal vesicles **(Mandal and Bhattacharyya, 2009)**. In a normal situation, nearly the whole sample is transformed into the coagulated state, and only a minor part remains liquefied. This

is generally regarded as the first portion of the ejaculate, containing the major part of the motile sperm fraction. In some cases, coagulation does not occur, and this may be attributed to the congenital absence of the vas deferens and the seminal vesicles, as the coagulating enzymes originate from the seminal vesicles, and is then associated with the absence of fructose in the seminal plasma too **(Oehninger and Kruger, 2007)**.

- **Liquefaction:** In a normal sample, liquefaction occurs within 10–20 minutes. This is caused by a proteolytic enzyme fibrinolysin secreted by the prostate **(Amelar, 1962)**, as well as two other proteolytic enzymes, fibrinogenase and aminopeptidase **(Mann, 1971)**. Liquefaction therefore serves as an indicator of normal prostatic function. After complete liquefaction the sample will appear homogeneous in composition and color. Small roundish particles may still be present in some samples; however, this can be regarded as normal, and they will usually dissolve within an hour. If liquefaction takes more than 20 minutes or does not occur at all, it is a sign that the prostate is not functioning normally usually as a result of previous prostatitis **(Menkveld and Kruger, 1996)**. In some cases this non-liquefaction of semen may be a cause of infertility, as the spermatozoa are not released from the coagulum **(Oehninger and Kruger, 2007)**.
- **Viscosity:** this parameter refers to the fluid consistency of the semen after coagulation and liquefaction have occurred. Viscosity is normal if it is possible to pour the semen in a drop by drop fashion **(Morey and Shoskes, 2009)**. It is essential to distinguish between a delayed period of liquefaction (non-homogeneous appearance) and an increase in the viscosity (homogeneous but 'sticky'). Increased viscosity may be the result of abnormal prostatic function due to an infection in the genital tract, prostate or seminal vesicles **(Alshahrani *et al.*, 2013)**, or an artifact as a result of the use of an unsuitable plastic container type, frequent ejaculation or the patient psychological state. A constant increase in viscosity may be regarded as a cause of infertility for *in vivo* conception, and can have an adverse effect on the determination of spermatozoa concentration and motility too **(Oehninger and Kruger, 2007)**.
- **pH:** the pH of semen normally ranges from 7.2 to 8.0. A low pH implies absence or blockage of the seminal vesicles, as the ejaculate consists entirely of acidic prostatic fluid **(Morey and Shoskes, 2009)**. Preference should be given to pH measurement using a special pH indicator paper (range 6.4–8.0), for hygiene reasons and the

possibility that sexually transmitted diseases may be transferred when using the glass-electrode method too. After liquefaction, a drop of semen is placed on the indicator strip and immediately compared against a color scale. The pH of a normal ejaculate may vary between 7.2 and 7.8 **(Cardona, 2010)**.

- **Volume:** the most common method still used nowdays to determine the volume is by transferring the sample to a 15-ml graduated conical tube and reading the volume to the nearest 0.1ml. Determination of the volume can be performed by means of weighing samples, taking the total weight of the sample and container minus the container weight determined beforehand. The weight is expressed as the nearest 0.1 ml, taking 1g equal to 1 ml **(Kvist and Björndahl, 2002)**. The normal volume of an ejaculate after 3–5 days of sexual abstinence is 2–6ml. A normal volume is important for good buffering function of the seminal pool against the acid secretions of the vagina. If the volume of a semen sample is smaller than 1.0 ml, it will be primordial to establish whether a complete sample was collected or not. This point should be taken into account, as the first portion containing the major amount of sperm with the best motility is frequently lost. A low volume may, however, also be the result of an obstruction due to a previous infection of the genital tract, or of congenital absence of the seminal vesicles and vas deferens; this condition will be associated with the absence of fructose **(Zaneveld and Polakoski, 1977)**. In addition, a small volume may be due to retrograde ejaculation, especially if the patient has had any previous surgery of the prostate or the bladder neck. Retrograde ejaculation can be diagnosed by investigation of the urine after ejaculation **(Oehninger and Kruger, 2007)**.
- **Color:** By paying attention to the color of the semen sample, an indication of possible pathology of the semen can already be obtained. The color of normal semen as opaque and grayish, which will change to yellowish with an increase in the days of abstinence. Fresh blood will give semen a reddish color and old blood a brownish color, that may be caused by recent inflammation. In some inflammation cases, a more yellowish color may exist, while samples with a low sperm concentration will usually have a transparent and watery consistency **(Plessis, 2014)**. Certain types of drugs such as antibiotics might discolor the semen **(Schirren, 1972)**.
- **Odor:** although semen has a strong, distinctive odor, derived from the prostatic secretions, this parameter is seldom used. The odor is sometimes compared to that of the flowers of the chestnut or St John's bread tree. It is thought that the odor is caused

by oxidation of the spermine secreted by the prostate. Only with absence of the odor or when an uncharacteristic odor is present should a note be made, as this is usually associated with an infection **(Zaneveld and Polakoski, 1977)**, or is the result of a long period of abstinence **(Mortimer, 1985)**.

c. Microscopic analysis

There is a general thought that the routinely measured semen parameters (sperm number, motility, vitality, morphology) do not monitor sperm function and that they are inadequate for predicting fertility **(Lewis, 2007)**. Whereas, routine morphological staining may be complemented by specific lectin or immunocytochemical stains of particular sperm structures **(Gomez *et al.* 1996; Cardona, 2010)**, additional investigations, in which certain spermatozoon functions are assessed, may provide clinically useful prognostic or diagnostic information. These tests should distinguish between fertile and infertile men or indicate the reason for the infertility and hence be of use in suggesting a rational therapy **(Nieschlag *et al.*, 2010)**.

- **Agglutination and presence of other cells:** The sample is examined for the presence of sperm agglutination. Two types of agglutination can be observed; in the first instance, agglutination can be due to non-specific factors where, in most cases, non-motile spermatozoa adhere to cells present in the seminal plasma; when this occurs it is termed aggregation **(Mortimer, 1994)**. The second is a specific agglutination, caused by antisperm antibodies, which consists mostly of motile spermatozoa clumps with only minimal involvement of other cells or debris. Agglutination is described as negative (–), occasional (±), slight (+), moderate (++) or severe (+++) **(Menkveld and Kruger, 1996)**, or as an appropriate percentage to the nearest 5% **(Mortimer, 1994; Kvist and Björndahl, 2002)**. Presence of further cells, such as round cells, and the presence of spermine phosphate crystals are recorded in the same way as for agglutination. The presence of any organisms is recorded too.
- **Motility and forward progression:** The extent of progressive sperm motility is related to pregnancy rates. Motility is actually determined in one of two manners. The first, by manual observation of the sample with phase-contrast optics. More recently, Automated Computer-Assisted Semen analysis (CASA) techniques have

been introduced with varying degrees of success **(Oehninger and Kruger, 2007)**. Sperm motility within semen should be assessed as soon as possible after liquefaction of the sample, preferably at 30 minutes. Nevertheless, in any case within 1 hour, following ejaculation, to limit the deleterious effects of dehydration, pH or changes in temperature on motility **(Cardona, 2010)**. Spermatozoa are classified according to the rapidity of their forward progressive motility into four grades, from grade a to graded, as follows:

- Grade a = rapid progressive motility;
- Grade b = slow or sluggish progressive motility;
- Grade c = non-progressive motility ;
- Grade d = immotile.

At least 200 spermatozoa should be counted in five separate high-power magnification fields utilizing phase-contrast microscopy. The percentages of the different categories must add up to 100%. The count should be repeated on a separate wet preparation. The results of the two counts are then averaged **(Kvist and Björndahl, 2002)**.

Poor motility or asthenozoospermia could be caused by several factors. One reason may be artifacts caused by the wrong collection method, such as use of a condom, which may be sperm-toxic, contamination by vaginal secretions, the use of lubricants **(Goldenberg and White 1975)**, an incomplete sample, and a long delay in transportation of the sample to the laboratory or exposure to extreme temperatures **(Appell and Evans, 1977; Makler, 1978; Carruthers, 1981)**.

Poor motility could be due to structural abnormalities of the midpiece too **(Folgeri *et al.*, 1993)** or the short-tail **(Barthelemy *et al.*, 2009)** and immotile cilia or Kartagener's Syndrome. Unfavorable environmental conditions during spermatozoa formation and maturation before they are released from the Sertoli cells, or during transport through the epididymis and ductal system, or via abnormal functions of the prostate or seminal vesicles, caused by acute infections or inflammation of the accessory glands, represent the main reasons for poor motility too. Other factors that can cause poor motility are the presence of hematospermia, a varicocele, chromosomal aberrations, bacterial infections,

and an abnormal pH, as well as the presence of certain metals or metal ions **(Gannon and Walsh, 2015)**.

- **Sperm concentration:** The cutoff point of 20 million spermatozoa/mL has been repeatedly suggested as the lower normal value for sperm concentration in an ejaculate **(Lieberman, 1993)**. The observation of a low sperm concentration, oligozoospermia, is indicated when sperm concentration falls below $5–10 \times 10^6$/mL depending on the cutoff point used. It may be due to the loss of a portion of the ejaculate, partial obstruction of the genital tract, drugs or genetic abnormalities. Other factors include medications such as nitrofurantoin and excessive heat exposure. On the other hand, azoospermia, complete absence of spermatozoa, may be due to the obstruction of the sperm transport, hypogonadism, and iatrogenic causes, such as chemotherapy or idiopathic factors, that are most probably genetic in origin **(Jarow *et al.*, 1989)**.
- **Sperm morphology:** Sperm morphology is another measure of semen quality. By assessing the dimensions and shape characteristics of the sperm head, midpiece, and tail, sperm can be classified as "normal" or not. In the strictest classification system (Kruger morphology) **(Menkveld and Kruger, 1996)**, only 14% of sperm are considered normal. In fact, this number correlates with the success of egg fertilization *in vitro* (IVF) and thus is ascribed clinical significance. In addition, sperm morphology is an indicator of testicular health, because shape characteristics are determined during spermatogenesis. Sperm morphology complements other information to estimate the chances of fertility **(Morey and Shoskes, 2009)**.

  A normal spermatozoon is defined as one having an oval form with a smooth contour and a clearly visible and well-defined acrosome, with homogeneous light blue staining. The tail should be apically inserted without any abnormalities of the neck/midpiece region; there should be no tail abnormalities; and there should be no cytoplasmic residues at the neck region or on the tail **(Menkveld and Kruger, 1996; Nieschlag *et al.*, 2010; Parekattil and Agarwal, 2012)**. The latest WHO manuals recommend that spermatozoa should be classified only as normal or abnormal. A note should be made if a specific abnormality occurs in a frequency of >20%. However, an abnormal spematozoon can have only one specific abnormality or any combination of two or up to four abnormalities. To reflect this, the teratozoospermia index (TZI) was

introduced as an indication of the mean number of abnormalities per abnormal spermatozoon **(Lieberman, 1993)**.

- **Sperm viability:** Non-motile sperm may either be dead (necrospermia) or immobile as a result of an ultrastructural defect. Viability testing is indicated when sperm motility is less than 5–10%. A high viability with a low motility is suggestive of a structural defect, such as primary ciliary dyskinesia, that may be further evaluated with electron microscopy. Surgically retrieved testicular sperm are non-motile, because they do not pass through the epididymis. Viability testing may be used to determine which sperm are alive and suitable for intra cytoplasmic sperm injection ICSI **(Wilcox *et al.*, 1995)**, using either dye exclusion or by evaluation of hypo-osmotic sperm swelling. Flow cytometry has been applied to assess sperm membrane vitality too, using dual staining for simultaneous assessment of the plasma and mitochondrial membrane integrity **(Evenson *et al.*, 1982)**. Dye exclusion involves mixing spermatozoa with a supravital dye, such as eosin or trypan blue. Sperm with an intact cell membrane are able to exclude the stain and will not change color. Because this method kills the sperm, they cannot later be used for ICSI. The hypo-osmotic sperm swelling test involves placing sperm into a low osmotic media and assessing their ability to respond **(Jeyendran *et al.*, 1984; Wallach *et al.*, 1992)**. Because the hypo-osmotic media does not kill viable sperm, they can be used for ICSI **(Samplaski *et al.*, 2010)**.
- **Detection and role of leukocytes:** The ejaculate contains cells other than spermatozoa, some of which may be clinically relevant. These include epithelial cells from the genitourinary tract, as well as leukocytes and immature germ cells, the latter two collectively referred to as "round cells" **(Cardona, 2010)**. The inclusion of a test for the identification of granular white blood cells must now be regarded as part of the standard basic routine semen analysis, as the presence of leukocytes is associated with ROS production of **(Oehninger and Kruger, 2007)**.
- **Computer-Assisted Sperm Assessment:** CASA involves the use of computer analysis of video-micrography to assess a variety of sperm kinetic parameters, including motility, concentration, motion kinetics, and morphology **(Paston *et al.*, 1994)**. Hyperactivation, characterized by high velocity, large amplitude, and flagellar waves, creates a characteristic pattern that is quantified by CASA **(Sukcharoen *et al.*, 1995a)**. This has been found to correlate with IVF fertilization rates **(Overstreet,**

1994; Sukcharoen *et al.*, 1995b). Although positively correlated with fertilization rates in IVF **(Macleod and Irvine, 1995)**, the assessment of sperm motion characteristics by CASA cannot reliably predict fertilization outcome **(Oehninger *et al.*, 2009)** and its role in fertility prediction is primarily investigational **(Samplaski *et al.*, 2010)**.

1.2.3.3 Investigations for Anti-Sperm Antibodies

The presence of Anti-Sperm Antibodies (ASA) has been documented to impede human fertility via several mechanisms **(Sabanegh, 2011)**. ASA are present in 4–8% of infertile men. Their formation is induced by breaching of the blood-testis barrier, either during development, traumatic disruption, surgery, or infection. Sperm agglutination on a semen analysis, severely impaired sperm motion, an abnormal postcoital test, or abnormalities of cervical mucus interaction or penetration may prompt ASA testing **(Samplaski *et al.*, 2010)**.

Nevertheless, the testing for ASA is controversial due to variations between different testing methodologies and the interpretation of the results in the context of male infertility. Most of the methods, previously described for the detection of ASA, are now obsolete due to the relatively high inter assay variability and their limited clinical benefits. Only two methods are now accepted to test for the presence of ASA, the mixed antiglobulin reaction (MAR) test and the immunobead test (IBT) (Figure 1.10) **(Sabanegh, 2011)**.

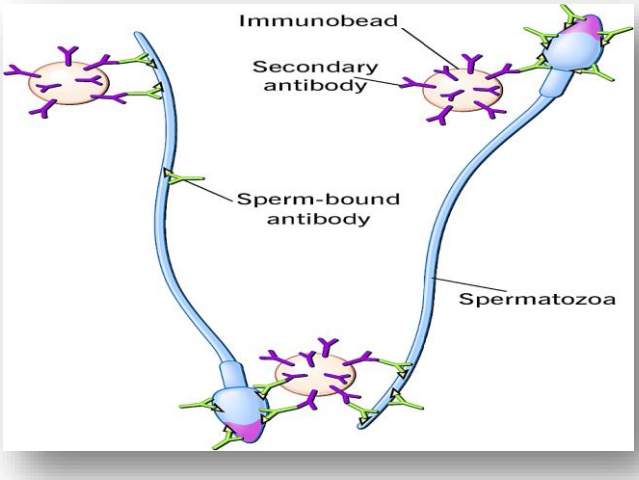

Figure 1.10: Immunobead test. Spermatozoa are mixed with beads that have been coated with IgG class-specific secondary anti- bodies. Antibodies are considered clinically significant when greater than 50% of spermatozoa are coated, or when the spermatozoa are unable to penetrate the pre-ovulatory human cervical mucus, or show impaired fertilizing capacity **(Samplaski *et al.*, 2010)**

1.2.3.4 Sperm function tests

The male infertility diagnosis is frequently based on the descriptive assessment of human semen, including the number of spermatozoa that are present in the ejaculate, their motility, and their morphology. However, it is not so much the absolute number of spermatozoa that determines fertility, but their functional competence **(Aitken, 2006)**.

Sperm function testing is used to determine if the sperm have the biologic capacity to perform the tasks necessary to reach and fertilize ova and ultimately result in live births. These tasks include penetrating the cervical mucus, reaching the ova, undergoing capacitation and the acrosome reaction, zona pellucida penetration, and ooplasm incorporation. Defects in any of these steps may result in infertility **(Kremer and Jager 1992; Johnson *et al.* 1995; Muller, 2000; Agarwal *et al.* 2008b; Sigman and Zini, 2009).**

1.2.3.5 Evaluation of oxidative stress

Oxidative stress (OS) can be measured during the clinical evaluation of the infertile male using direct and indirect assays. Indirect assays measure the net oxidative sum of the balance between ROS production and the intra- and extracellular antioxidants that scavenge ROS, by assessing oxidation of the sperm cell membrane. The most widely used assay measures malondialdehyde (MDA), one of the final products of sperm cell membrane lipid peroxidation (LPO). Quantification of sperm DNA damage has also been used as an indirect assay of intracellular ROS-induced oxidant injury, although it is unable to distinguish oxidant-induced DNA damage from non-oxidative mechanisms **(Sharma *et al.* 2001; Agarwal *et al.* 2008c; Esteves and Agarwal, 2011)**.

To accurately quantify oxidative stress, levels of ROS and antioxidants should be measured in fresh samples. Direct methods such as pulse radiolysis and electron-spin resonance spectroscopy have been useful for several body systems, even they have limitations in their use in the male reproductive system. These methods are confronted with issues of a relatively low volume of seminal plasma, short life span of ROS, and the need to perform the evaluation in fresh samples **(Agarwal *et al.*, 2007)**. Thus, another method is needed that avoids the issues encountered by the direct methods. Recently, one of the most widespread methods of measuring ROS is chemiluminescence assay (Figure 1.11) that seems to quantify

both intracellular and extracellular ROS. It uses sensitive probes such as luminol (5-amino-2, 3, dihydro 1, 4, phthalazinedione) and lucigen for quantification of redox activities of spermatozoa **(Aitken *et al.*, 2004a)**. The reaction of luminol with ROS results in production of a light signal that is converted to an electrical signal (photon) by a luminometer. Levels of ROS are assessed by measuring the luminal-dependent chemiluminescence with the luminometer **(Makker *et al.*, 2009)**.

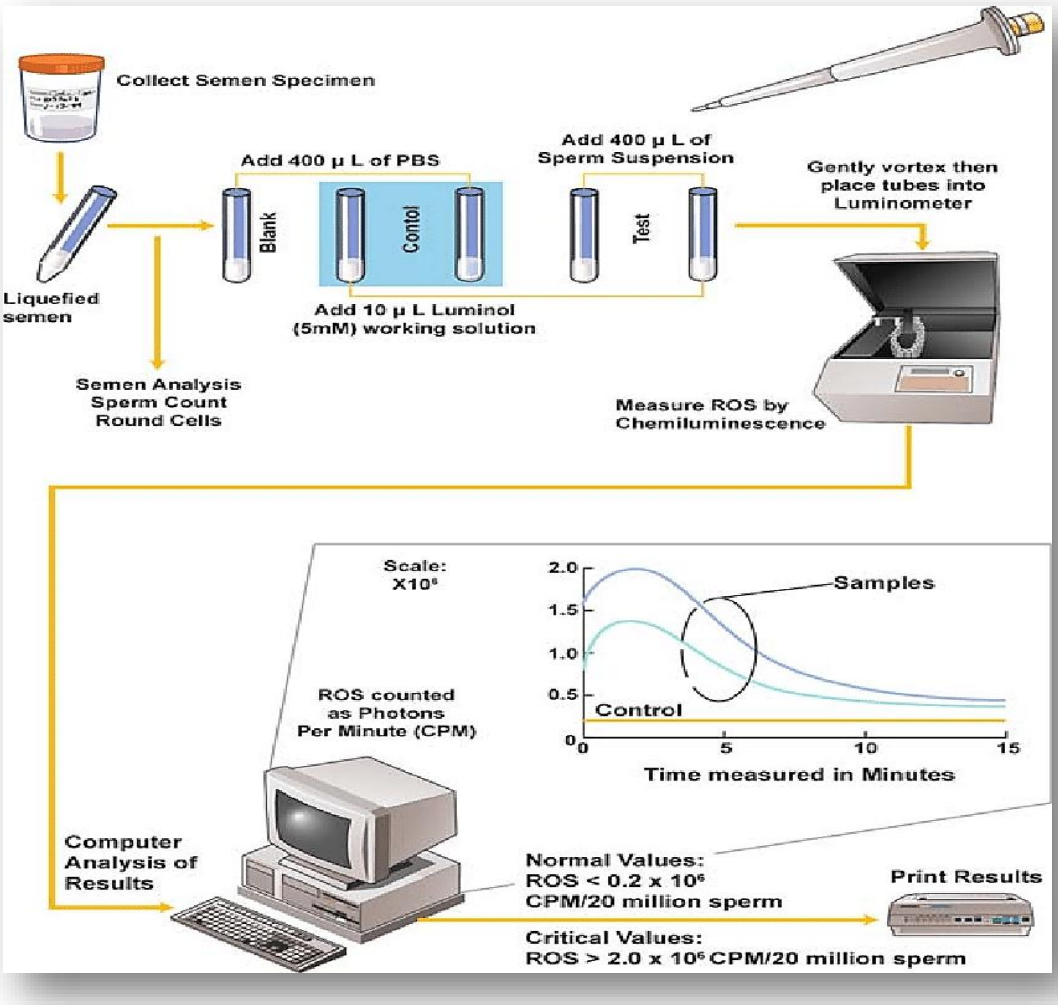

Figure 1.11: Measurement of reactive oxygen species (ROS) in sperm suspensions by chemiluminescence assay (**Agarwal *et al.*, 2014**)

Recently, oxidative stress has become the focus of interest as a potential cause of male infertility. Generally, equilibrium exists between reactive oxygen species (ROS) production and antioxidant scavenging activities in the male reproductive organs **(Vijayprasad *et al.*, 2014)**.

Chapter 2

Oxidative Stress, Mechanisms and Treatment

Chapter 2

Oxidative Stress, Mechanisms and Treatment

2.1 Oxidative Stress, Mechanisms and Manifestation

2.1.1 Definition

Aerobic metabolism is associated with the prooxidant molecules generation called free radicals or reactive oxygen species (ROS) that include the hydroxyl radicals, superoxide anion, hydrogen peroxide, and nitric oxide. There is a complex interaction of the prooxidants and antioxidants, resulting in the maintenance of intracellular homeostasis. Whenever there is an imbalance between the prooxidants and antioxidants, a state of oxidative stress is initiated **(Agarwal *et al.*, 2006)**.

Oxidative stress (OS) has been identified as one factor that affects fertility status and thus, has been extensively studied in recent years. Spermatozoa, like any other aerobic cell, are constantly facing the "oxygen-paradox" **(Sies, 1993)**. Oxygen is essential to sustain life as physiological levels of reactive oxygen species (ROS) are necessary to maintain normal cell function. Conversely, breakdown products of oxygen such as ROS can be detrimental to cell function and survival **(De Lamirande and Gagnon, 1995)**. Reactive oxygen species are present as free radicals. Examples of ROS include the hydroxyl ion, superoxide, hydrogen peroxide, peroxyl radical, and hypochlorite ion. These are the common forms of ROS that have been considered injurious to sperm survival and function when present in abundance (Figure 2.1).

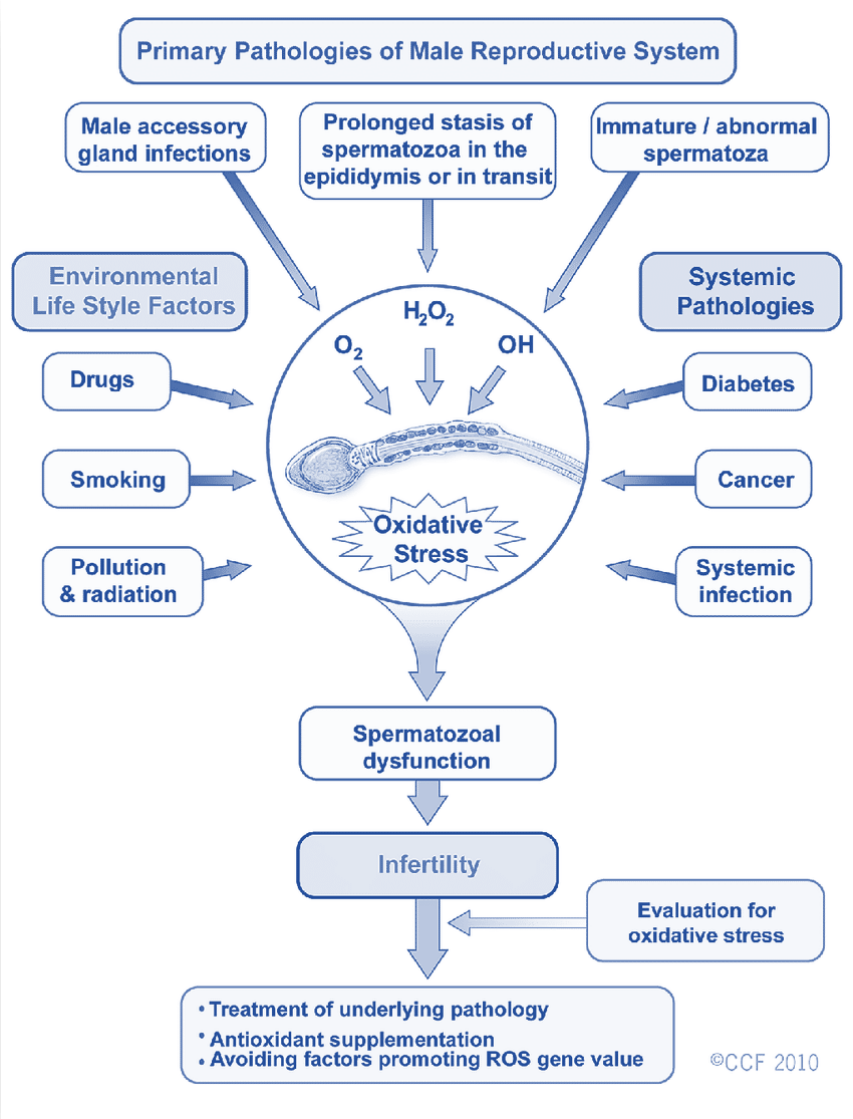

Figure 2.1 : Factors contributing to oxidative stress-induced male infertility **(Esteves and Agarwal, 2011)**

OS is a consequence of an imbalance between the production of ROS and the body's antioxidant defense mechanisms (Figure 2.2). OS also has been implicated in the pathogenesis of many other human diseases such as atherosclerosis, cancer, diabetes, liver damage, rheumatoid arthritis, cataracts, VHI, inflammatory bowel disease, central nervous system disorders, Parkinson's disease, motor neuron disease, and conditions associated with premature birth **(Makker *et al.*, 2009)**.

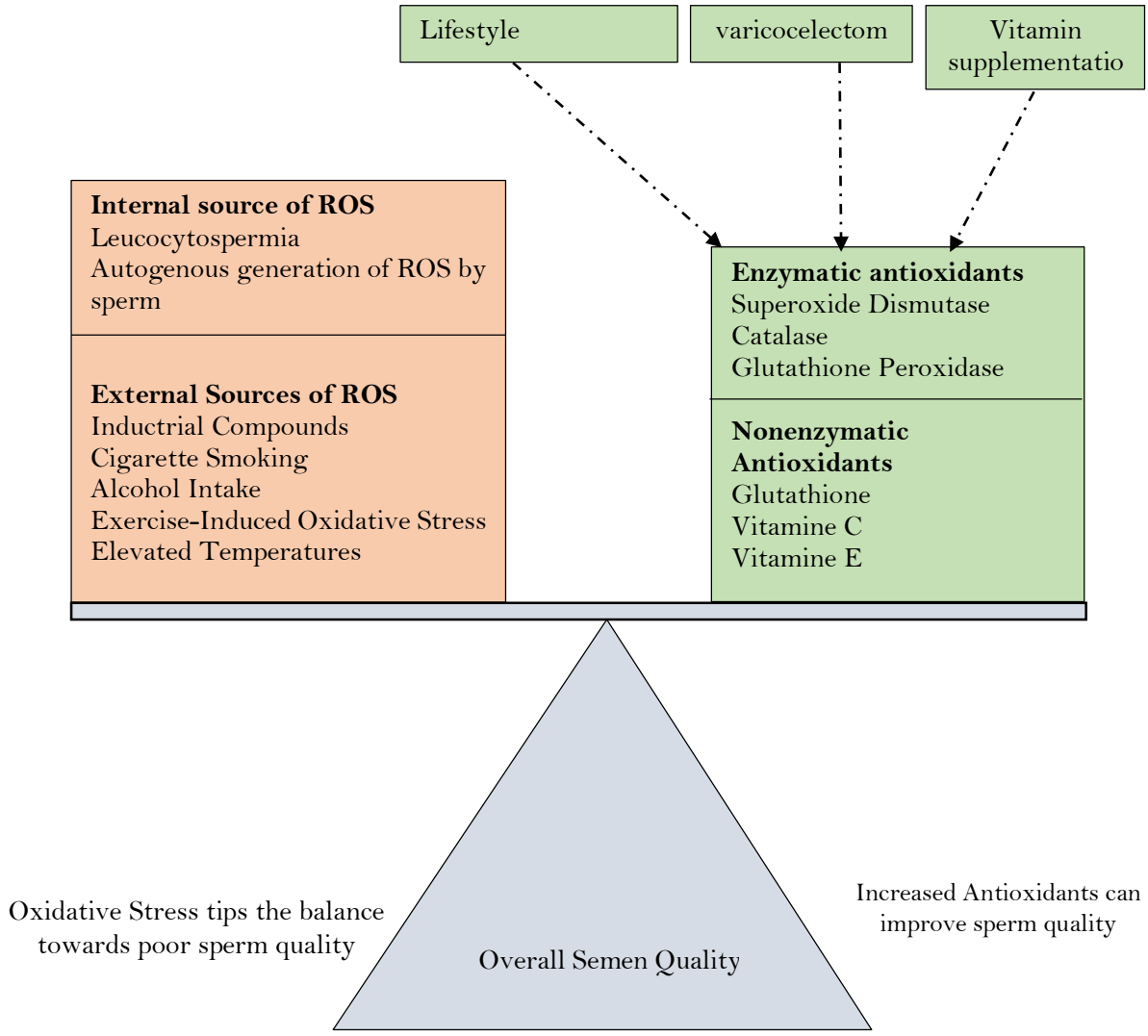

Figure 2.2: Oxidants and antioxidants balance influencing overall sperm quality. In a system with high levels of oxidants, sperm quality will be poor overall. Conversely, a system with increased antioxidant capacity can keep the oxidants in balance, leading to improved sperm quality **(Kefer *et al.*, 2009)**

The general sources, mechanisms, and consequences of OS on male fertility are summarized on Figure 2.3. Clinical conditions related to OS include idiopathic infertility, leukocytospermia, varicocele, genitourinary tract infection, environmental and lifestyle factors. OS acts through several mechanisms that lead to subfertility, such as LPO, DNA damage, and apoptosis. OS can lead to several consequences related to male fertility, both in an in vivo and in vitro setting **(Said *et al.*, 2012)**.

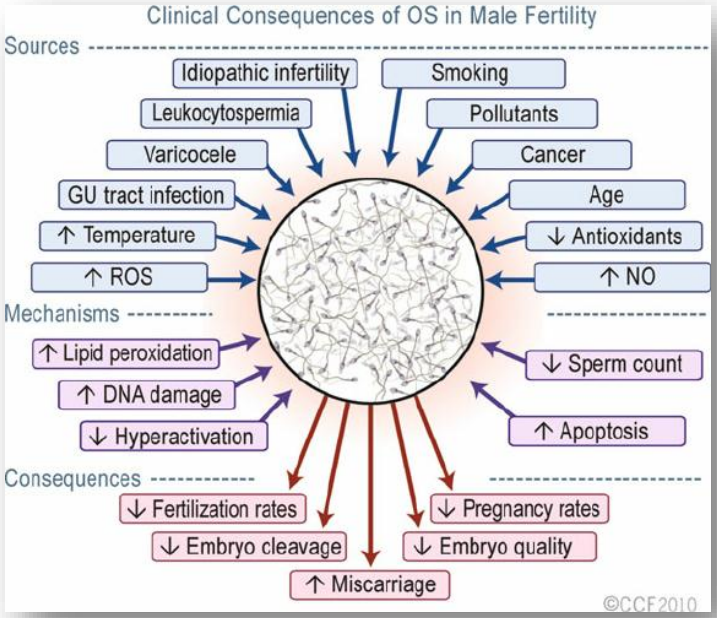

Figure 2.3 : General sources, mechanisms, and consequences of OS on male fertility **(Said *et al.*, 2012)**

2.1.2 Reactive Oxygen Species (ROS)

ROS, commonly known as free radicals, have at least one unpaired electron. They are oxidizing agents generated as byproducts from the oxygen metabolism. Due to the unpaired electron in the outer shell, they form highly reactive molecules **(Henkel, 2010; Miranda-Vilela *et al.*, 2010)**. ROS represents a collection of a broad range of radicals (e.g., hydroxyl ion [OH^-], superoxide ion [O_2^-], nitric oxide [NO], peroxyl [RO_2], lipid peroxyl [LOO], Thiyl [RS]) and non-radical molecules (singlet oxygen [$^1O_2$], hydrogen peroxide [H_2O_2], hypochloric acid [HOCL], lipid peroxide [LOOH], and ozone [O_3]) **(Bansal and Bilaspuri, 2011)**.

ROS generation in spermatozoa may occur via two methods: (1) the nicotinamide adenine dinucleotide phosphate (NADP) oxidase system at the level of the sperm plasma membrane and/or (2) the nicotinamide adenine dinucleotide-dependent oxidoreductase reaction at the mitochondrial level. The latter mechanism appears to be the main source of ROS. Spermatozoa are rich in mitochondria because a constant supply of energy is required for

their motility **(Henkel, 2010)**. Therefore, the presence of dysfunctional spermatozoa in the semen significantly increases the ROS production, which in turn affects its mitochondrial function and subsequently, sperm function such as motility **(Agarwal *et al.*, 2014)**.

A majority of ROS generated in human spermatozoa is O_2^-. This electron-reduced product of O_2^- reacts with itself via dismutation to generate H_2O_2. In the presence of transition metals such as iron and copper, H_2O_2 and O_2^- undergo the Haber-Weiss reaction to generate the extremely reactive and destructive OH^- (Figure 2.4). OH^- radicals are exceptionally potent initiators of the LPO cascade and can lead to a loss of sperm function from the disruption of membrane fluidity **(Chen *et al.*, 2013, Sikka, 2001)**.

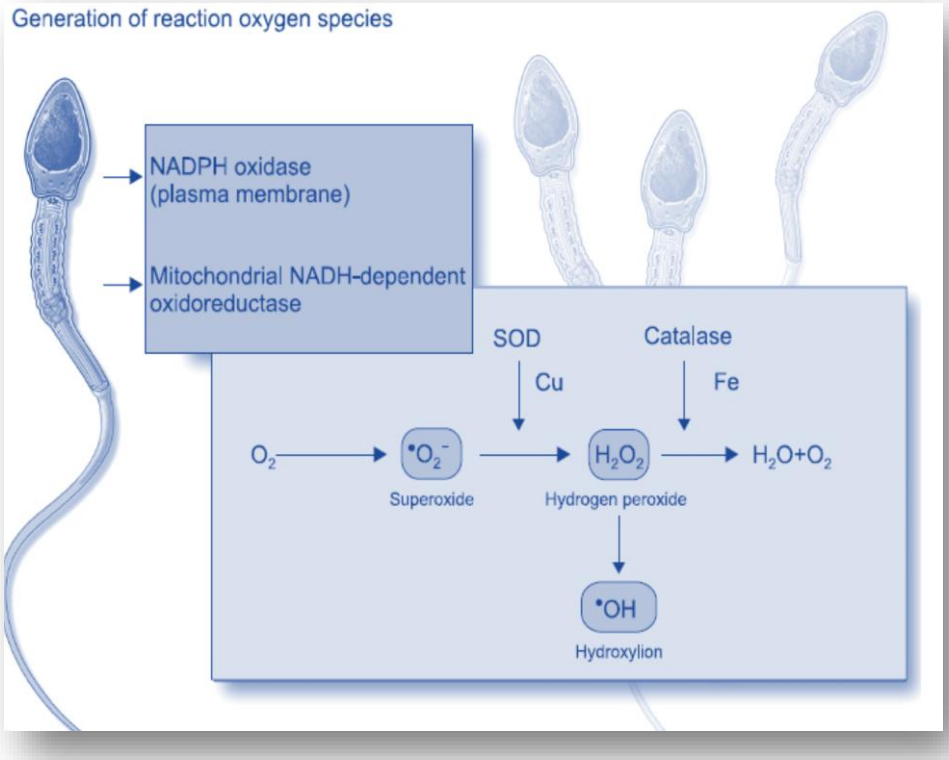

Figure 2.4: ROS Generation. NADPH: nicotinamide adenine dinucleotide phosphate, NADH: reduced nicotinamide adenine dinucleotide, SOD: superoxide dismutase, Cu: copper, Fe: Iron **(Agarwal *et al.*, 2014)**

2.1.3 ROS Sources in Seminal Plasma

ROS are products of normal cellular metabolism. The majority of energy produced by aerobic metabolism utilizes oxidative phosphorylation within mitochondria during the enzymatic reduction of oxygen to produce energy, free radicals form as a byproduct. A free radical is an oxygen molecule containing one or more unpaired electrons. Normally, molecular oxygen has two unpaired electrons, and this electronic structure makes oxygen especially susceptible to radical formation. For example, the addition of an extra electron to molecular oxygen (O_2) forms a superoxide anion radical (O_2^-), the primary form of ROS. This superoxide anion can then be directly or indirectly converted to secondary ROS such as the hydroxyl radical (•OH), peroxyl radical (ROO•) or hydrogen peroxide (H_2O_2) (Figure 2.5). Free radicals induce cellular damage when they pass this unpaired electron onto nearby cellular structures, resulting in the oxidation of cell membrane lipids, amino acids in proteins or within nucleic acids **(Kefer *et al.*, 2009)**.

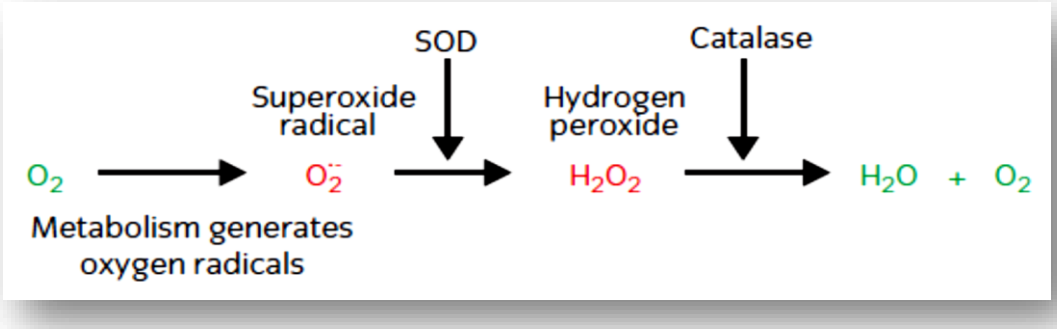

Figure 2.5 : Antioxidant scavenging pathways of free radicals by superoxide dismutase (SOD) and catalase **(Kefer *et al.*, 2009)**

ROS found in seminal plasma derive from various endogenous and exogenous sources. The human ejaculate consists of different cells types, including mature and immature cells, round cells from different stages of spermatogenesis, leukocytes, and epithelial cells. Of these, leukocytes-mainly neutrophils and macrophages- and immature spermatozoa are considered as the main endogenous ROS sources, while several lifestyle factors such as excessive smoking and alcohol consumption, and environmental factors such as radiation and toxins can contribute to exogenous ROS as shown on Figure 2.6 **(Gharagozloo and Aitken, 2011, Choudhary *et al.*, 2010, Esteves, 2002)**.

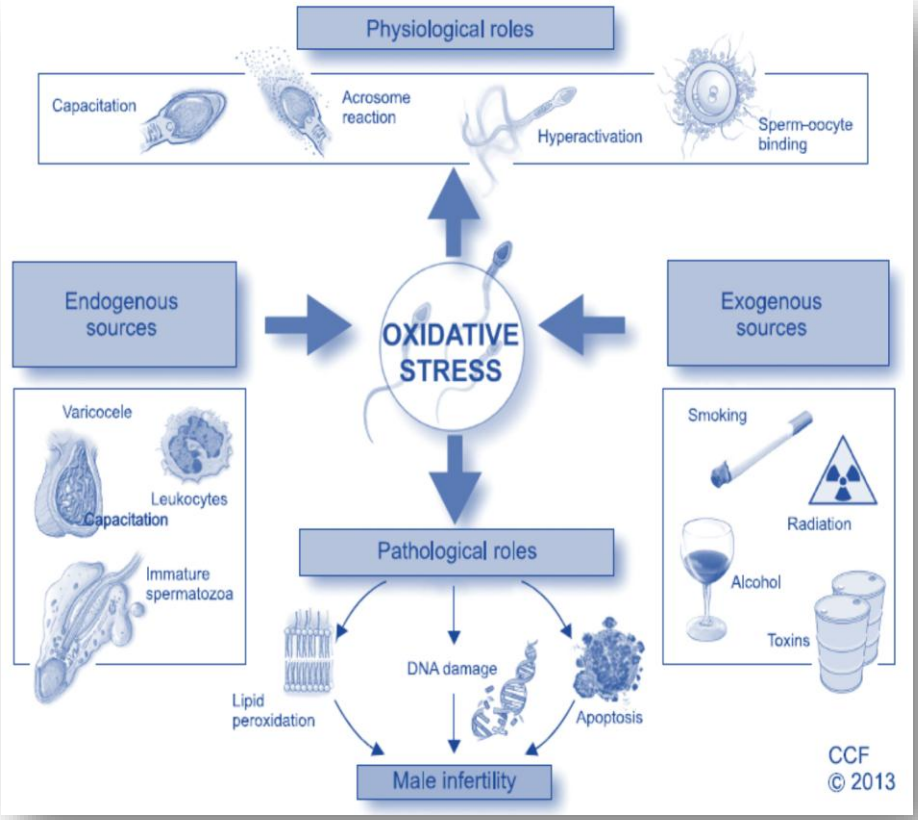

Figure 2.6 : Oxidative stress in male reproduction **(Agarwal *et al.*, 2014)**

2.1.3.1 Endogenous sources of ROS

a. Leukocytes

Peroxidase-positive leukocytes include polymorphonuclear leukocytes, which represent 50 to 60 % of all seminal leukocytes, and macrophages, which represent another 20 to 30 % **(Thomas *et al.*, 1997)**. The prostate gland and the seminal vesicles are the main sources of these peroxidase-positive leukocytes in human ejaculate **(Wallach and Wolff, 1995)**. Leukocytes may be activated in response to various stimuli such as infection and inflammation **(Pasquallotto *et al.*, 2000)**, and these activated leukocytes can produce up to 100-fold higher amounts of ROS compared with non-activated leukocytes **(Plante *et al.*, 1994)**. This is mediated by an increase in NADPH production via the hexose monophosphate shunt. The myeloperoxidase system of both polymorphonuclear leukocytes and macrophages is activated too, leading to a respiratory burst and production of high ROS levels. Sperm damage from ROS that is produced by leukocytes, occurs if seminal leukocyte concentrations are

abnormally high, such as in leukocytospermia **(Shekarriz *et al.*, 1995)** or if seminal plasma is removed during sperm preparation for assisted reproduction **(Ochsendorf, 1999)**.

d. Immature spermatozoa

Spermatozoa have also been found to generate reactive oxygen species independent of leukocytes **(Baker *et al.*, 2003)**, and the ability of sperm to generate ROS is dependent on the maturation level of the sperm. During spermatogenesis, developing spermatozoa extrude their cytoplasm in order to prepare for fertilization. However, damaged spermatozoa retain excess cytoplasm around the midpiece due to an arrest in spermiogenesis; this condition is known as excess residual cytoplasm (ERC). ERC activates the NADPH system by means of the hexose-monophosphate shunt, which spermatozoa considere as a source of electrons for ROS generation and potentially, OS **(Rengan *et al.*, 2012)**. Hence, ERC ultimately affects sperm motility, morphology, and fertilization potential, which may lead to male infertility **(Hampl *et al.*, 2012)**.

e. Varicocele

It has been shown that the level of seminal ROS is associated with the grade of varicocele; that is, the higher the grade of varicocele is, the greater is the level of ROS detected **(Agarwal *et al.*, 2014)**.

2.1.3.2 Exogenous sources of reactive oxygen species

a. Radiation

Radiation, a natural source of energy, has significant clinical effects on humans. With respect to male reproductive health, several studies have implicated radiation emitted from mobile phones in the increase of the production of ROS in human semen with impaired semen quality **(Aitken *et al.*, 2005; Agarwal *et al.*, 2008d)**. *In vitro* studies have demonstrated that electromagnetic radiation induces ROS production and DNA damage in human spermatozoa, which further decreases the motility and viability of sperm cells as well as their concentration depending on the duration of exposure to radiation **(De Iuliis *et al.*, 2009; Lavranos *et al.*, 2012)**.

b. Toxins

Toxins released from structural materials or industrial products accumulate in the human body and increase ROS production in the testes, negatively impacting the sperm structure and function **(Esfandiari *et al.*, 2002)**. Phthalates, found in a variety of plastic objects used for domestic and industrial purposes, have been sufficiently studied **(Latini *et al.*, 2006; Pant *et al.*, 2008)**. They have been found to impair spermatogenesis and induce sperm DNA damage **(Kasahara *et al.*, 2002)**. Furthermore, it was demonstrated that workers who were regularly exposed to toxins in the form of metals such as cadmium, chromium, lead, manganese, and mercury were more likely to have decreased sperm quality, count, volume, and density **(Jurasović *et al.*, 2004)**.

c. Cigarette smoking

Not surprisingly, exposure to cigarette smoke generates high levels of oxidative stress, directly increasing both seminal leukocyte concentrations and seminal ROS generation **(Arabi and Mosthagi, 2005)**, and decreasing seminal levels of the antioxidant enzyme SOD **(Pasquallotto *et al.*, 2008)**. Smoking has been found to decrease the seminal plasma antioxidants Vitamin C and E concentrations **(Sofikitis *et al.*, 1995; Vine, 1996)**, thereby reducing the oxidant scavenging capacity of the spermatozoa and seminal fluid **(Kefer *et al.*, 2009)**.

d. Alcohol consumption

Alcohol is known as a ROS production promoter and interferes with the body's antioxidant defense mechanism, particularly in the liver. Many factors are involved in causing alcohol-induced OS. When acetaldehyde, one of the byproducts of ethanol metabolism, interacts with proteins and lipids, ROS is formed and therefore damage proteins, lipids, and DNA **(Agarwal and Prabakaran, 2005; Saalu, 2010)**.

e. Elevated temperatures

Several researchers demonstrated that increased intrascrotal temperatures in rodent models leads to decreased sperm counts and motility, and increased sperm DNA damage.

Increased intrascrotal temperatures have been suggested as a possible cause of poor sperm quality in humans as well **(Banks *et al.*, 2005; Perez-Crespo *et al.*, 2007)**.

f. Exercise-induced oxidative stress

Some studies indicated that chronic intensive exercise training can lower testosterone levels, or interfere with the hypothalamic-pituitary-testis axis involved in reproduction **(Hackney, 2001)**. As testosterone plays a major role in the development and maturation of sperm during spermatogenesis, this decrease in testosterone may decrease sperm quality **(Kefer *et al.*, 2009)**.

2.1.4 Physiological Roles of ROS in Seminal Plasma

Until recently, ROS was considered toxic, exclusively to human spermatozoa **(Aitken, 1997)**. Substantial evidence exists to suggest that small amounts of ROS are necessary for spermatozoa to acquire fertilizing capabilities **(Gagnon *et al.*, 1991)**. Low levels of ROS have been shown to be essential for fertilization, acrosome reaction, hyperactivation, motility, and capacitation **(Griveau and Le Lannou, 1997; Agarwal *et al.*, 2004)**. Co-incubation of spermatozoa with low concentrations of hydrogen peroxide has been shown to stimulate sperm capacitation, hyperactivation, acrosome reaction, and oocyte fusion **(Kodama *et al.*, 1996)**. ROS such as nitric oxide (NO) and the superoxide anion have been shown to promote capacitation and the acrosome reaction too **(Griveau *et al.*, 1995)**. Furthermore, ROS have been implicated in sperm oocyte interaction **(Agarwal *et al.*, 2007)**.

2.1.4.1 Capacitation

Capacitation is the penultimate process in the maturation of spermatozoa and is required to render them competent to successfully fertilize the ovum **(Choudhary *et al.*, 2010)**. Controlled ROS production occurs in spermatozoa during the capacitation process, initiating various molecular modifications. The first step involves an increase in cyclic adenosine 3',5'-monophosphate (cAMP). As known, the cAMP pathway is necessary for many living organisms and life processes as it can activate enzymes and regulate gene expression **(Tsai *et al.*, 2013)**. This pathway involves the activation of protein kinase A (PKA) and the phosphorylation of PKA substrates (arginine, serine, and threonine). This subsequently leads

to the phosphorylation of MEK (extracellular signal regulated kinase) like proteins and threonine-glutamate-tyrosine, and finally tyrosine phosphorylation of fibrous sheath proteins. This increase in cAMP causes spermatozoa hyperactivation (Figure 2.7). Only hyperactivated spermatozoa have increased motility to undergo acrosome reaction and acquire the characteristics required for successful fertilization. From these findings, it was deduced that ROS was involved **(De Lamirande and O'Flaherty, 2008; Kothari *et al.*, 2010; Chen *et al.*, 2013)**.

2.1.4.2 Hyperactivation

Hyperactivation is a specific state of sperm motility when spermatozoa become highly motile (Figure 2.7). The process of hyperactivation is essential for successful fertilization and is considered as a capacitation subcategory. Hyperactive spermatozoa exhibit features of high amplitude, asymmetric flagellar movement, increased side-to-side head displacement, and non-linear motility **(Suarez, 2008)**. The role of ROS in the initiation of hyperactivation has been well documented *in vitro* when spermatozoa were incubated with low concentrations of OH^- induced hyperactivation **(Agarwal *et al.*, 2014)**.

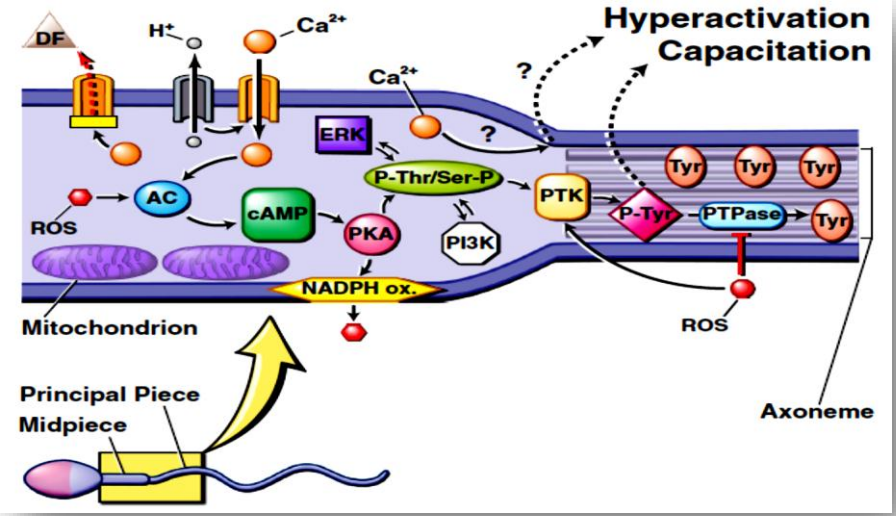

Figure 2.7: Biochemical pathway proposed to regulate sperm capacitation and hyperactivation. The process is initiated by an influx of Ca2+ and HCO_3^-, possibly caused by the inactivation of an ATP-dependent Ca^{2+} regulatory channel (PMCA) and alkalization of the cytosol. Both Ca^{2+} and reactive oxygen species (ROS), specifically O_2^-, activate adenylate cyclase (AC), which produces cyclic adenosinemonophosphate (cAMP). cAMP activates downstream protein kinase A (PKA). PKA triggers a membrane bound NADPH oxidase to stimulate greater ROS production. In addition, PKA triggers phosphorylation of serine (Ser) and tyrosine (Tyr) residues that, in addition to other inter-connected pathways, lead to the activation of protein tyrosine kinase (PTK). PTK phosphorylates Tyr residues of the fibrous sheath surrounding the axoneme, the cytoskeletal component of the flagellum. ROS, specifically hydrogen peroxide (H_2O_2), increases the amount of Tyr phosphorylation by promoting PTK activity and inhibiting phosphotyrosine phosphatase (PTPase) activity, which normally de-phosphorylates Tyr residues. The enhanced Tyr phosphorylation observed in capacitation is the last known step in the process, but intermediate steps or other (in)direct methods may be involved **(Du Plessis *et al.*, 2015)**

2.1.4.3 Acrosome reaction

Once the hyperactivated spermatozoon passes the *cumulus oophorus*, it binds to the zona pellucida (ZP) of the oocyte and initiates an exocytotic release of proteolytic enzymes, creating a pore in ZP's extracellular matrix. The spermatozoa then penetrate this physical zona barrier and fuse with the oocyte **(De Lamirande and O'Flaherty, 2008)**. The molecular events of the acrosome reaction overlap substantially with those of capacitation, including phosphorylation of similar tyrosine proteins, influx of Ca^{2+}, and increased cAMP and PKA levels (Figure 2.8). The role of ROS in the *in vivo* acrosome reaction involves the

spermatozoa's actions on the ZP via phosphorylation of three plasma membrane proteins. *In vitro* activation of the AR was observed when physiological concentrations of O_2^-, H_2O_2, and NO were added to the seminal plasma **(Bansal and Bilaspuri, 2011)**.

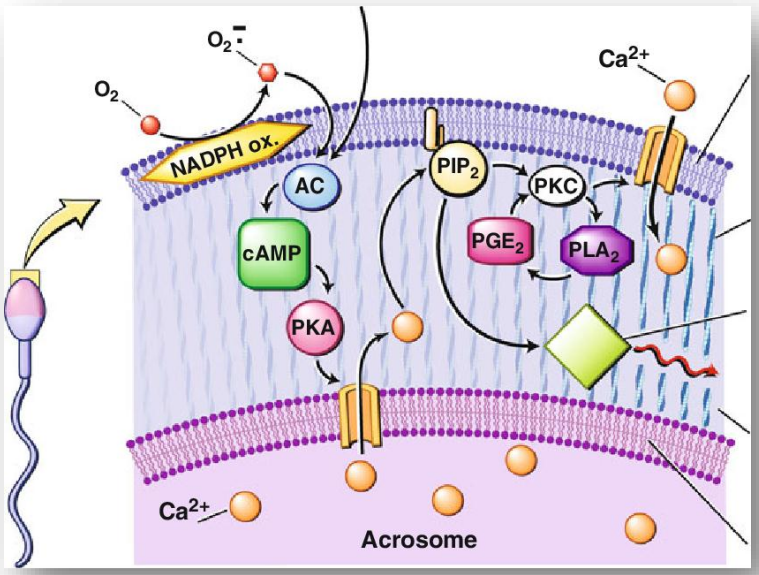

Figure 2.8: Biochemical pathway proposed to regulate the acrosome reaction (AR). Induction of the AR can occur by physiological and non- physiological activators, including the zona pellucida (ZP), progesterone, or reactive oxygen species (ROS). Subsequent release of Ca2+ from the acrosomal calcium store generated during capacitation causes the cleavage of phosphatidylinositol-4, 5-biphosphate (PIP_2), which forms diacylglycerol (DAG) and inositol triphosphate (IP3). The latter activates actin-severing proteins, which leads to the fusion of the acrosomal and plasma membranes, and eventual acrosomal exocytosis. DAG later activates protein kinase C (PKC), causing a second, greater influx of Ca^{2+} and activation of phospholipase A_2 (PLA_2). The release of large amounts of membrane fatty acids increases the fluidity of the plasma membrane necessary for later fusion with the oocyte **(Du Plessis *et al.*, 2015)**

2.1.4.4 Sperm-oocyte fusion

For successful fertilization, the spermatozoa must penetrate the ZP and fuse with the oocyte. High amounts of PUFAs, particularly docosahexaenoic acid (DHA), play a major role in regulating membrane fluidity in sperm. In studies of human spermatozoa, ROS has been shown to increase the membrane fluidity and rates of sperm-oocyte fusion, which occurs during the biochemical cascade of capacitation and acrosome reaction. Throughout capacitation, ROS inhibits protein tyrosine phosphatase activity and prevents

dephosphorylation and deactivation of phospholipase A_2 (PLA_2). PLA_2 cleaves the secondary fatty acid from the triglycerol backbone of the membrane phospholipid and increases the membrane's fluidity **(Calamera *et al.*, 2003; Khosrowbeygi and Zarghami, 2007)**.

2.1.5 Pathological Roles of ROS

All cellular components, including lipids, proteins, nucleic acids, and sugars are potential targets of OS. The extent of OS-induced damage depends not only on the nature and amount of ROS involved, but also on the duration of ROS exposure and on extracellular factors such as temperature, oxygen tension, and the composition of the surrounding environment **(Makker *et al.*, 2009)**.

2.1.5.1 Lipid peroxidation

Spermatozoa are particularly vulnerable to LPO because they contain high concentrations of polyunsaturated fatty acids (PUFA), particularly docosahexaenoic acid with six double bonds per molecule **(Shannon and Curson, 1982)**. The latter are vulnerable to free radical attack because the carbon hydrogen dissociation energies are lowest at the bisallylic methylene position. As consequence, the hydrogen abstraction event that initiates LPO is promoted, generating a carbon-centered lipid radical that then combines with oxygen to generate peroxyl (ROO•) and alkoxyl (RO•) radicals that, in order to stabilize, abstract hydrogen atoms from adjacent carbons. These chemical reactions create additional lipid radicals that then perpetuate the LPO chain reaction, culminating in the generation of small molecular mass electrophilic lipid aldehydes such as 4-hydroxynonenal (4HNE), acrolein and malondialdehyde **(Aitken *et al.*, 2014)**. This later byproduct has been used in various biochemical assays to monitor the degree of peroxidative damage sustained by spermatozoa. Results of such an assay exhibit an excellent correlation when examining the relationship between impaired sperm function, discussed in terms of motility, and the capacity for sperm-oocyte fusion **(Makker *et al.*, 2009)**.

2.1.5.2 DNA damage

ROS also can cause various types of gene mutations such as point mutations and polymorphism, resulting in decreased semen quality. Other mechanisms such as denaturation

and DNA base - pair oxidation also may be involved. A common byproduct of DNA oxidation, 8-hydroxy-2-deoxyguanosine (8-OH-2-deoxyguianosine), has been considered a key biomarker of this oxidative DNA damage **(Makker *et al.*, 2009)**. DNA damage is a contributory factor to apoptosis, poor fertilization rate, high frequency of miscarriage, and morbidity in offspring **(Agarwal *et al.*, 2014)**.

2.1.5.3 Apoptosis

Apoptosis is an ongoing physiological phenomenon that leads to the elimination of abnormal spermatozoa in order to limit the number of male germ cells that can be maintained by Sertoli cells in the testes. Apoptosis is initiated in spermatozoa when high levels of ROS damage the mitochondrial membranes, and cytochrome c proteins are then released. This activates caspases 9 and 3, which play essential roles in apoptosis. High levels of cytochrome c and caspases have been correlated with increased levels of sperm DNA damage such as single and double-stranded DNA strand breaks. Caspases have been implicated in the decrease of sperm motility too **(Agarwal *et al.*, 2008c ; Said *et al.*, 2012)**.

2.1.5.4 Impaired semen parameters

OS and excessive ROS production have been associated with impaired sperm motility, concentration, and morphology. These parameters are the most important predictors of an individual's potential to produce viable sperm **(Agarwal *et al.*, 2014)**.

a. Decreased motility

One of the first functions affected by oxidative stress and LPO is sperm motility. Correlations between lipid peroxide formation and sperm movement have been repeatedly observed in a variety of different species **(Aitken *et al.*, 2014)**.

However, the exact mechanism through which this occurs is not understood. One hypothesis suggests that H_2O_2 diffuses across the membranes into the cells, and inhibits the activity of some vital enzymes, such as glucose-6-phosphate dehydrogenase (G6PD), via the hexose mono-phosphate shunt controls the intracellular availability of NADPH, which is then used as a source of electrons by spermatozoa, to fuel the generation of ROS by an enzyme system known as NADPH oxidase. Another hypothesis involves a series of interrelated events

resulting in a decrease in axonemal protein phosphorylation and sperm immobilization. Both of which are associated with a reduction in membrane fluidity that is necessary for sperm-oocyte fusion **(Agarwal *et al.*, 2008c)**.

b. Sperm morphology

Caspase-mediated apoptosis and increased OS have a positive relationship with increased sperm damage and abnormal sperm morphology. In addition, there is a higher incidence of abnormal sperm morphology in conditions related to OS. However, DNA damage and ROS production has been found to correlate with abnormal head morphology and cytoplasmic retention in immature sperm, but not in mature sperm. This may be a result of OS affecting the regulation of spermiogenesis, the final stage of spermatogenesis where immature spermatids develop into mature spermatozoa. Morphologically abnormal and immature sperm can lead to even higher levels of ROS production during sperm migration, and consequently lead to OS-related damage in mature sperm. Abnormal morphology related to OS is not limited to immature spermatids and can extend to mature spermatozoa **(Said *et al.*, 2012)**.

2.2 Antioxidant Treatment of Male Infertility

A series of antioxidant enzymes and numerous endogenous and dietary antioxidant compounds maintain defenses against OS by scavenging ROS. The susceptibility of cells to OS is the function of the overall balance between the degree of ROS formation and their antioxidant defense capability. Overproduction of ROS depletes enzymatic and non-enzymatic antioxidants leading to additional ROS accumulation and cellular damage. The primary antioxidant enzymes include, but are not limited to, superoxide dismutase (SOD), catalase, and glutathione and thioredoxin peroxidases. The non-enzymatic antioxidants include, among others, vitamin C (ascorbic acid), vitamin E (α-tocopherol), ß-carotene, and reduced glutathione **(Musicki and Burnett, 2012)**.

In view of the male germ cell's high vulnerability to extrinsic and intrinsic ROS as well as lack of its own protection, spermatozoa have to receive protection against OS through relevant scavengers by the male and/or female reproductive tracts. Therefore, in the male, the testis and the accessory sex glands that are producing the seminal plasma have to provide substances and mechanisms for such protection. Indeed, seminal plasma is the biological fluid

containing highest concentrations of antioxidant substances, even more than in any other physiological fluid. This anti-oxidative protection can be separated into two systems, interceptive and preventive. Among the scavenging antioxidants, another distinction between enzymatic and non-enzymatic systems can be performed **(Henkel, 2012)**.

2.2.1 Definition of Antioxidant

Spermatozoa are protected by various antioxidants and antioxidant enzymes in the seminal plasma or in spermatozoa itself to prevent oxidative damage. An antioxidant that reduces OS and improves sperm motility could be useful in the management of male infertility. Antioxidants are the agents, which break the oxidative chain reaction, thereby, reduce the OS. Antioxidants, in general, are the compounds and reactions which dispose, scavenge, and suppress the formation of ROS, or oppose their actions **(Bansal and Bilaspuri, 2011)**.

Oral antioxidant supplements have been reported to increase the scavenging capacity of seminal plasma thereby reducing levels of ROS within the semen. The oral antioxidants vitamin E, vitamin C, β-carotene, and acetyl-cysteine are all potent scavengers of ROS and have all been shown to reduce seminal ROS levels **(Parekattil and Agarwal, 2012)**.

2.2.2 Antioxidant Pathways

Each antioxidant during its activity generates or can generate species that could promote oxidation, hence all antioxidants possess either directly or indirectly pro-oxidant properties. In fact, a good antioxidant is one that generates compounds having a low propensity to promote peroxidative processes. Indeed, the pro-oxidant effect of antioxidants has been the object of several studies that have shown antioxidants to be harmful when administered in high doses in an ordinary diet. Therefore, it should be focused that it is possible for a compound to show both pro- and anti-oxidant properties depending on concentrations, experimental conditions, stage of oxidation, and the presence of a reaction partner (co-antioxidant).

Following is a treatise on some of the different mechanisms by which antioxidants work and the possible pro-oxidant effects that can be generated during their activity. It is important to underline here that not all antioxidants exert their inhibitory action through the same

mechanism and that the same antioxidant may inhibit free radical processes by more than one mechanism (**Damiani *et al.*, 2008**).

2.2.2.1 Hydrogen transfer inhibition

Hydrogen atom transfer represents the key reaction through which a chain- breaking antioxidant exerts its action. This is the typical mechanism for phenolic antioxidants, as well as for other antioxidants such as carotenoids.

Most synthetic and naturally occurring antioxidants are phenolic compounds for which the generic term ArOH will be used since, by definition, they contain at least one hydroxyl group attached to a benzene ring. They owe their activity to the donation of the phenolic hydrogen atom to the chain carrying peroxyl radicals by reaction (1), which is a much faster reaction than the attack of the peroxyl radicals on the organic substrate, reaction (2), the propagating step of the peroxidative process.

$$ROO^{\bullet} + ArOH \xrightarrow{k_{inb}} ROOH + ArO^{\bullet} \ldots (1)$$

$$ROO^{\bullet} + RH \xrightarrow{K_2} ROOH + R^{\bullet} \left(\xrightarrow{O_2} ROO^{\bullet}\right) \ldots (2)$$

Theoretically, ArOH could donate its phenolic hydrogen atom to carbon-centred radicals formed in the initiation step of peroxidative processes (Figure 2.9 a) and hence acting as an antioxidant also in this case but, practically, these radicals, once formed, immediately react with molecular oxygen leading to peroxyl radicals, reaction close to be diffusion controlled.

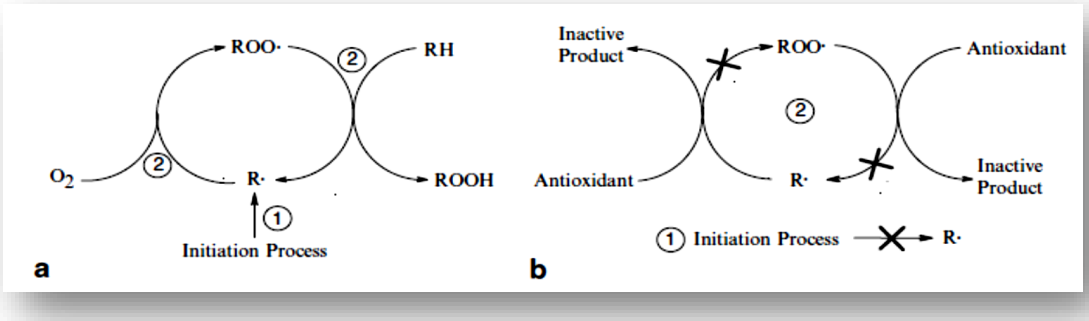

Figure 2.9: The peroxidative process (a) and it's inhibition (b) by preventive antioxidants ①, and chain-breaking antioxidants ② **(Damiani *et al.*, 2008)**

The antioxidant properties of Vitamin E and of all the other phenolic antioxidants is a result of the relatively weak bond dissociation enthalpy (BDE) of the O-H bond. The phenolic hydrogen atom of Vitamin E is rapidly extracted in an exothermic reaction by ROO• to provide an alkyl hydroperoxide ROOH having a BDE (O-H) **(Burton and Ingold, 1986; Wright *et al.*, 1997)**.

In fact, it is well known that electron releasing groups, especially if in conjugated positions, reduce the BDE O-H value in a substituted phenol **(Brigati *et al.*, 2002)**. Moreover, the influence of a substituent (hydroxyl or alkoxyl) is also due to the possible formation of intramolecular hydrogen bonds with the phenolic O-H thus stabilizing the starting phenol and/or the corresponding phenoxyl radical.

Moreover, Vitamin E may be easily regenerated by several co-antioxidants, namely ascorbic acid (Vitamin C), $Q_{10}H_2$, etc. Vitamin C, which at physiological pH, is largely ionized in the ascorbate form, may easily reduce tocopheryl radical back to tocopherol **(Davies *et al.*, 1988; Bowry and Ingold, 1995)** via either a hydrogen atom transfer or a concerted electron and proton transfer mechanism **(Bisby, 1995)**.

2.2.2.2 Electron "E" transfer inhibition

Antioxidants may inhibit peroxidative processes by the transfer of electrons to or from radical or non-radical species involved in oxidative stress, according to the redox potentials of the species involved.

Nitroxide radicals as shown on Figure 2.10 can act as preventive antioxidants through this mechanism. These compounds are among the most stable free radicals known that have been widely exploited as biophysical probes, spin labels and contrast agents for many biophysical/medical studies. Because of their versatile ability to deactivate free radicals and of their low toxicity **(Damiani *et al.*, 2008)**, nitroxides have also been thoroughly investigated as antioxidants for all those systems subject to oxidation **(Mitchell *et al.*, 1997)**.

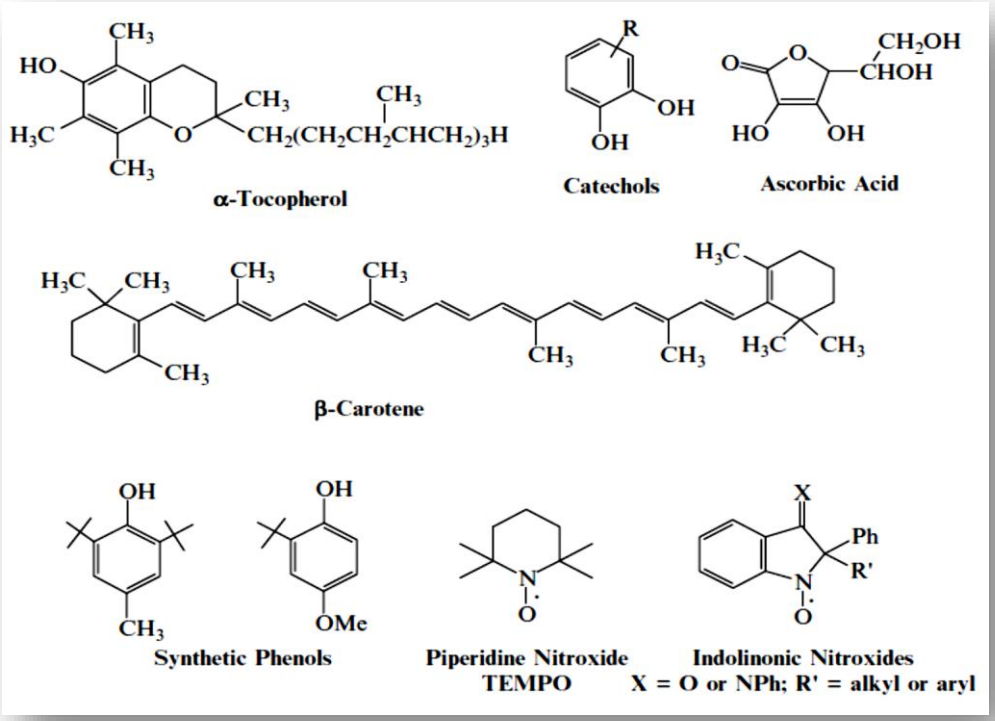

Figure 2.10: Selected natural and synthetic antioxidants **(Damiani *et al.*, 2008)**

Via single electron transfer reactions, nitroxides can be reduced to hydroxylamines or oxidized to oxoammonium cations, thus they can afford protection by acting both as oxidazing and as reducing agents, and in some cases they can be continuously recycled (Figure 2.11).

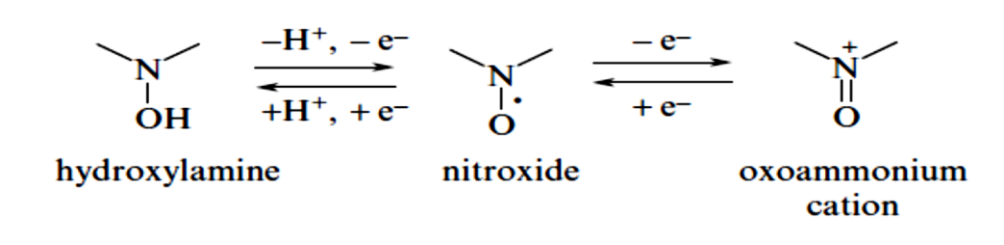

Figure 2.11: Reduction of nitroxide to hydroxylamine (1) and it's oxidation to oxammonium cation (2) by single electron transfer **(Damiani *et al.*, 2008)**

2.2.2.3 Radical scavenging inhibition

All radicals taking part in a peroxidative process may be inactivated by coupling reactions with other radical species. These reactions have too low activation energies therefore their rates are almost diffusion controlled. Nitroxide radicals, mentioned earlier, can deactivate radicals also through this mechanism thereby acting as chain-breaking antioxidants. These compounds, similarly to phenoxy radicals, are unreactive towards non-radical molecules. However, they react with carboncentred radicals at an almost diffusion controlled rate leading to the formation of alkoxyamines as shown in figure 2.12 for TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl), one of the most widely studied aliphatic nitroxides **(Chateauneuf *et al.*, 1988)**.

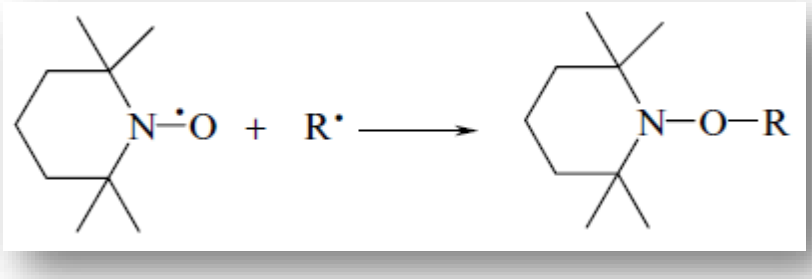

Figure 2.12: Nitroxide radical scavenging **(Damiani *et al.*, 2008)**

2.2.3 Role of Antioxidant Therapy in Male Infertility

Treatment strategies to reduce seminal OS levels may enhance natural conception and the outcome of assisted reproductive technologies. Antioxidants are the most important defense against free radical- induced infertility. Standard semen analysis and use of the sperm deformity index have been utilized to identify infertile males with high ROS levels **(Agarwal *et al.*, 2004)**.

Several clinical trials have examined the potential of antioxidant supplementation to treat oxidative- stress-induced male factor infertility **(Ross *et al.*, 2010)**. The low cost and

relatively low risk of toxicity of the following antioxidants is appealing to both patients and clinicians.

2.2.3.1 Enzymatic antioxidants

Three groups of enzymes play significant roles as oxidant scavengers. Superoxide dismutases (SOD) are metal-containing enzymes that catalyze the conversion of two superoxides into oxygen and hydrogen peroxide, which is less toxic than superoxide (Figure 2.5). Catalase, an enzyme found in peroxisomes, degrades hydrogen peroxide to water and oxygen, thereby completing the reaction started by SOD (Figure 2.5). Glutathione peroxidase also acts to degrade hydrogen peroxide (Figure 2.13). Other enzymes, such as glutathione transferase, ceruloplasmin or hemoxygenase may participate in enzymatic control of oxygen radicals and their products too **(Tremellen, 2008)**.

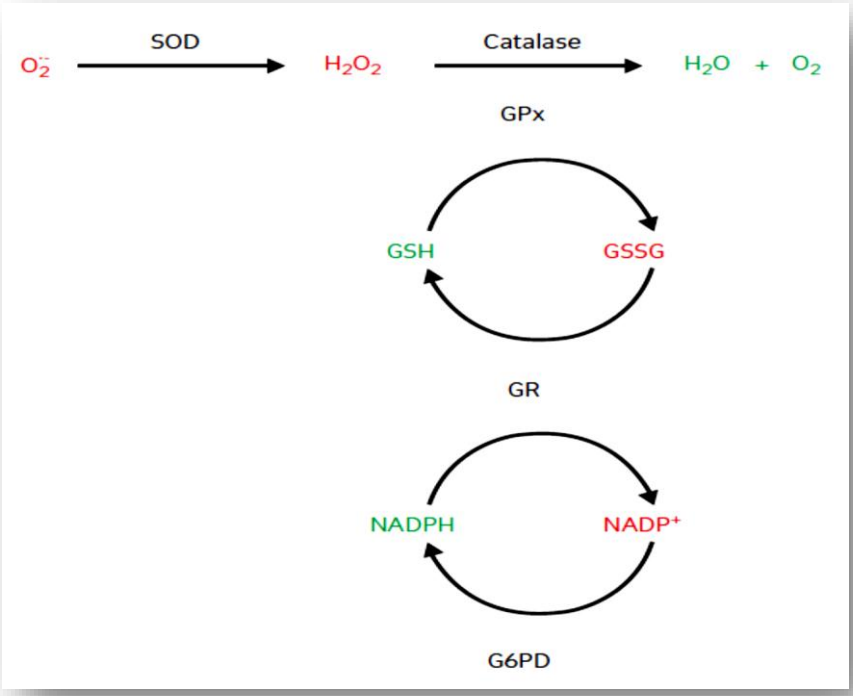

Figure 2.13: Glutathione peroxidase (GPx) also scavenges hydrogen peroxide (H2O2), along with glutathione (GSH), which becomes oxidized and is reduced/regenerated by glutathione reductase (GR) to allow further antioxidant function. **G6PD**, glucose 6 phosphate dehydrogenase; **GSSG**, glutathione disulfide; **NADPH**, nicotinamide adenine dinucleotide phosphate; **SOD**, superoxide dismutase

a. Glutathione peroxidase

Glutathione (GSH) represents the most abundant reducing agent found in human body, protecting lipids, proteins and nucleic acids against oxidative damage. GSH combines with vitamin E and Selenium (Se) to give birth to glutathione peroxidase. GSH/glutathione peroxidase acts as scavenging antioxidants in the epididymis and testes **(Mora-Esteves and Shin, 2013)**. Their modification of the spermatozoa membrane confers protection on the lipid constituents, thus preserving sperm viability and motility **(Lanzafame *et al.*, 2009)**. Previous *in vitro* studies showed that GSH preserves the tail-beat frequency, reduces LPO, and improves the sperm membrane characteristics **(Griveau and Le Lannou, 1994)**.

b. Superoxide dismutase and catalase

SOD protects sperm from superoxide anions by catalyzing the conversion of superoxide into oxygen and H_2O_2, thereby preventing LPO and improving motility **(Agarwal *et al.*, 2004)**. On the other hand, catalase aids in the decomposition of H_2O_2 into water and oxygen **(Mora-Esteves and Shin, 2013)**. Thus, both SOD and catalase assist in removing ROS that has the potential to damage sperm **(Agarwal *et al.*, 2014)**.

2.2.3.2 Non-enzymatic antioxidants

a. Carnitine

Carnitine is a water-soluble antioxidant usually covered via dietary sources. Carnitine may participate in sperm motility as a fuel source by assisting free fatty acid utilization and preventing lipid oxidation. Therefore, carnitine protects the sperm DNA and membranes from oxidative damage, and maintains the sperm viability and motility **(Sharma and Agarwal, 1996)**.

b. Carotenoids

Although carotenoids have short half-lives, they are highly effective and efficient singlet molecular oxygen quenchers. Two major carotenoids worth mentioning are β-carotene, which prevents lipids in the cell membrane from being peroxidized, and lycopene, which is the most

potent and readily available carotenoid that prevents peroxidation in the seminal plasma **(Agarwal *et al.*, 2014)**.

c. Cysteines

Cysteines are precursors of intracellular GSH and therefore, increase the amount of GSH synthesized. GSH subsequently scavenges oxidants and prevents oxidative damage to the cell membrane and DNA. N-acetyl-L-cysteine works via two mechanisms: (1) by boosting the amount of reducing agent produced and (2) ridding the spermatozoa of free radicals, thereby proserving sperm motility **(Mora-Esteves and Shin, 2013).**

d. Vitamin E

Vitamin E (α-tocopherol) is a chain breaking antioxidant found in the sperm's cell membrane and acts by neutralizing H_2O_2 and quenching free radicals, hence halting chain reactions that produce lipid peroxides and protecting the membrane against the damage induced by ROS. Furthermore, it improves the activity of other scavenging oxidants **(Mora-Esteves and Shin, 2013)**. In these ways, vitamin E helps to preserve both sperm motility and morphology **(Agarwal *et al.*, 2014)**.

e. Vitamin C

Vitamin C has a unique function as an antioxidant. When vitamin C loses one electron (oxidation), it remains highly stable, allowing it to gain an electron from a more aggressive free radical while also not damaging critical cellular structures. When vitamin C is oxidized, it is recycled back to an antioxidant form via the NADPH pathway, as well as by the glutathione pathways **(Parekattil and Agarwal, 2012)**.

Vitamin C plays a significant role (up to 65%) in combatting OS in the seminal plasma. It reacts with OH^-, O_2^-, and H_2O_2 in the extracellular fluid, thus protecting sperm viability and motility. However, vitamin C is only a weak ROS scavenger in the cell membrane and, hence, has almost no effect within the cell **(Agarwal *et al.*, 2014)**.

It is well known that vitamins C and E are naturally occurring antioxidants, working in synergy with each other and against different types of free radicals. Their levels are known to be suppressed in diabetes **(Mohasseb *et al.*, 2011)**. The effects of this desease on human male

reproductive function have been largely neglected beyond concerns about impotence. In the following chapter, the diabetes impact on male infertility is outlined.

Chapter *3*

Diabetes Mellitus and Male Infertility

Chapter 3

Diabetes Mellitus and Male Infertility

3.1 Diabetes Mellitus at a Glance

Diabetes mellitus (DM) is a heterogeneous metabolic disorder characterized by the presence of hyperglycemia due to impairment of insulin secretion, defective insulin action or both **(Punthakee et al., 2018)**. Moreover, DM is associated with severe disturbances of carbohydrate, fat and protein metabolism. Diabetes is rapidly emerging as a major public health challenge and demands special attention towards its management. **(Jangir and Jain, 2014)**. The chronic hyperglycemia of diabetes is associated with relatively specific long-term microvascular complications affecting the eyes, kidneys and nerves, as well as an increased risk for cardiovascular disease (CVD) **(Punthakee et al., 2018)**.

DM may present with characteristic symptoms such as thirst, polyuria, blurring of vision, and weight loss. In most severe forms of DM, ketoacidosis or a non-ketotic hyperosmolar state may develop and lead to stupor, coma and, in absence of effective treatment, death. Often symptoms are not severe, or may be absent, and consequently hyperglycaemia of sufficient degree to cause pathological and functional changes may be present for a long time before the diagnosis is made **(Alberti and Zimmet, 1998)**

3.1.1 Diabetes Mellitus Prevalence

3.1.1.1 In the world

Diabetes is one of the largest global health emergencies of the 21st century (Figure 3.1). Diabetes is among the top 10 causes of death globally and together with the other three major non communicable diseases (NCDs) (cardiovascular disease, cancer and respiratory disease) account for over 80% of all premature NCD deaths. In 2015, 39.5 million of the 56.4 million deaths globally were due to NCDs. A major contributor to the challenge of diabetes is that

30-80% of people with diabetes are undiagnosed. Population-wide lifestyle change, along with early detection, diagnosis and cost-effective treatment of diabetes are required to save lives and prevent or significantly delay devastating diabetes related complications. Only multi-sectoral and coordinated responses with public policies and market interventions within and beyond the health sector can address this issue **(International Diabetes Federation, 2017)**.

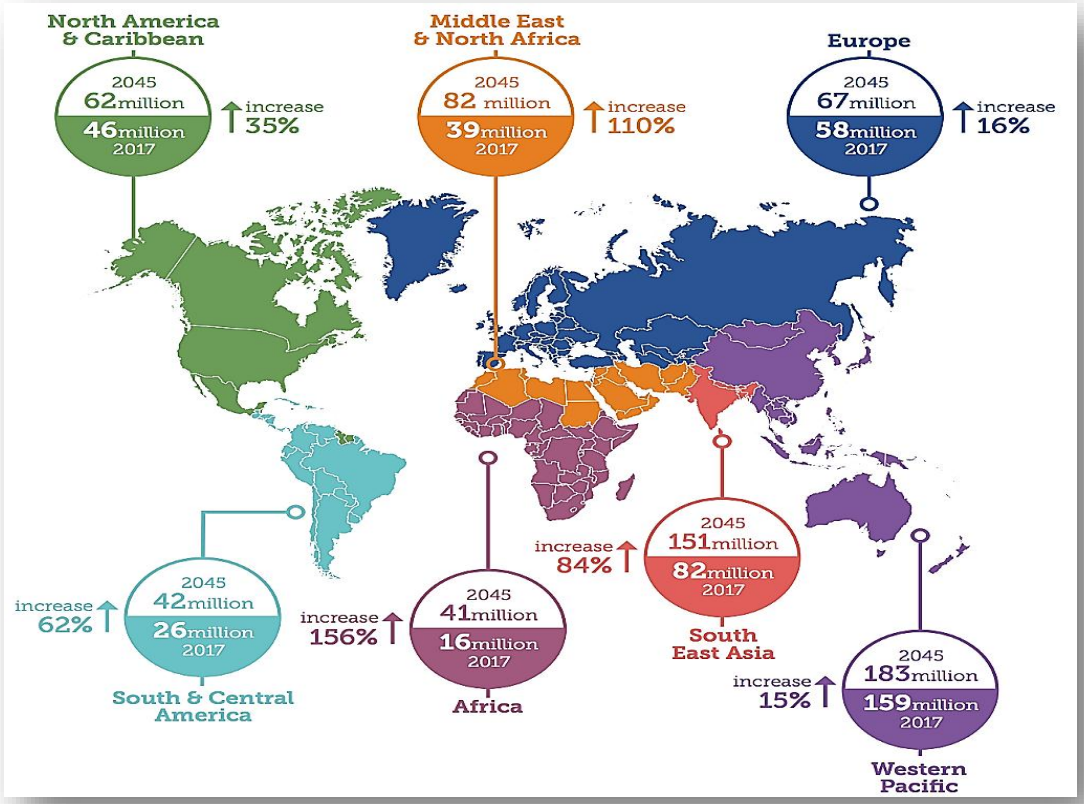

Figure 3.1: Number of people with diabetes worldwide and per region in 2017 and 2045 (20-79 years) **(International Diabetes Federation Atlas, 2017)**

It has been estimated that globally as many as 212.4 million people or half (50.0%) of all people 20-79 years with diabetes are unware of their disease (Figure 3.2). It is better for people with diabetes to be diagnosed as early as possible since the chances of preventing harmful and costly complications will be higher. Since half of the people with diabetes are undiagnosed, there is a global urgent need to screen, diagnose and provide appropriate care to people with diabetes. People with undiagnosed diabetes are also subject to higher usage of healthcare services compared to people without diabetes, and consequently likely to incur larger

healthcare expenditures. Based on one American study, the total economic cost of undiagnosed diabetes was 33 billion USD in 2012 **(Dall *et al.*, 2012)**.

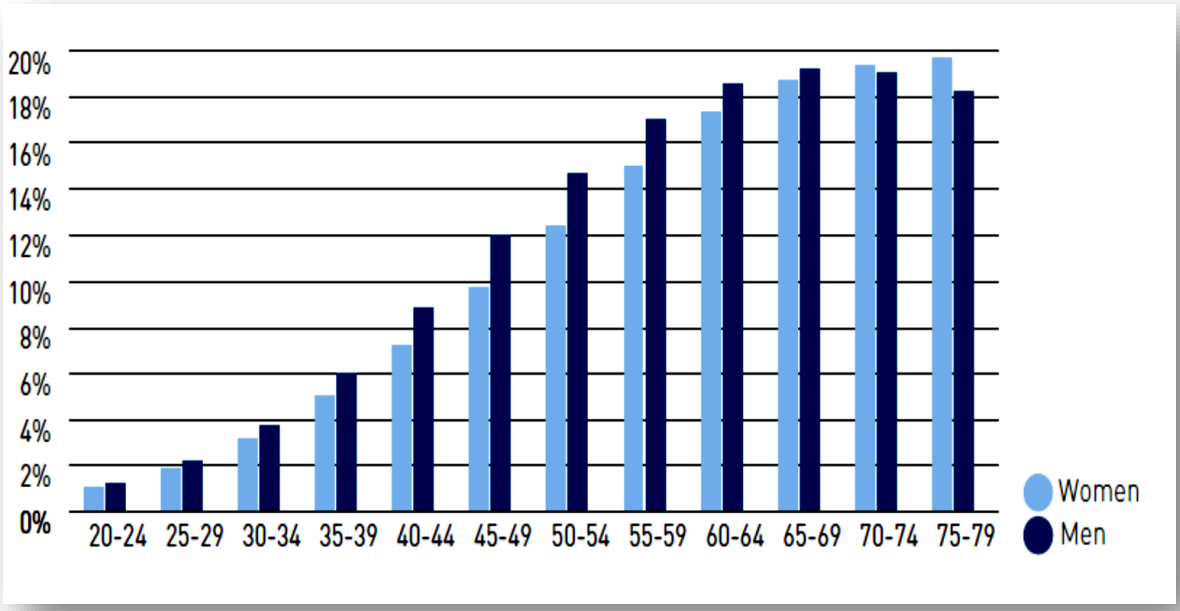

Figure 3.2: Prevalence of people with diabetes by age and sex, 2017

(International Diabetes Federation Atlas, 2017)

3.1.1.2 Diabetes Mellitus Prevalence in Middle East and North Africa (MENA)

In 2017, approximately 38.7 (27.1-51.4) million people, or 9.6% (6.7-12.7) of adults aged 20-79 years are living with diabetes in MENA. About 49.1% of these are undiagnosed. Although 55.5% of all adults in the region live in urban areas, 67.3% of people with diabetes live in urban environments. The vast majority of people with diabetes in the region are living in low or middle income countries (83.8%). Countries with highest age adjusted comparative diabetes prevalence in MENA are Saudi Arabia (17.7%), Egypt (17.3%) and UAE (17.3%), The countries with the largest number of adults aged 20-79 years with diabetes are Egypt (8.2 million), Pakistan (7.5 million) and Iran (5.0 million). A further 33.3 million people age 20-79 years in the region, or 8.2% of the adult population, are estimated to have impaired glucose tolerance (IGT) and are at high risk of developing diabetes. It is estimated that the number of people with diabetes in the region will double to 82 million by 2045 **(International Diabetes Federation, 2017)**.

3.1.2 Diabetes Mellitus Classification

3.1.2.1 Diabetes Type 1 (DT1)

Type 1 indicates the processes of beta-cell destruction that may ultimately lead to diabetes mellitus in which 'insulin is required for survival' to prevent the development of ketoacidosis, coma and death **(Alberti and Zimmet, 1998)**. It is instigated by injury or cellular-controlled autoimmune destruction of the pancreatic -cells. Heredity, race or ethnicity, age and gender are some of the associated risk factors that play a role in ascertaining the susceptibility of the insulin producing cells to abrasion. It may develop swiftly over a period of a few days or weeks, following this sequence: (1) decreased insulin; (2) elevated usage of fats for energy and for formation of cholesterol by the liver; (3) reduction of the body's proteins **(Temidayo and Du Plessis, 2018)**.

3.1.2.2 Diabetes Type 2 (DT2)

Type 2 diabetes is the most common type of diabetes, accounting for around 90% of all cases of diabetes. In type 2 diabetes, hyperglycaemia is the result of an inadequate production of insulin and inability of the body to respond fully to insulin, defined as insulin resistance. During a state of insulin resistance, insulin is ineffective and therefore initially prompts an increase in insulin production to reduce rising glucose levels but over time a state of relative inadequate production of insulin can develop. Type 2 diabetes is most commonly seen in older adults, but it is increasingly seen in children, adolescents and younger adults due to rising levels of obesity, physical inactivity and poor diet **(International Diabetes Federation, 2017)**.

3.1.2.3 Other Specific Types of Diabetes

Other Specific Types (Table 2.1) are less common causes of diabetes mellitus, but are those in which the underlying defect or disease process can be identified in a relatively specific manner. They include, for example, fibrocalculous pancreatopathy, a form of diabetes, which was formerly classified as one type of malnutrition-related diabetes mellitus **(American Diabetes Association, 2014)**.

Table 3.1: Other specific types of diabetes **(American Diabetes Association, 2014)**

Genetic defects of beta-cell function	Chromosome 20, HNF4? (MODY1) Chromosome 7, glucokinase (MODY2) Chromosome 12, HNF1α (MODY3) Chromosome 13, IPF-1 (MODY4) Mitochondrial DNA 3243 mutation Others
Genetic defects in insulin action	Type A insulin resistance Leprechaunism Rabson-Mendenhall syndrome Lipoatrophic diabetes Others
Diseases of the exocrine pancreas	Fibrocalculous pancreatopathy Pancreatitis Trauma/pancreatectomy Neoplasia Cystic fibrosis Haemochromatosis Others
Endocrinopathies	Cushing's syndrome Acromegaly Phaeochromocytoma Glucagonoma Hyperthyroidism Somatostatinoma Others
Infections	Congenital rubella Cytomegalovirus Others
Uncommon forms of immune-mediated diabetes	Insulin autoimmune syndrome (antibodies to insulin) Anti-insulin receptor antibodies 'Stiff man' syndrome Others

3.2 Male Infertility and Diabetes Mellitus

A closer look into fertility rates of modern societies reveals that the increased incidence of DM has been closely associated with falling birth rates and fertility. This could be a result

of a disturbing increase of diabetic men in reproductive age. The bulk of patients with type 1 diabetes (DT1) are diagnosed before the age of 30 and there is an alarming number of childhood and adolescent with DT1 and type 2 diabetes (DT2). Moreover, western diets, lifestyle habits, and obesity in young individuals strongly contribute for the increasing incidence of DT2 among youth individuals **(Alves *et al.*, 2013)**.

Although there is no doubt that the disease is responsible for several pathological and biochemical alterations that reduce male fertility, the real impact of DM on male reproductive health remains obscure and is a matter of large debate. Sexual disorders, such as erectile dysfunction or retrograde ejaculation, are known to occur in diabetic individuals and usually end-up in a reduction of sexual appetite that is often attributed to lethargy and a certain degree of tiredness associated to the hyperglycemic state. In view of this, it is essential that a logical and rigorous scientific analysis of the effects of diabetes on male reproductive function be performed **(Agbaje *et al.*, 2007)**.

3.2.1 Glucose Metabolism in Sperm

Serum glucose concentrations depend on and alter several organs function and tissues. Liver and fat are usually known to suffer a tight control from glucose fluctuations, especially because they are known to play key roles in the use and storage of nutrients by hormonally regulated mechanisms. In testes, glucose metabolism is a pivotal event too.

Moreover, spermatogenesis maintenance *in vivo* depends upon glucose metabolism although there are low levels of this sugar in tubular fluid **(Zysk *et* al., 1975; Robinson and Fritz, 1981)**. Therefore, blood-to-germ cells transport of glucose and other metabolic intermediates is highly controlled, particularly due to the presence of the blood–testis barrier (BTB). This barrier not only physically divides the seminiferous epithelium in two compartments but is responsible for the maintenance of different levels of substances and metabolites between rete testis fluid and the lymph or plasma too. One of the most relevant testicular cells for all the functions of BTB is the Sertoli cell (SC). These cells have such an important role on male reproductive function (see Chapter 1), that their number is usually associated with testis size **(Sharpe *et al.*, 2003)** and play a broad spectra of functions in the spermatogenic event. Besides being responsible for water transport from the interstitial space to the lumen, they control the seminiferous fluid pH and ionic composition too. Furthermore,

SCs are known as "nurse cells" because they provide physical and nutritional support for the developing germ cells, which is pivotal for a normal spermatogenesis. SCs are well known for their ability to produce lactate at a high rate that is consumed, together with pyruvate, by pachytene spermatocytes and round spermatids **(Alves *et al.*, 2013)** (Figure 3.3).

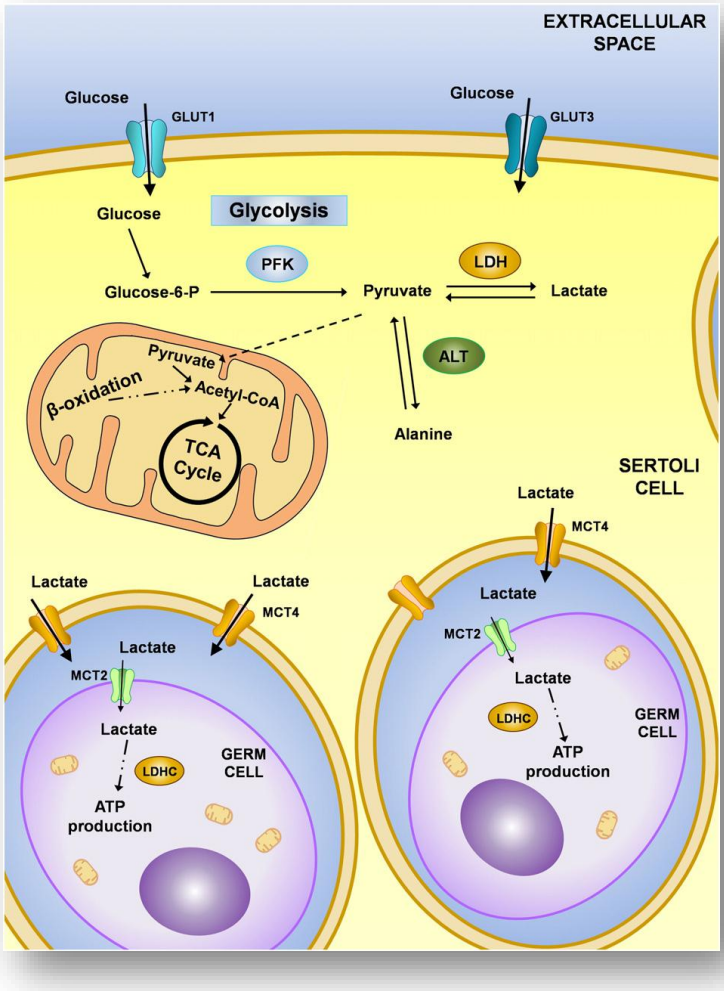

Figure 3.3: Schematic illustration of the metabolic cooperation between Sertoli and germ cells. The glucose from the interstitial fluid enters the Sertoli cells through glucose transporters, mainly GLUT1 and GLUT3, and is converted into glucose-6-phosphate that is then converted into pyruvate by phosphofructokinase (PFK). The pyruvate can then be: a) transported into the mitochondrial matrix to form acetyl-CoA; b) converted into lactate-by-lactate dehydrogenase (LDH); or c) converted into alanine-by-alanine aminotransferase (ALT). The lactate produced by Sertoli cells is exported to the intratubular fluid by proton linked plasma membrane transporters (MCTs), mainly MCT4. The germ cells uptake the lactate produced by Sertoli cells through MCTs, which is then directed for ATP production **(Alves *et al.*, 2013)**

Sperm cell is the most differentiated mammalian cell. The main goal of sperm consists of transferring male haploid DNA to female DNA through a series of mechanisms that imply their displacement along the female genital tract and fertilizing ability **(Rodriguez-Martinez, 2007)**.

Energy in sperm cells is mainly used to maintain the motility to complete capacitation and subsequent acrosome reaction **(Yanagimachi, 1994; Flesch and Gadella, 2000)**. Sperm cells need energy to acquire and maintain motion competence after epididymal maturation because they are actually immotile in testis **(Yanagimachi, 1994)**. Much adenosine triphosphate (ATP) in sperms is consumed for maintaining the motility. Except some metabolites, such as lactate and citrate, sperm mainly utilize sugars as an energy fuel including glucose, mannose, and fructose. The two main metabolic pathways involved in energy generation are anaerobic glycolysis and oxidative phosphorylation **(Bucci *et al.*, 2010)**.

Inhibitors of either oxidative metabolism or glycolysis in many species show that either pathway alone can maintain mobility independently **(Storey, 2008)**. Sperm metabolism can proceed through glycolysis, mitochondrial oxidative phosphorylation or the pentose phosphate pathway. The predominant pathway used depends on species, oxygen content and/or hexose availability. In fact, sperm have a mitochondrial sheath in the midpiece, where the oxidative processes may take place **(Bucci *et al.*, 2010)**.

Therefore, the most important glycolytic enzymes are mainly located at the principal piece of the tail, which is connected to the fibrous sheath **(Eddy *et al.*, 2003; Miki, 2007; Bucci *et al.*, 2010)**.

Carbohydrates are polar molecules that are rich in alcoholic (-OH) groups and can passively cross the lipidic bilayer in a too slow and inefficient manner. Therefore, carriers are required when cells uptake glucides **(Bucci *et al.*, 2010)**. An important role of supplying cells with energy is realized by different membrane proteins that can actively (sodium-dependent glucose transporters [SGLT]) or passively (glucose transporters [GLUT]) transport hexoses through the lipidic bilayer **(Joost and Thorens, 2001; Scheepers *et al.*, 2004)**.

The proteins of the SGLT family are active transporters of sugars, particularly glucose **(Scheepers *et al.*, 2004)**. GLUTs are 13 proteins of a family that facilitate glucose transport and show a peculiar distribution in different tissues, as well as a particular affinity for

substrates. Other hexoses (fructose, mannitol), vitamins, and amino sugars as glucosamine can also be transported by GLUTs **(Angulo *et al.*, 1998)**.

Glucose passively transport across the blood-testis barrier, which is mediated by GLUTs, is an important event in spermatogenesis. GLUTs also exist in mature sperm cells, which, in fact, require carriers for uptake energetic sources that are important for maintaining basic cell activity, as well as specific functions, such as motility and fertilization ability **(Bucci *et al.*, 2010)**.

In human sperm cells, GLUT1 and GLUT2 were in the acrosomal region and the principal and end pieces of the tail, whereas GLUT3 was found in the midpiece **(Angulo *et al.*, 1998)**. GLUT4 did not show any immunologic positivity and GLUT5 was detected in the subequatorial region and the mid and principal pieces **(Bucci *et al.*, 2010)**.

GLUT8 was firstly identified in the testes as well as other tissues **(Doege *et al.*, 2000)**. GLUT8 localizes in the midpiece and principal piece as well as in the acrosomal region of the sperm. Immunoelectron microscopic analysis shows that GLUT8 is strongly detectable at the acrosome and neck region of the sperm. In the midpiece, GLUT8 localizes at the outer dense fibers (ODF) as well as at the circumference of the spiral mitochondria. In the principal piece, GLUT8 localizes at the ODF **(Sung and Moley, 2007)**. GLUT8 has an endosomal and lysosomal targeting motif, and is only translocated to the plasma membrane in response to insulin in blastocysts. Therefore, it might only transport hexoses across endosomes and lysosomes intracellularly. Similar to the lysosome, the acrosome of sperm cells contains lysosomal proteins under a low pH. GLUT8 might play a role in the acrosome reaction upon sperm binding to the oocyte **(Huang *et al.*, 2015)**.

GLUT9 has been recently identified to have a high homology degree comparing to GLUT5 that transports fructose as well as glucose **(Manolescu *et al.*, 2007)**. GLUT9 has two isoforms: a long form containing 12-transmembrane domains (GLUT9a) and a short form without two transmembrane domains (GLUT9b) that exist in sperm cells. Both GLUT9a and GLUT9b are expressed in the midpiece, whereas GLUT9b is also found in the acrosome and principal piece **(Sung and Moley, 2007)**. The two isoforms are also high-capacity urate transporters **(Caulfield *et al.*, 2008)**.

Uric acid inhibits peroxynitrite (ONOO-) generation in sperm and can interact with other ROS such as hydroxyl radicals **(Aitken *et al.*, 2004b)**. Because ROS generated by sperm cells are involved in capacitation, in addition to the role of providing energy substrates, GLUT9 may regulate the redox state and capacitation (Figure 3.4).

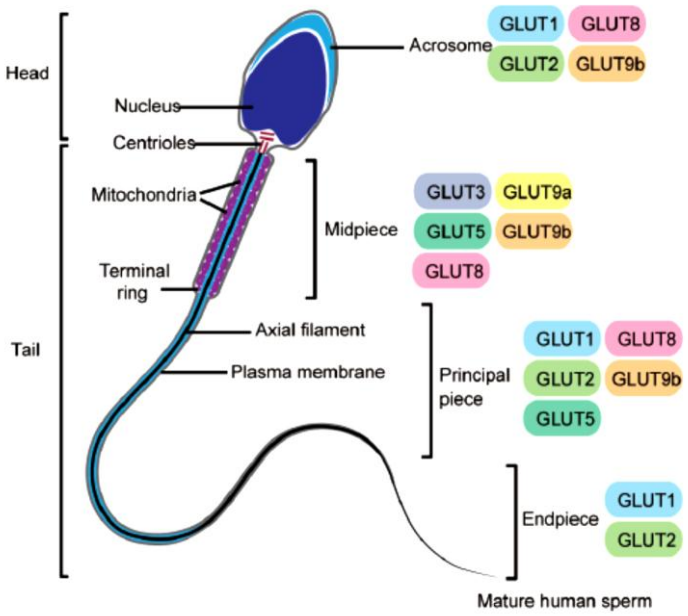

Figure 3.4: Schematic representation of human sperm cell and localization of several glucose transporter isoforms in the distinct parts of the spermatozoon, namely head (which includes the nucleus and acrosome) and tail (comprehending the midpiece, principal piece and endpiece). GLUT1: glucose transporter 1; GLUT2: glucose transporter 2; GLUT3: glucose transporter 3; GLUT5: glucose transporter 5; GLUT8: glucose transporter 8; GLUT9a: glucose transporter 9a; GLUT9b: glucose transporter 9b **(Huang *et al.*, 2015)**

The molecular basis of glucose metabolism and the importance of its sub products in testis are far beyond the simple maintenance of the germ cells nutritional status. In fact, a vast majority of patients suffering from subfertility and infertility exhibit issues in sperm function rather than lack of sperm. The ATP levels in sperm are maintained by several glycolytic and non-glycolytic substrates since both glycolysis and mitochondrial respiration are active in mammalian sperm. However, sperm capacitation is known to be stimulated by glucose, evidencing the regulatory role that this ose can exert in the overall male reproductive

function. Importantly, the loss of sperm functional competence is often associated to oxidative stress that arises from sperm metabolism, since sperm capacitation is achieved by biochemical and metabolic modifications **(Alves *et al.*, 2013)**.

3.2.2 Molecular Mechanisms of Testicular Glucose Metabolism in Diabetic Conditions

There is no doubt that spermatogenesis is a metabolically active process that depends upon strict metabolic cooperation between the several testicular cell types. During spermatogenesis, spermatozoa are produced within the seminiferous tubule in a process that takes several days and is under endocrine and paracrine control through the SCs **(Sexton and Jarow, 1997)**. In addition, SCs are responsible of glucose conversion, a non- metabolized substrate by developing germ cells, in lactate that is the preferential substrate for those cells.

The molecular mechanisms of testicular glucose metabolism in diabetic conditions are far from being disclosed. Nevertheless, *in vitro* and morphological studies of human biopsies from diabetic men allowed the collection of small and vital information concerning those molecular mechanisms.

Human biopsies from diabetic men show that there are morphological changes in testicular cells, namely in SCs, which present extensive vacuolization and have a high degree of degeneration. Moreover, germ cells exhibit a normal morphology, but the seminiferous tubules are depleted, and the number of Leydig cells vary considerably, with these cells presenting lipid droplets and variable number of vacuoles **(Cameron *et al.*, 1985)**. All these changes certainly have dramatic consequences to testicular cells glucose metabolism and to the overall metabolic cooperation between SCs and developing germ cells.

In fact, glucose metabolism impairment is frequently related with increased fatty acid metabolism. Early studies reported that DM caused an increased endogenous oxygen uptake and reduced lactate production by testicular cells. DM increased cholesterol, non-esterified fatty acids, triglycerides and phospholipids in rat testis tissues **(Sharaf *et al.*, 1978)**.

According to **Hutson (1984),** who investigated the biochemical responses of rat SCs and peritubular cells cultured under simulated diabetic conditions and reported that the *in vitro*

metabolic functioning of these testicular cells was highly sensitive to glucose concentrations. In fact, SCs cultured with high glucose concentration increased lactate secretion. At that time, it had already been reported that lactate enhanced respiration rates, protein and RNA synthesis in isolated pachytene spermatocytes, and round spermatids **(Jutte *et al.*, 1981)**, by interacting in other metabolic pathways and producing ATP **(Nakamura *et al.*, 1981)**.

Besides, lactate was also described as a modulator of NADH oxidation and the pentose phosphate pathway in those cells **(Grootegoed *et al.*, 1986; Courtens, 1999)** described new functions for the lactate produced in the testis. Intratesticular lactate infusion was reported to improve the spermatogenesis in adult cryptorchid rat testis and later, it was described that germ cell death is inhibited in a dose-dependent way by lactate **(Erkkila *et al.*, 2002)**. Therefore, any condition that promotes an alteration in testicular lactate levels or lactate production by SCs may compromise germ cell development that can occur through several distinct mechanisms.

3.2.3 Diabetes Impact on Male Fertility

3.2.3.1 Effects on spermatogenesis: role of endocrine disorder

Under normal circumstances, the hypothalamus releases gonadotropin-releasing hormone, thereby stimulating the anterior pituitary to secrete luteinizing hormone (LH) and follicle stimulating hormone (FSH). LH stimulates the Leydig cells to secrete testosterone and dihydrotestosterone, while FSH stimulates the Sertoli cells of seminiferous tubules to assist spermatogenesis process **(Temidayo and Du Plessis, 2018)**. Unarguably, spermatozoa are capable to utilize glycolysis and/or oxidative phosphorylation to generate energy. They are as well structured to be capable of using external hexoses (glucose, fructose and mannose), and smaller substrates (lactate, citrate amino acids, and lipids) for energy production **(Ballester *et al.*, 2004)**. However, spermatozoa are known to secret their own insulin since they are sensitive to hormonal fluctuations **(Carpino *et al.*, 2009)**. Therefore, deficiency of insulin or insensitivity to insulin in DM alters the endocrine pathway (negative feedback mechanism), resulting in impaired male reproductive function.

Animal studies on induced hyperglycemia revealed some adverse effects on male reproductive function relative to altered endocrine control. Additionally, decreased Sertoli cell vacuolization **(Roessner *et al.*, 2012)**, decreased sperm production **(Cameron *et al.*, 1990;**

Amaral *et al.*, 2006; Rama Raju *et al.*, 2011), decreased fertility (**McGaw *et al.*, 2007; Jelodar *et al.*, 2009**), alteration of epididymis morphology and density (**Soudamani *et al.*, 2005**), decreased LH, FSH, and testosterone serum levels (**Seethalakshmi *et al.*, 1987; Scarano *et al* ., 2006**), decreased number of Leydig and Sertoli cells and decreased number of spermatogonia (**Jelodar *et al.*, 2009**) were observed in induced diabetes.

The decreased Leydig cell number linked to the decrease in serum LH, which in part explained the LH stimulatory effects on Leydig cells (**Ballester *et al.*, 2004**). This indicates that the Leydig cells production involving insulin and insulin-like growth factor 1 (ILGF-1) signal mechanisms is mediated by LH (**Baccetti *et al.*, 2002; Yu *et al.*, 2006**). While the impaired cell function was measured by the loss of tyrosine phosphorylation, as well as decreased expression of GLUT-3 receptors, androgen receptors and ILGF-1 receptors (**Mangoli *et al.*, 2013**). These findings are supported by several other animal studies that investigated the effect of DM on male fertility (**Soudamani *et al.*, 2005; Singh *et al.*, 2009; Mora-Navarro-Casado *et al.*, 2010; Esteves and Shin, 2013**). Moreover, DM alters spermatogenesis through an FSH-related mechanism (**Temidayo and Du Plessis, 2018**).

Insulin deficiency present in DT1 does not appear to affect spermatogenesis through a direct effect on the epithelium of seminiferous tubules. Nevertheless, through an alteration in serum FSH levels. Decreased FSH is followed by a reduction in tubular FSH receptors in STZ induced DT1, thus causing a diminished epithelium response of the seminiferous tubules to FSH stimulation. Therefore, DM alters spermatogenesis by disrupting the insulin modulating effect on the regulation of serum FSH levels (**Steger and Rabe, 1997; Ballester *et al.*, 2004**,).

Likewise, glucose has been shown to be important for spermatogenesis and the AR. This was evidenced when a medium deprived of glucose inhibited the spontaneous AR, which was swiftly restored after the subsequent glucose addition (**Urner and Sakkas, 1996**). These substrates are conveyed into the cell by GLUTs (**Lampiao and Du Plessis, 2010**). As previously mentioned, GLUT family consists of 14 members and can be divided into three groups based on their sequence similarities (**Scheepers *et al.*, 2004**). GLUT8 belongs to class 3 transporters and is expressed predominantly in the testis (**Schürmann *et al.*, 2002; Gómez *et al.*, 2006**). Glucose transported into the cell is converted to energy, which is needed for spermatogenesis and motility (**Temidayo and Du Plessis, 2018**).

The GLUT8 activity disruption caused by decreased insulin resultes in reduced sperm motility and impaired fertilization **(Gawlik *et al.*, 2008)**. This can as well be a consequence of lower gonadotropin response to gonadotropin releasing hormone in diabetics **(Baccetti *et al.*, 2002)**.

3.2.3.2 Effect on sperm parameters: role of OS and Advanced Glycated End products (AGEs)

Effect of DM on male reproductive function can also be explained through the impact of OS, caused by the inequality between ROS production and antioxidant defense mechanisms **(Agarwal *et al.*, 2014)**. The main origins of ROS in the male reproductive system are known to be the immature spermatozoa and leukocytes **(Leclerc *et al.*, 1997; Agarwal *et al.*, 2014)**.

Additionally, mechanisms that involve repeated mild changes in cellular metabolism may result in tissue damage within a brief hyperglycemia occurrence. An enormous bulk of data give priority to certain metabolic pathways as being dominant contributors to hyperglycemic induced cell damage, e.g. elevated glycolysis, glucose autoxidation, increased polyol pathway flux, increased AGE formation, activation of protein kinase C isoforms, and increased hexosamine pathway flux **(Ahmed, 2005; Rolo and Palmeira, 2006)**. It has been shown that excessive production of O_2 by mitochondria in hyperglycemia is the trigger propel these pathways. Excessive production of O_2 momentously inhibits glyceraldehyde-3-phosphate dehydrogenase activity, which in turn activates all the pathways of hyperglycemic damage by diverting upstream glycolytic metabolites to these pathways **(Ahmed, 2005)**.

Furthermore, when the highly potent ROS exceeds the seminal antioxidant defense ability, many cascades of reactions will occur, which can lead to sperm DNA damage and mitochondrial DNA fragmentation, then altered sperm parameters and subsequently male infertility. In OS, there is excessive production of NO^-, which is detrimental to sperm motility. NO^- may react with O_2 or H_2O_2 to form $ONOO^{--}$ or OH^-, which might cause oxidation of sperm membrane lipids and thiol proteins **(Yu *et al.*, 2006)**. A decreased ATP levels could be affected too, thereby affecting spermatozoa kinematics.

The high PUFA contents in the sperm plasma membrane are susceptible to ROS, its invasion thereof, leads to LPO **(Huang *et al.*, 2015)** that occurs in three stages: initiation,

propagation, and termination. During initiation, free radicals react with fatty acid chains to form the lipid peroxyl radical. Peroxyl radicals in turn react with fatty acids to generate free radicals and the reaction is thus propagated. In termination, the two radicals react with each other, which lead to lipid break down **(Huang *et al.*, 2015)**.

Furthermore, glucose oxidation by OH^- has been shown to be the main cause of DNA strand breaks. Oxidative damage can further cause base degradation, DNA fragmentation, and proteins cross-linking. **Ceriello (2000)** found that the DNA strand break proportion is increased in the sperm of infertile diabetic men.

Apoptosis can be instigated by ROS-induced oxidative damage. High levels of ROS alter integrity of mitochondrial membrane **(Doege *et al.*, 2000; Joost and Thorens, 2001)**, resulting in mitochondria DNA (mtDNA) damage and subsequently affects negatively sperm functions.

In addition, elevated ROS production has been implicated in the generation of AGEs. AGEs are products of non-enzymatic reaction between sugar and the amino groups of proteins, lipids and DNA under hyperglycemic conditions **(Unoki *et al.*, 2007; Singh *et al.*, 2014)**.

AGEs can alter the normal functioning of macromolecules directly, by generating ROS independently, or indirectly, by activating the receptors for advanced glycated end products (RAGE). AGEs may play a key role in instigating harm and further act as mediator of damage to reproductive system of diabetic men **(Yamagishi, 2008; Karimi *et al.*, 2011)**.

RAGE is a ligand binding receptor that increases cellular dysfunction in inflammatory disorders such as DM. RAGE is expressed at low levels in normal tissues. However, in pathological conditions such as DM, its increased expression leads to tissue damage **(Schmidt *et al.*, 2001; Chavakis *et al.*, 2004)**.

Furthermore, it has been shown that seminal plasma has important antioxidant systems that can supply the spermatozoa with a defensive environment against OS **(Yu *et al.*, 2006)**. However, it was demonstrated that men with diabetes have significantly lower seminal total antioxidant capacity (TAC) levels compared to their non-diabetic counterparts. This was supported by another study that showed that seminal TAC has an effect on male fertility and

that increased ROS levels leads to low TAC levels (**Mahfouz *et al.*, 2009**). The reduced TAC level in DM is consistent with higher malonaldehyde levels, which suggests a possible role for AGEs in instigating LPO levels (**Temidayo and Du Plessis, 2018**).

Part II: Experimental Study

Chapter 4

Material and Methods

Chapter 4

Materiel and Methods

4.1 Ethical Consideration

All the experimental protocols were conducted with reference to the Guide for the Care and Use of Laboratory Animals. All animals received humane care according to this guide, of the Institute for Laboratory Animal Research Eighth edition **(National Reasearch Concil, 2011)**.

4.2 Objectives

In the current study, we aimed to assess the efficiency of supplementation with vitamin C and E in the case of whether DM is the cause of infertility or if the infertile men suffer from diabetes by inducing diabetes on male Wistar rats using a single dose of streptozotocin (STZ). Hence, we studied the efficacy of vitamin C, and E, either separated or combined, on semen parameters, and hormonal levels of hyperglycemic rats aiming to have reliable and comprehensive results.

4.3 Place of Study

The study was conducted at the level of Djilali LIABES University of Sidi-Bel-Abbes, Algeria. Rats were housed and sacrificed at Djilali LIABES Animal house. Sperm parameters were analyzed at Djilali LIABES University Biology Department (Development Biology Laboratory). Hormonal assays were performed in a private laboratory.

4.4 Experimental Study

The experiment was performed on 30 adults male Wistar rats, weighing 253 ± 4 gr. The animals were provided from the central animal house of Pasteur Institute of Algiers, Algeria. The experiment rats were housed in a plastic cage under a well- regulated light dark (12 h: 12 h) at room temperature (21± 2 °C).

The body weight was weekly assessed during the whole protocol period, and the dose of vitamins was adjusted accordingly. All animals were fed water, and a standard commercial ruminant pellet (ingredient shown in table 4.1) *ad libitum*. Supplementation of vitamins was undertaken per gavage to ensure that each animal receives equal amounts of supplements, which was prepared fresh on a daily basis.

After two weeks of acclimatization period, the animals were divided into five groups. Each group was composed of six rats.

Table 4.1: Ingredients of standards commercial ruminant pellet

Additives	
Vitamin A	6000 mg/kg
Vitamin E	15 mg/kg
Vitamin D3	2000 mg/kg
Oligo-elements	
Copper	15 mg/kg
Analytic complements	
Humidity	14%
Proteins	19%
Fats	3%
Ash	7.90%
Cellulose	5%

4.4.1 Induction of Experimental Diabetes and Administration of Vitamins C and E

4.4.1.1 Diabetes Mellitus induction

➔ **Group 1:** normoglycemic control group was given an intraperitoneal injection of a citrate buffer 0.01 M (pH=4.5).

The four other groups were rendered diabetic with an intraperitoneal injection of a single dose of STZ (Sigma Aldrich) (40mg/kg) in sodium citrate buffer 0.01M (pH=4.5) to overnight fasted animals **(Fernandes *et al.*, 2011)**.

By measuring the glucose levels using glucose test strip (Contour Plus, Bayer Health Care) five days after the induction, the diabetic state was confirmed. Animals were considered diabetic at 300mg/dL and above of blood glucose levels **(Guneli *et al.*, 2008)**.

➔ **Group 2:** was considered as hyperglycemic control.

The first and the second groups were given orally the vehicles (corn oil and water), while the third, the fourth, and the fifth groups were supplemented with vitamins by gavage as follows:

➔ **Group 3:** hyperglycemic supplemented with 250 mg/kg/day of vitamin C **(Sönmez *et al.*, 2005)**.

➔ **Group 4:** hyperglycemic supplemented with 250 mg/kg/day of vitamin E **(Sönmez *et al.*, 2005)**.

➔ **Group 5:** hyperglycemic supplemented with 250 mg/kg/day of vitamin C + 250 mg/kg/day of vitamin E **(Sönmez *et al.*, 2005)**.

4.4.1.2 Preparation and administration of vitamins

Vitamin C (L-ascorbic acid, Sigma-Aldrich) and E (α- Tocopherol, Sigma-Aldrich) were prepared daily by diluting the required quantity in warm water and corn oil respectively. Both vitamins were stored in dark containers for protection against light. Vitamins were administered by gavage once daily for a period of 4 weeks.

4.4.2 Animals Sacrifice

At completion of the four weeks of experiment period, the rats were weighed and fasting blood glucose levels were assessed after four hours of fasting using tail prick method. Animals were than anesthetized with chloroform for five minutes and sacrificed by cardiac puncture and blood was collected in heparinized tubes.

4.4.3 Parameters Investigated

4.4.3.1 Reproductive organ weights

It is essential that the weight of the testes and the epididymis be determined together and then separately **(De Kretser and O'Donnell, 2013)**.

The testes and the epididymis of sacrificed rats were excised, washed in phosphate buffer, and weighed with digital balance (Denver Instrument, USA): 0,0001 gr sensitivity.

4.4.3.2 Reproductive hormones

Blood samples, from each rat, were collected by cardiac puncture, in which a large gauge needle attached to a 5 ml syringe was inserted into the heart under deep anesthesia. Serum follicle stimulating hormone (FSH) and luteinizing hormone (LH), which stimulate the testis to produce sperm, and the male steroid hormone, testosterone (T) were dosed. Measurement of these parameters required access to specific and sensitive radioimmunoassay techniques **(Mohammadi *et al.*, 2017)**.

a. Testosterone

Testosterone *in vitro* competitive ELISA (Enzyme-Linked Immunosorbent Assay) kit was used for the testosterone levels assessment.

The determination of Testosterone was performed in plasma. The assay was undertaken on the same day of sample collection while the specimen was kept at 4°C. The kits reagents were brought to room temperature before use.

A 96-well plate was precoated with anti-Testosterone antibodies. Samples and the Testosterone-HRP conjugate were added to the wells, where Testosterone in the sample competes with the added Testosterone-HRP for antibody binding. After incubation, the wells were washed to remove unbound material and TMB substrate was then added which was catalyzed by HRP to produce blue coloration. The reaction was terminated by addition of Stop Solution that blocks the color development and produced a color change from blue to yellow. The intensity of signal was inversely proportional to the amount of Testosterone in the sample and the intensity was measured at 450 nm.

b. Luteinizing Hormone LH

Luteinizing Hormone Human ELISA Kit is intended for the quantitative determination of luteinizing hormone (LH) concentration in blood serum. The assay is based on the principle of a solid phase enzyme-linked immunosorbent assay.

The assay system utilizes a mouse monoclonal anti-α-LH antibody for solid phase (microtiter wells) immobilization and a mouse monoclonal anti-β-LH antibody in the antibody-enzyme (horseradish peroxidase) conjugate solution. The kits reagents were brought to room temperature before use.

The test sample was allowed to react simultaneously with the antibodies, resulting in LH molecules being sandwiched between the solid phase and enzyme-linked antibodies. After a 45-minute incubation period at room temperature, the wells were washed with water to remove unbound-labeled antibodies. A solution of tetramethylbenzidine (TMB) reagent was added and incubated for 20 minutes, resulting in the development of a blue color. The color development was stopped with the addition of Stop Solution, and the color was changed to yellow and measured spectrophotometrically at 450 nm. The LH concentration was directly proportional to the color intensity of the test sample.

c. Follicle Stimulating Hormone FSH

Follicle Stimulating Hormone in vitro ELISA (Enzyme-Linked Immunosorbent Assay) kit is designed for the accurate quantitative measurement of Follicle Stimulating Hormone in Human serum.

A 96-well plate was precoated with anti-Follicle Stimulating Hormone antibodies. Samples and standards were added to the wells, where FSH in the sample and standards binded to the precoated antibody. After incubation and washing, added Anti-Follicle Stimulating Hormone HRP conjugate binded to the antibody-FSH complex. After incubation, the wells were washed to remove unbound material and TMB substrate was then added which was catalyzed by HRP to produce blue coloration.

The reaction was terminated by addition of Stop Solution, which stopped the color development and produced a color change from blue to yellow. The intensity of signal was directly proportional to the FSH amount in the sample and the intensity was measured at 450 nm.

4.4.3.3 Quantitative assessment of sperm

It is difficult to accurately determine whether germ cell numbers are reduced by a simple histological analysis. A simple method to quantify spermatogenesis is the measurement of elongated spermatid content. This technique is based on the principle that beyond ~step 17 of spermatid development, the nuclei of the elongated spermatids are resistant to destruction by detergents. The following method can be applied to the measurement of the Elongated Spermatid Content of testis or epididymis.

For the determination of the number of elongated spermatids in the testes, one testis was weighed and decapsulated, and added to an appropriate volume of 0.05% Triton X-100 in PBS. A 2:1 ratio of buffer (ml) to testis weight (g), was used but smaller or larger volumes could be used if sperm were less or more concentrated.

For epididymal tissue, a ratio of 2:1–4:1 buffer to organ weight was used. The epididymal tissue was chopped with small scissors prior to buffer addition to facilitate homogenization. Tissue was homogenized in a tissue homogenizer for ~20 s (longer for epididymis) until the tissue was evenly dispersed. Homogenates could be stored for 2–3 days at 4°C prior to counting.

An aliquot of homogenate was diluted in PBS and loaded into a cover- slipped Thoma cell counting chamber. Since sperm heads were readily settle in solution, it was essential that all homogenates and dilutions be well vortexed prior to use. Once the sperm have been loaded

into the Thoma cell-counting chamber, the sperm heads were allowed to settle for ~2 min prior to counting.

Five squares from the four corners and the center of each chamber were counted; the homogenate was diluted accordingly so that approximately 100 sperm were counted in each chamber (therefore roughly 20 sperm per square). When each square was counted, sperm landing on the left and bottom edges were excluded (exclusion boundary) and those landing on the top and right edges were included. The number of sperm counted in the five squares were added up to calculate the total per chamber, and the average per chamber was calculated by adding both totals together and dividing by "2".

The average was multiplyed by the dilution factor (e.g., 100) to calculate the average sperm count per chamber. The elongated spermatid content was calculated as follows (1):

Average sperm count per chamber × 50,000 × Z ……. (1)

(Z = no. mls homogenization buffer + tissue weight (g)) **(De Kretser and O'Donnell, 2013)**.

4.4.3.4 Histopathological analysis

The right testis (six per experimental group) were removed and fixed in 10% formalin until processed for histopathological examination.

To fix whole testis following removal from the animals, gently prick the tough tunica albuginea in multiple places with a 22 g needle to facilitate the penetration of the formalin's fixative into the parenchyma of the testis and place them in the fixative for 5 h at room temperature. Testis were transferred to 70% ethanol. They are placed into the following solutions with a duration of 2 h in each: 70% Ethanol, 90% Ethanol, 100% Ethanol, 100% Ethanol, 50:50 Ethanol: Xylene-based solvent, Xylene-based solvent, Xylene-based solvent, Paraffin wax (60°C), Paraffin wax (60°C). While the tissues were at 60°C, they were removed from processing cassette and placed into a histology mold containing melted paraffin wax. Solidified on a cold plate until the paraffin wax block could be removed from the mold **(De Kretser and O'Donnell, 2013)**. Deparaffined and rehydrated sections were stained with hematoxylin-eosin and examined using a light microscopy.

4.4.3.5 Histopathological assessement of spermatogenetic activity

The spermatogenesis was categorized by using the Johnsen's score. A grade from 1 to 10 to each tubule cross section is applied according to the following criteria:

- 10 = complete spermatogenesis and perfect tubules;
- 9 = many spermatozoa present and disorganized spermatogenesis;
- 8 = only a few spermatozoa present;
- 7 = no spermatozoa but many spermatids present;
- 6 = only a few spermatids present;
- 5 = no spermatozoa or spermatids but many spermatocytes present;
- 4 = only a few spermatocytes present;
- 3 = only spermatogonia present;
- 2 = no germ cells but only Sertoli cells present;
- 1 = no germ cells and no Sertoli cells present.

The counting is performed with 10x objectives exposing several tubules in one field of vision. Tubules having their major portion in this field are scored, and the slide is than moved sideward to bring the adjacent area within the field. When coming to the edge of the biopsy, the slide is moved up to bring the structure at the bottom edge to the top edge and scoring continued. Damaged tubule at the edge of the biopsy specimen are not included.

In order to calculate a mean score, the number of tubuli recorded at each score is multiplied with the score and the sum of all 10 multiplications is divided by the total number of tubuli **(Johnsen, 1970)**.

4.5 Data Analysis

The data were statistically analyzed using the Statistical Package for Social Science (SPSS) version 20.0. One Way Analysis of Variance (ANOVA) followed by the Least Significant Difference test (LSD test) were used. The values were expressed as means ± SEM (Standard Error of Means) with accepted significance level at $p<0.05$.

Chapter 5

Results & Discussion

Chapter 5

Results & Discussion

5.1 Blood Glucose Level Before and After Streptozotocin Injection

Figure 5.1 shows the fasting glucose levels in all five groups before and after diabetes was induced. No significant difference ($p>0.05$) was observed among all groups before STZ injection. However, a significative ($p<0.05$) increase was noticed in the treated groups after inducing diabetes when compared to the normoglycemic control group over 5 days (Figure 5.1).

The final blood glucose levels, were maintained higher after 4 weeks of experiment period when compared to the initial blood glucose levels before STZ injection.

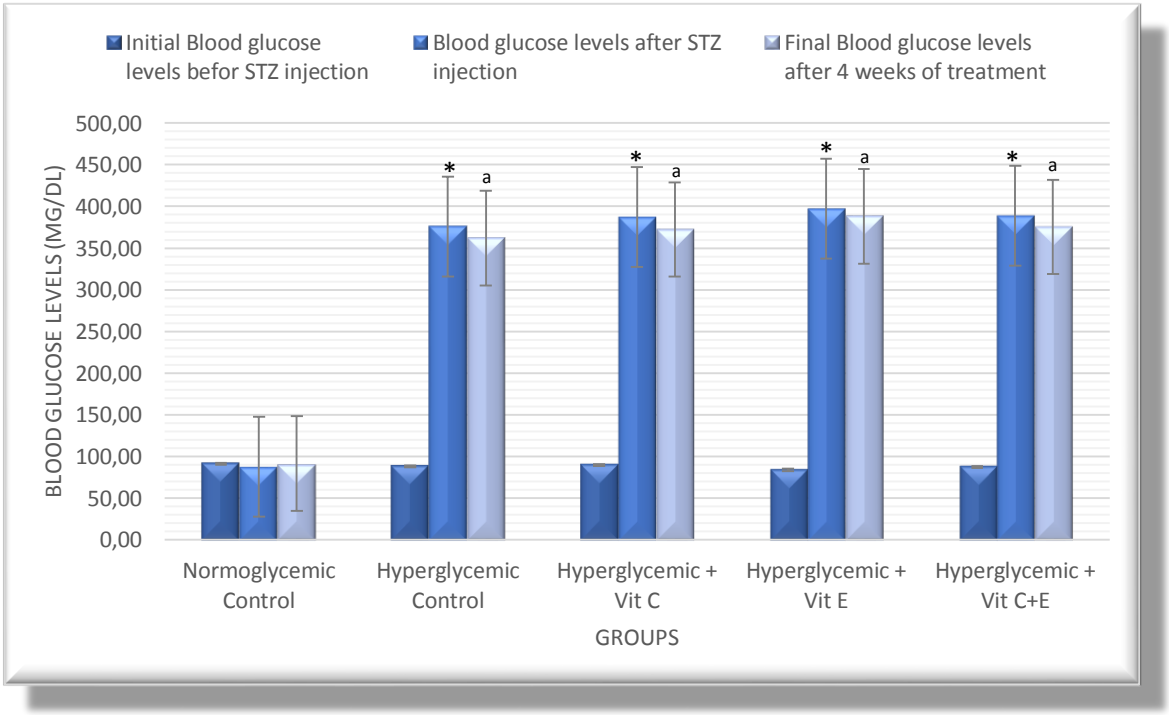

Figure 5.1: Blood glucose levels of the different groups before and after diabetes induction. $P<0.05$ is considered significant. (*) as compared to normoglycemic control group, (a) as compared to Hyperglycemic Control group. ANOVA test, with post hoc LSD test

STZ is a compound synthesized by *Streptomyces achromogenes*. It has been mostly used in the treatment of cancers **(Weiss, 1982; Dolan, 1997)** and in medical research studies to induce either DT1 or DT2, depending on the STZ dosage used **(Szkudelski, 2001; Islam and Loots du, 2009; Wei *et al.*, 2011)**. STZ is known to exhibit pancreatic cell toxicity and has diabetogenic properties, which may manifest through various mechanisms. These mechanisms include, STZ targeting the GLUT-2 receptor on pancreatic β-cells **(Raza and John, 2012)** or by inducing oxidative damage by releasing NO and increasing free radical production **(Hosokawa *et al.*, 2001; Friederich *et al.*, 2009)**. At low doses, STZ was observed to cause β-cell dysfunction and at higher doses, β-cell necrosis was observed **(Liu *et al.*, 2007)**. DT2 is normally induced by giving multiple low doses of STZ over a period of time that will trigger an immune response, and cause an inflammatory reaction. This reaction is usually linked to the release of glutamic acid and decarboxylase auto antigens **(Dufrane *et al.*, 2006)**.

In the current study, hyperglycemia was observed 5 days after a single intraperitoneal administration of STZ in adult wistar rats. This was confirmed by significant high blood glucose levels of the hyperglycemic group when compared to the initial fasting blood glucose of all five groups (Figure 5.1 and Table 5.1). Thus, we can assume that the diabetic rat model was successfully created.

Table 5.1: Blood glucose levels of wistar rats before diabetes induction, 5 days after diabetes induction, and 4 weeks after treatment

	Initial Blood glucose levels before STZ injection	**Blood glucose levels 5 days after STZ injection**	**Blood glucose levels after 4 weeks of treatment**
Normoglycemic Control	91,01±2,43	87,64±3,09	91,35±3,09
Hyperglycemic Control	87,87±3,97	375,89±14,13*	362,19±14,64[a]
Hyperglycemic +Vit C	89,41±2,20	387,14±9,12*	372,32±12,39 [a]
Hyperglycemic +Vit E	83,80±3,69	397,26±13,88*	388,34±14,58 [a]
Hyperglycemic +Vit (C+E)	87,18±4,26	388,81±8,83*	375,37±11,34 [a]

Data are expressed as mean ± SEM. $P<0.05$ is considered significant. (*) as compared to normoglycemic control group, (a) as compared to Hyperglycemic Control group. ANOVA test, with post hoc LSD test.

The results of the current study was supported by previous studies of diabetes induction in wistar rats by intravenous STZ injection, confirmed hyperglycemia 24 hours after induction **(Ramos-Lobo *et al.*, 2015)** and another study confirmed hyperglycemia 7 days later **(Li *et al.*, 2014)**.

5.2 Body and Reproductive Organ Weights

5.2.1 Body Weight

The hyperglycemic control group showed a significant decrease ($p<0.05$) in the final body weight, when compared with their initial one. While, there was a significant increase ($p<0.05$) in the final body weight of the normoglycemic control, hyperglycemic vitamin C treated and hyperglycemic vitamin C+E treated groups. Whereas, no changes were observed in the hyperglycemic vitamin E treated group (Figure 5.2).

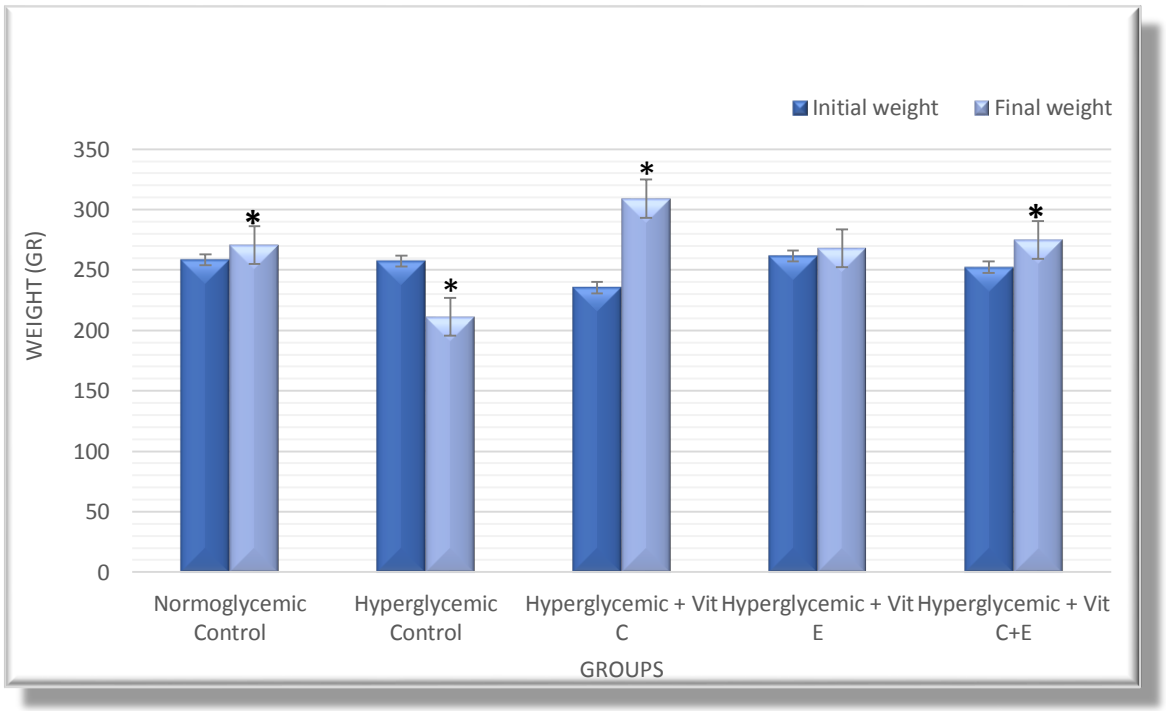

Figure 5.2: Effect of diabetes mellitus and vitamins supplementation on the initial and final body weight of the adult male rats. Values expressed as Mean ± SEM. $P<0.05$ is considered significant. (*) as compared to normoglycemic control group. ANOVA test, with post hoc LSD test

The body weight gained, at the end of the experiment, was maximum, in case of hyperglycemic vitamin C treated (73.5 g), and followed by hyperglycemic vitamin C + E (22.67 g), then control rats (12.16 g). The body weight gain was the least in the hyperglycemic vitamin E treated group (6.5 g) (table 5.2). Figure 5.2 shows that there was a significant difference ($p<0.05$) in final body weight ($F= 14.62$, $df= 29$, $p<0.05$) of experimental animals, when compared to control ones.

Other studies, reporting similar results underlined a decrease in body weight after inducing diabetes with an intraperitoneal injection of 40 mg/kg body weight of STZ **(Naziroglu 2003, Naziroglu *et al.*, 2011)**. **Soudamani *et al.* (2005)** suggested that the reduced body weight, observed in STZ induced diabetic rats, could be correlated to a cumulative effect of diminished insulin, growth hormone, and thyroid hormones. However, **Hassan (2001)** found a significant increase in body weight of hyperglycemic rats and supposed that it can be explained on the basis of altered metabolism in the diabetic state.

Table 5.2: Body weight change results upon sacrifice wistar rats after treatment of respective groups with vitamins C or/and E

	Initial weight (gr)	**Final weight (gr)**	**Weight change (gr)**
Normoglycemic control	258.50 ± 6.89	270.66 ± 7.60	+12.16
Hyperglycemic control	257.50 ± 6.83	211.33 ± 8.70*	- 46.17
Hyperglycemic + vit C	235.50 ± 13.18	309.00± 8.77*[a]	+73.5
Hyperglycemic + vit E	261.50 ± 4.83[b]	268.00 ± 5.00[ab]	+6.5
Hyperglycemic +vit (E+C)	252.33 ± 9.73	275.00± 3.65[ab]	+22.67

Values expressed as Mean ± SEM. $P<0.05$ is considered significant. (*) as compared to normoglycemic control group, (a) as compared to hyperglycemic control group, (b) as compared to hyperglycemic vitamin C treated group, (c) as compared to hyperglycemic vitamin E treated group. ANOVA test, with post hoc LSD test.

In our study, the final body weight of hyperglycemic treated groups significantly increases, as compared to hyperglycemic and normoglycemic control groups. Consistent with our results, **Naziroglu (2003)** observed that vitamin E supplementation increased the final body weight of hyperglycemic rats. Later, the same author reported that a combination dietary of vitamins E and C increased the final body weight of the hyperglycemic animals too **(Naziroglu *et al.*, 2011)**.

5.2.2 Epididymis and Testicular Weight

The STZ-induced hyperglycemia produced a significant ($p<0.05$) decrease in the epididymis weight, compared to the normoglycemic animals. However, there was a significant increase ($p<0.05$) among the epididymis weight of hyperglycemic vitamin C treated and hyperglycemic vitamin C+E treated groups, when compared to the hyperglycemic control group (Figure 5.3).

Table 5.3: Epididymal and testicular tissue weights results upon sacrifice wistar rats after treatment of respective groups with vitamins C or/and E

	Testis weight (gr)	**Epididymis weight (gr)**
Normoglycemic control	2.89 ± 0.10	1.69 ± 0.06
Hyperglycemic control	3.10 ± 0.14	1.35 ± 0.10*
Hyperglycemic + vit C	3.18 ± 0.11	1.69 ± 0.05[a]
Hyperglycemic + vit E	2.90 ± 0.08	1.61 ± 0.06
Hyperglycemic +vit (E+C)	3.15 ± 0.11	1.69 ± 0.15[a]

Values expressed as Mean ± SEM. P <0.05 is considered significant. (*) as compared to normoglycemic control group, (a) as compared to hyperglycemic control group. ANOVA test, with post hoc LSD test.ANOVA test, with post hoc LSD test.

These results corroborate with those obtained by **Badr *et al.* (2011)** who observed a significant increase of epididymal weight in vitamin C treated diabetic group.

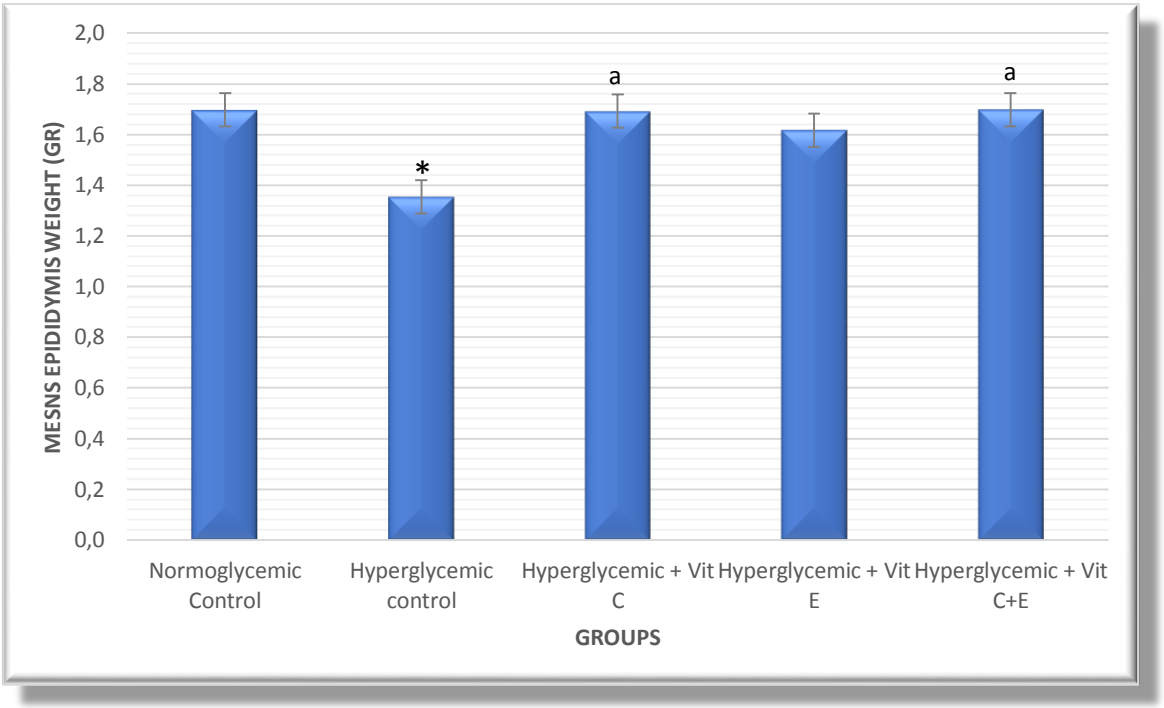

Figure 5.3: Effect of diabetes mellitus and vitamins supplementation on epididymis weight of adult male rats. Values expressed as Mean ± SEM. $P < 0.05$ is considered significant. (*) as compared to normoglycemic control group, (a) as compared to hyperglycemic control group. ANOVA test, with post hoc LSD test

Some studies reported that hyperglycemia might cause a decrease in testis and epididymal weight **(Cai *et al.*, 2000, Navarro-Casado *et al.*, 2010)**. Therefore, low testosterone levels and reduced androgenic stimuli, in the epididymis, may attribute adverse changes in the growth and development of rat epididymis **(Soudamani *et al.*, 2005)**.

Other studies reported that, no changes were observed in testis weight of treated animals with vitamin C **(Sönmez *et al.*, 2005; Fernandes *et al.*; 2011)**. **Lei *et al.* (1997)** observed that, the testis weight was similar to the control animals, after a treatment with vitamin E.

Concerning testicular weights, no significant ($p>0.05$) difference among all groups was observed (Figure 5.4, table 5.3).

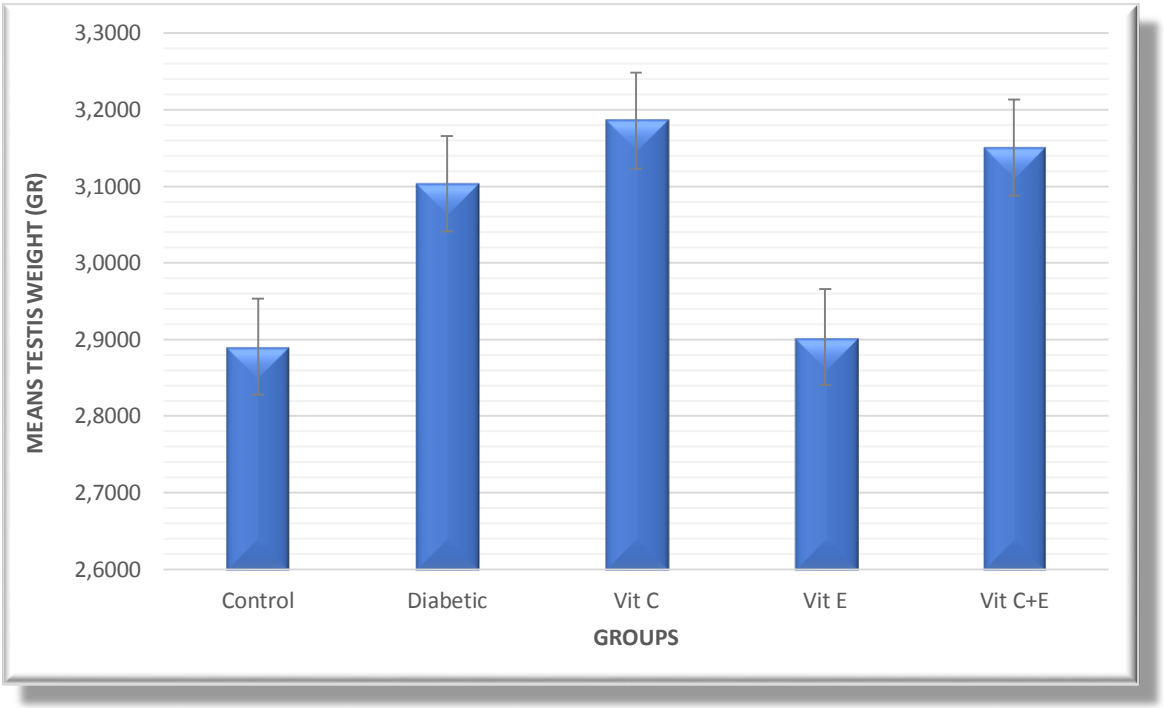

Figure 5.4: Effect of diabetes mellitus and vitamins supplementation on testis weight of adult male rats. Values expressed as Mean ± SEM. No significant difference among all groups

5.3 Hormonal Blood Levels

The testosterone levels, in hyperglycemic control rats, were significantly ($p<0.05$) lower than those in the normoglycemic animals. Significantly, higher levels were observed in vitamin C, and vitamin C+E supplemented hyperglycemic animals (Figure 5.5, table 5.4).

Table 5.4: Blood testosterone, LH, and FSH concentrations of male Wistar rats after supplementation compared to control groups

	FSH (ng/mL)	LH (ng/mL)	Testosterone (ng/mL)
Normoglycemic control	0.11 ± 0.00	0.13 ± 0.01	8.63 ± 0.49
Hyperglycemic control	0.10 ± 0.00	0.10 ± 0.00*	0.25 ± 0.12*
Hyperglycemic + vit C	0.13 ± 0.01*[a]	0.13 ± 0.01[a]	12.53 ± 0.68*[a]
Hyperglycemic + vit E	0.11 ± 0.00[a]	0.11 ± 0.00	1.46 ± 0.22*[b]
Hyperglycemic +vit (E+C)	0.13 ± 0.00*[a]	0.13 ± 0.00[a]	8.61 ± 2.46[abc]

Values expressed as Mean ± SEM. P <0.05 is considered significant. (*) as compared to normoglycemic control group, (a) as compared to hyperglycemic control group, (b) as compared to hyperglycemic vitamin C treated group, (c) as compared to hyperglycemic vitamin E treated group. ANOVA test, with post hoc LSD test.

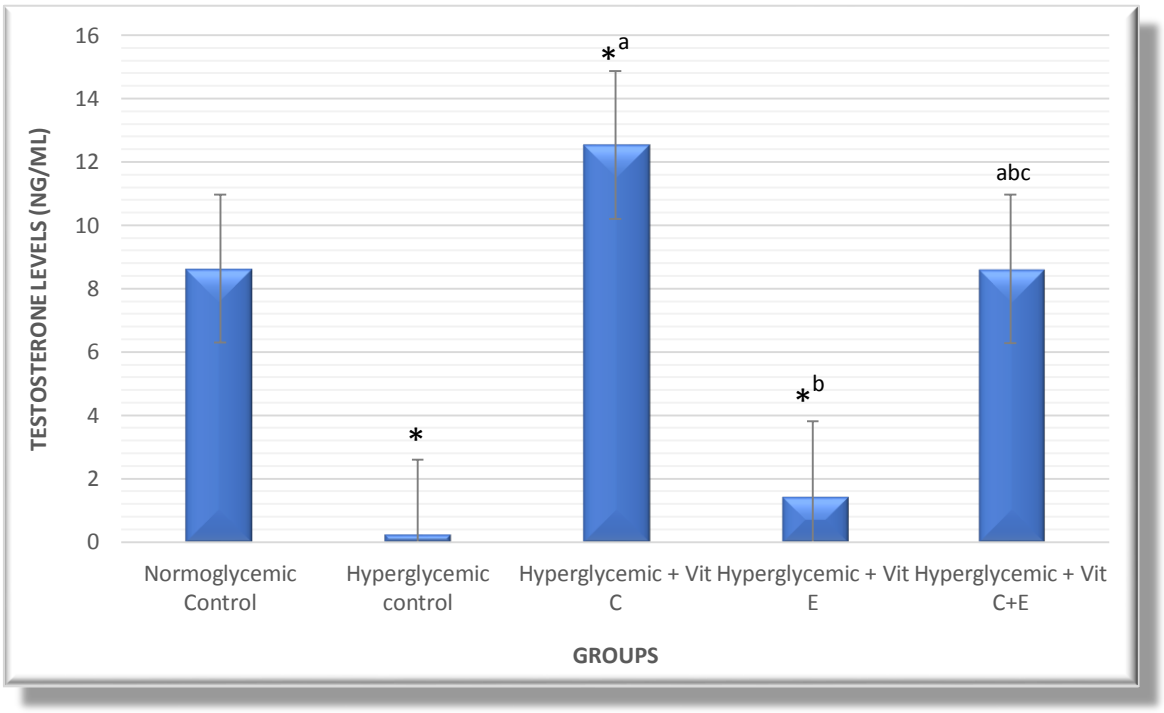

Figure 5.5: Effect of diabetes mellitus and vitamins supplementation on testosterone blood levels of adult male rats. Values expressed as Mean ± SEM. P <0.05 is considered significant. (*) as compared to normoglycemic control group, (a) as compared to hyperglycemic control group, (b) as compared to hyperglycemic vitamin C treated group, (c) as compared to hyperglycemic vitamin E treated group.. ANOVA test, with post hoc LSD test

It should be noticed that in STZ induced diabetes animals, the FSH levels were not affected. Nevertheless, there was a significant increase ($p<0.05$) in plasma FSH levels in the hyperglycemic vitamins treated animals (Figure 5.6).

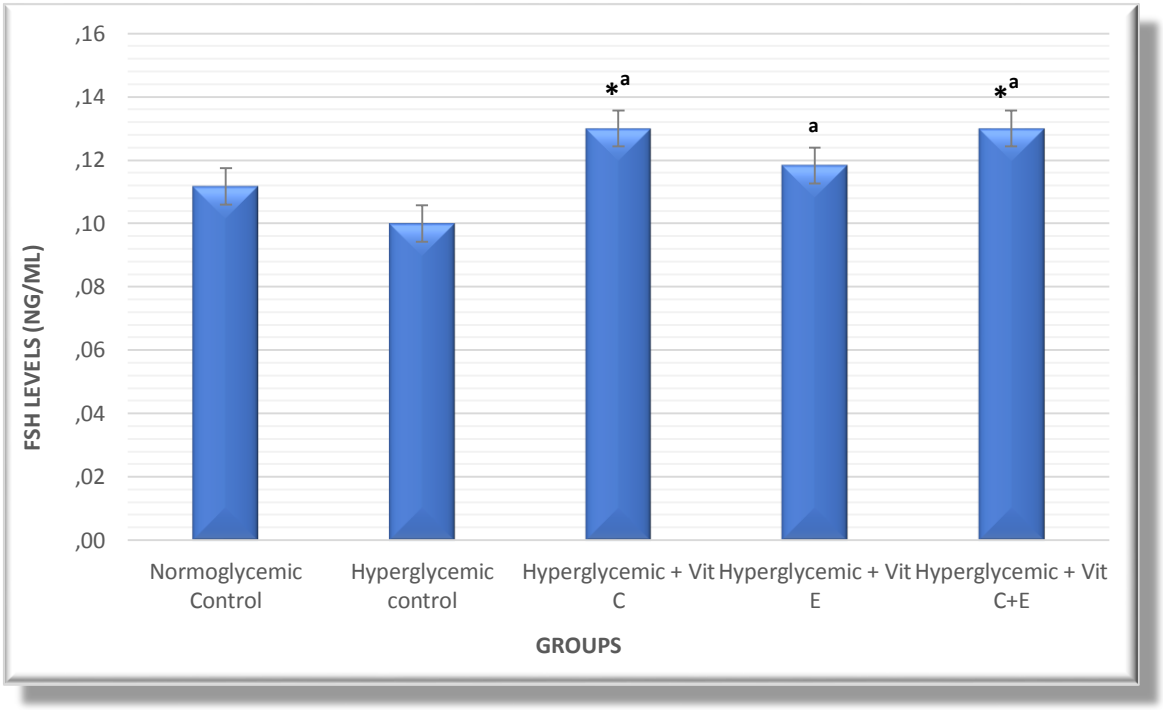

Figure 5.6: Effect of diabetes mellitus and vitamins supplementation on FSH blood levels of adult male rats. Values expressed as Mean ± SEM. $P<0.05$ is considered significant. (*) as compared to normoglycemic control group, (a) as compared to hyperglycemic control group. ANOVA test, with post hoc LSD test

LH, in hyperglycemic control group, decreased significantly ($p<0.05$), compared to the normoglycemic control group. Whereas, there was a significant increase in LH levels among the hyperglycemic vitamin C, and hyperglycemic vitamin C+E treated animals (Figure 5.7).

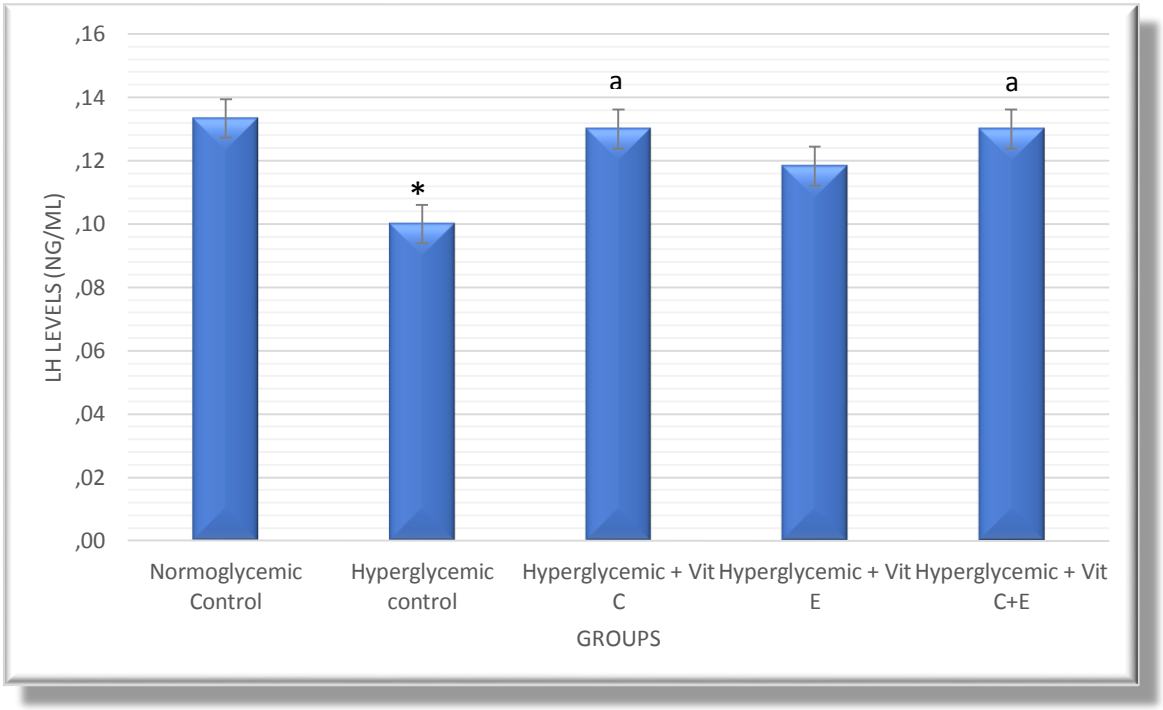

Figure 5.7: Effect of diabetes mellitus and vitamins supplementation on LH blood levels of adult male rats. Values expressed as Mean ± SEM. *P* <0.05 is considered significant. (*) as compared to normoglycemic control group, (a) as compared to hyperglycemic control group. ANOVA test, with post hoc LSD test

Androgen is the essential regulator of growth and function of the epididymis. Testicular testosterone is crucial for spermatogenesis and high testosterone level is essential for the normal physiology of seminiferous tubules. In our study, a significant decrease in testosterone, FSH, and LH levels were seen in the hyperglycemic control group compared with normal one. Our results corroborate with those of some authors, who found that the testosterone levels decreased in hyperglycemic animals **(Sanguinetti *et al.*, 1995, Mohammadi *et al.*, 2017)**. Other authors found a diminution in testosterone levels **(Scarano *et al.*, 2006; Navarro-Casado *et al.*, 2010; Zhao *et al.*, 2010)**, FSH, and LH levels **(Ballester *et al.*, 2004; Zhao *et al.*; 2010)**. **Aybek *et al.* (2008)** suggested that low levels of testosterone in diabetic was

correlated with an elevated testis MDA (Malondialdehyde). After antioxidant supplementation, the levels of testosterone increased to the control levels.

In their recent published article, **Aguirre-Arias *et al.* (2017)** have proposed that the reduction in testosterone levels is linked to alteration in spermatogenesis cycle. In diabetic rats, a decreased in 3ß-hydroxysteroïd dehydrogenase (HSD) and 17α (HSD) enzyme activities in Leydig cells are linked to the testosterone diminution, because of oxidative stress. However, a supplementation in vitamin C can correct this deficiency and increases hormones levels to those similar to control levels.

As mentioned previously, the increased level of FSH and LH, which was observed among vitamin C, E, and C+E treated animals, corroborates with **Fernandes *et al.*** findings. Those authors suggested that vitamin C is a vitaminergic transmitter that recovers LH levels by activating the releasing of LH and FSH hormones from the anterior pituitary gland **(Fernandes *et al.*, 2011)**.

5.4 Quantitative Assessment of Sperm

Compared to normoglycemic control group, the hyperglycemic one presented no significant difference ($p>0.05$) in epididymis sperm number as shown in table 5.3. However, the hyperglycemic vitamins supplemented groups presented a significant increase ($p<0.05$) in sperm count as compared to the normoglycemic and hyperglycemic control animals (Figure 5.8).

Table 5.5: Epididymal sperm count and testicular Johnsen score results of male Wistar rats after treatment compared to control groups

	Sperm count ($x10^6$)	**Johnsen score**
Normoglycemic control	3.37 ± 0.49	9,00±0,36*
Hyperglycemic control	2.72 ± 4.61	5,17±0.30
Hyperglycemic + vit C	9.53 ± 0.73*a	8,17±0.40*
Hyperglycemic + vit E	6.89 ± 0.53*ab	8,17±0.30*
Hyperglycemic +vit (E+C)	6.75 ± 0.68*ab	8,33±0,49*

Values expressed as Mean ± SEM. $P<0.05$ is considered significant. (*) as compared to normoglycemic control group, (a) as compared to hyperglycemic control group, (b) as compared to hyperglycemic vitamin C treated group. ANOVA test, with post hoc LSD test.

Our result differs from other studies, in which some authors reported that the sperm count diminished within epididymis and testis due to the damage of sperm themselves caused by the diabetes oxidative effects **(Hassan *et al.*, 1993, Scarano *et al.*, 2006)**.

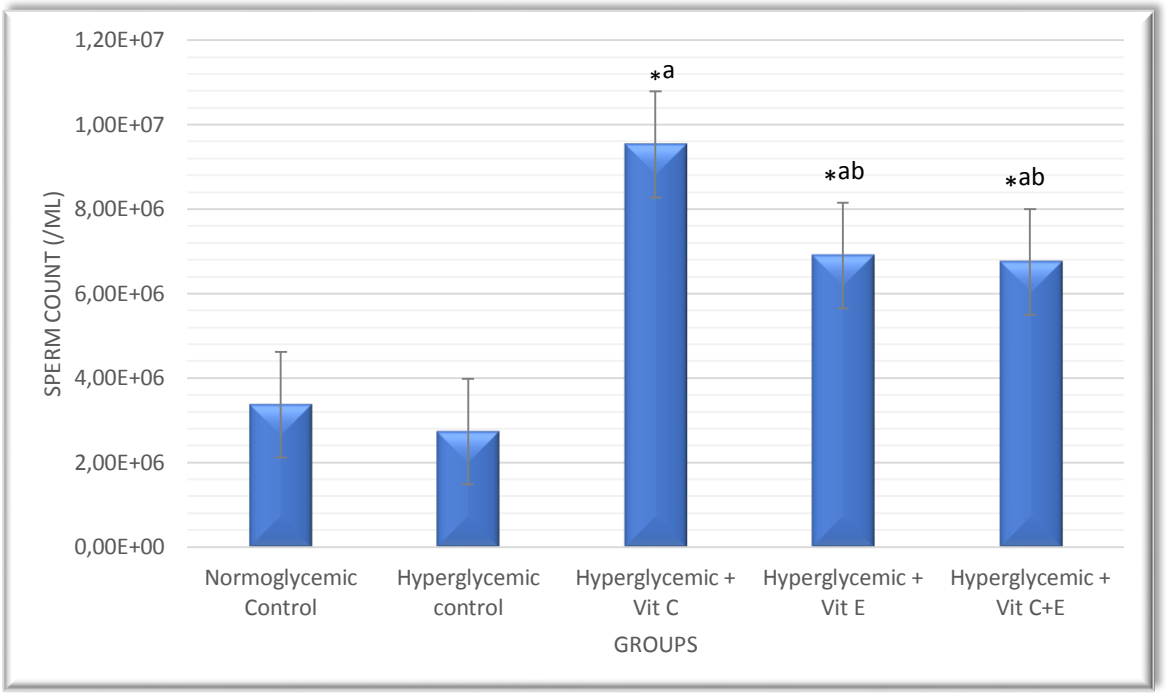

Figure 5.8: Effect of diabetes mellitus and vitamins supplementation on epididymal sperm number of adult male rats. Values expressed as Mean ± SEM. $P < 0.05$ is considered significant. (*) as compared to normoglycemic control group, (a) as compared to hyperglycemic control group, (b) as compared to hyperglycemic vitamin C treated group. ANOVA test, with post hoc LSD test

The epididymal sperm concentration, obtained in the vitamin C supplemented rats, was significantly higher than those of rats supplemented with vitamin E or a combination of both vitamins C and E. Vitamin C is an effective scavenger of reactive oxygen species within the aqueous environment of the cytosol and extracellular fluids. This vitamin constitutes the unique endogenous antioxidant that protects the lipids against oxidative damage, initiated by non-lipid peroxyl radicals generated in the aqueous phase **(Rebec, 2015)**. However, vitamin E representes another endogenous antioxidant, which does not possess this effect. Apparently, all peroxyl radicals, generated in the aqueous phase, are captured by vitamin C before diffusing into lipid phase. Thereby, vitamin C acts as the first line of antioxidant defense **(Ball, 2004)**. According to **Vijayprasad *et al.* (2014)**, the administration of a high dose of vitamin C showed significant improvement in sperm count. **Takhshid (2012)**, underlined that vitamins combination could provide more potent protection. He suggested that this could be attributed to generation of vitamin E by vitamin C.

5.5 Histopathological Investigation

The spermatogenic cells and sertoli cells, in the seminiferous tubules of the vitamin-supplemented groups and control rats, were structurally normal.

Histopathological examination of normoglycemic rats' testes showed normal histological structure of active mature functioning seminiferous tubules, associated with complete spermatogenic series as demonstrated on figure 5.9 (a).

The hyperglycemic control rats' testes revealed marked degeneration of most seminiferous tubules with absence of spermatogenic series in tubular lumen as shown on figure 5.9 (b). Testis histopathological sections showed shrunken and distorted seminiferous tubules containing only spermatogonia with enlarged lumen.

The reduction of seminiferous tubular diameter corroborates with other results, in which this impairment, in streptozotocin-induced hyperglycemia, was obtained after three and twenty days **(Shrilatha, 2007, Guneli *et al.*, 2008)**, or six weeks **(Altay *et al.*, 2003, Navarro-Casado *et al.*, 2010),** and six months **(Cai *et al.*, 2000)**. In contrast, Ballester and colleagues reported similarity in the seminiferous tubular diameters in hyperglycemic and normoglycemic rats, after three months of streptozotocin-induced hyperglycemia **(Ballester *et al.*, 2004)**.

Several cellular and molecular mechanisms are proposed for cell apoptosis in diabetic subjects. It is well known that diabetes mellitus induces oxidative stress and LPO that damages the biological membrane in the testes. This in turn may cause spermatogenic and leydig cells degeneration, which disrupts spermatogenesis and reduces sperm counts. Previous studies showed that diabetic rats present severe damages in seminiferous tubules together with increased apoptosis **(El-Missiry, 1999; Koh, 2007; Kilarkaje *et al.* 2014; Xu *et al.* 2014)**.

Hyperglycemia leads to cell death, through increase oxidative stress, and due to release of intracellular $Ca^{2+,}$ activation of mitogenic agents, and impairment of protein kinases cell signaling pathways **(Shojaeii *et al.*, 2013)**. On the other hand, STZ has been shown to induce

cytotoxicity by increasing ROS/RNS (reactive oxygen species and reactive nitrogen species) production, oxidative stress, and mitochondrial dysfunction **(Raza and John, 2012)**.

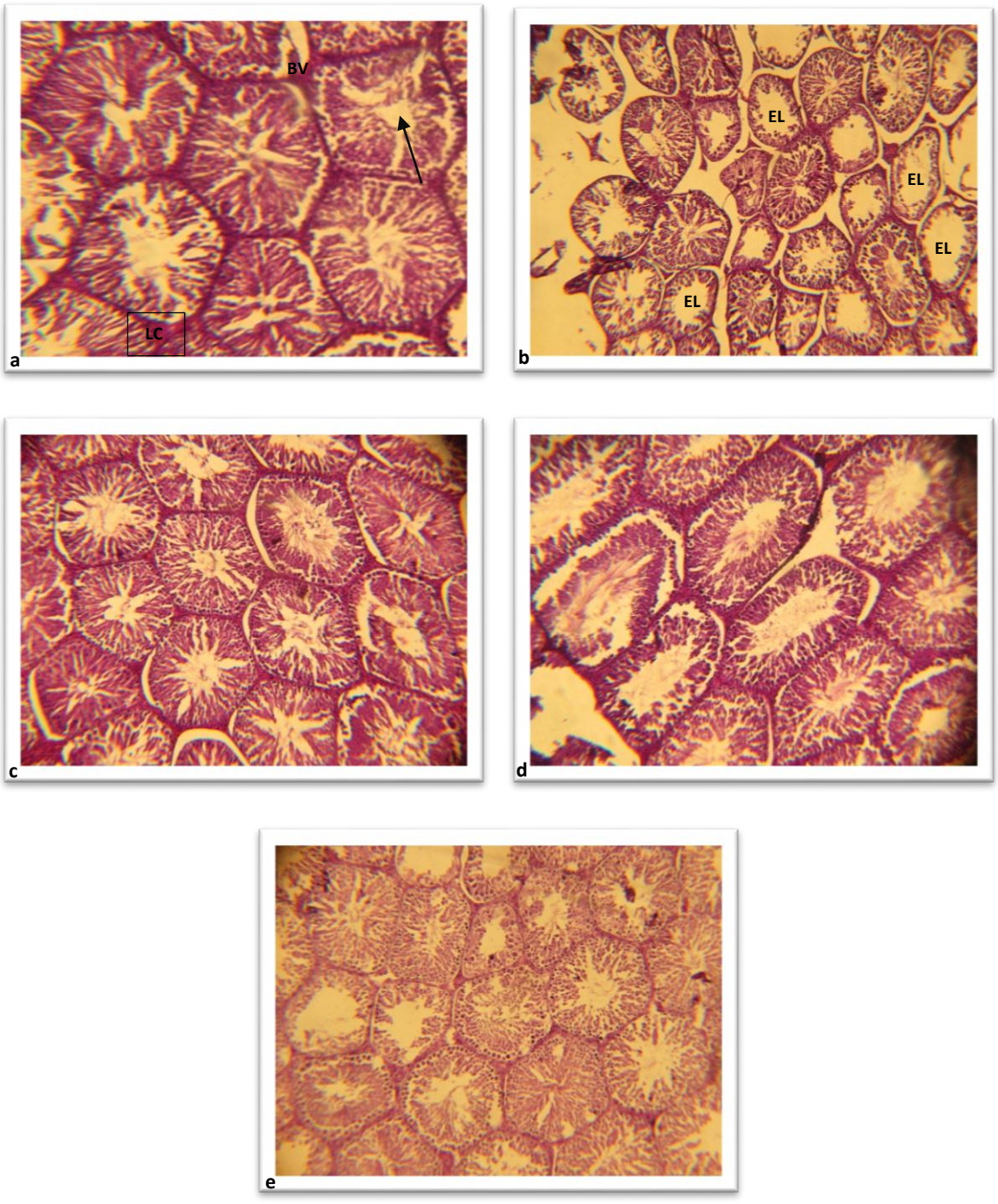

Figure 5.9: Paraffin section photograph 400 ×. (a)-Section of testis of control group showing normal seminiferous tubules with all the cells of spermatogenetic series. Blood vessels (BV) and Leydig cells (LC) also normal; (b)-Shows shrunken and distorted seminiferous tubules containing only spermatogonia with enlarged lumen (EL). (c, d, e)-Section of testis from the vitamin treated groups (C, E, C+E) respectively revealing normal characteristics

Microscopic examination of supplemented rats' testes revealed normal histological structure of most seminiferous tubules with normal spermatogenic series, as illustrated on figures 5.9 c, d, and e.

In both normoglycemic and hyperglycemic vitamin supplemented groups, Leydig cells were found in the interstitial connective tissue, between the seminiferous tubules and the tubules appeared to be uniform in size and shape. (Figure 5.9. (a, c, d, and e)).

Vitamin C is a known antioxidant compound present in the testis with the precise role of protecting the latter from the oxidative damage. Furthermore, vitamin C contributes to the support of spermatogenesis at least in part through its capacity to maintain this antioxidant effect in an active state. It has long been established as an agent to play a crucial role in the differentiation process of the spermatogonial cells to sperm **(Vijayprasad *et al.*, 2014)**.

Previous studies showed that vitamins C and E supplementation reduces oxidative stress in diabetic animals. Vitamins C and E deficiency enhances the diabetogenicity of STZ in rat testes, whereas vitamins C and E supplementation has an opposite effect **(Naziroglu, 2003)**.

Vitamin E constitutes an important antioxidant, especially in maintaining the integrity of the cell membrane **(Traber and Atkinson, 2007)**. Vitamin E is a non-enzymatic chain-breaking antioxidant with a particular function of scavenging peroxyl radicals to prevent lipid peroxidation.

Jedlinska-Krakowska *et al.* (2006) indicated that vitamin E exhibits a protective action on the rats testis exposed to ozone through stabilization of Sertoli cell membrane. The same authors found that vitamin C does not exert the same effect as vitamin E.

Nagda and Bhatt (2011) reported that the antioxidants' combinations showed more profound effect as compared to their individual administration.

Aybek *et al.* (2008), in a similar study on the impact of vitamin E on oxidative stress in young and old rats that had diabetes by STZ induction, reported that no histopathological difference was found in any group. A result that is contrary to ours.

5.6 Histopathological Assessement of Spermatogenetic Activity (Johnsen Score)

In our study, histomorphologic changes, observed in diabetic rat testes, included degenerated cells with reduced sertoli cells numbers, and reduced spermatogenesis.

The mean Johnsen score value was 5.17 in the diabetic rats and it significantly improved in the Diabetic + vitamins group (Table 5.5, Figure 5.10). This result explores the protective effect of Vitamins C and E on spermatogenesis. **Ballester *et al.*, (2004)** suggested that the low level of testosterone in diabetic rats might be related to the decrease in Leydig cells or in androgen biosynthesis.

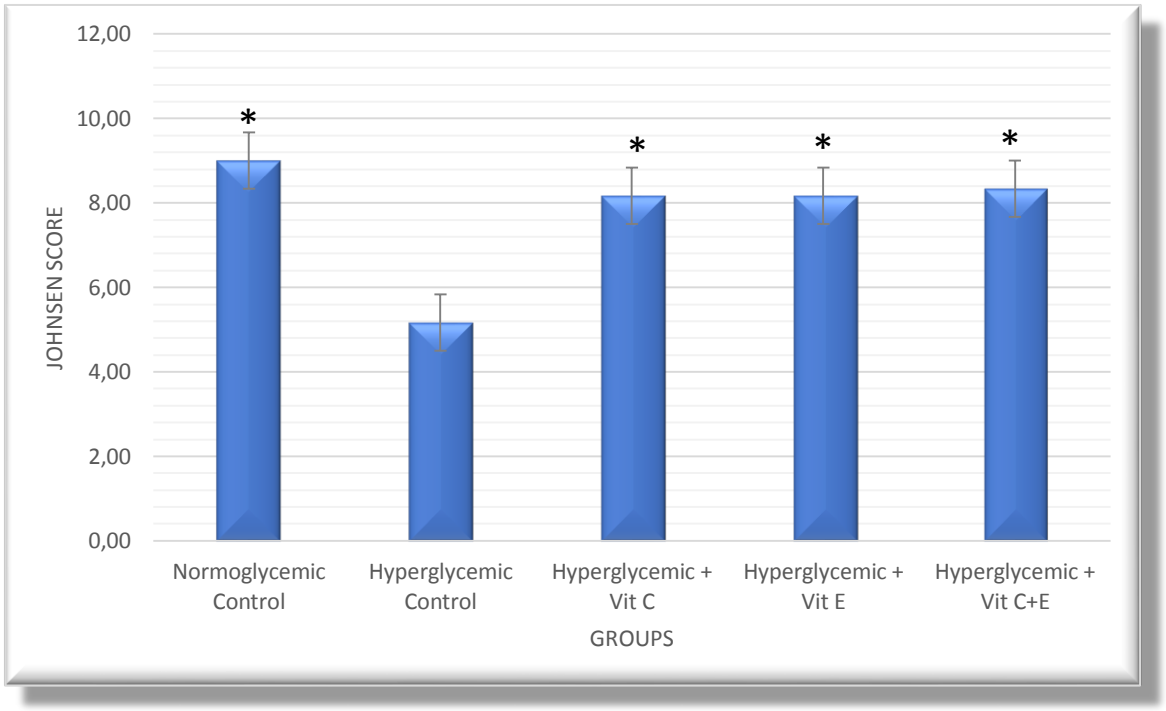

Figure 5.10: Effect of diabetes mellitus and vitamins supplementation on the Johnsen score. Values expressed as Mean ± SEM. * P<0.05 considered significant compared to control group. ANOVA test, with post hoc LSD test

In accordance with our results, **Koksal *et al.*, (2003)** showed that high levels of LPO were associated with severe pathologic changes in the testicular tissue. Their findings suggested that overproduction of reactive oxygen species (ROS) may play a role in the mechanism of testicular degeneration, associated with infertility. **Dkhil *et al.* (2016)** found that diabetes induction in rats resulted in reduction of Sertoli and spermatogenic cells and was associated with degeneration in the spermatogenic cell series and thickened interstitial vessel walls. Diabetes has also been reported to reduce the number of spermatogenic cells and the diameter of the seminiferous tubules by **Guneli *et al.* (2008)**, and to induce cell apoptosis and atrophy in the seminiferous tubules, which are both indicators of spermatogenesis failure **(Cai *et al.*, 2000; Dkhil *et al.*, 2016)**.

In the present study, administration of vitamins C and E was able to protect the diabetic rat testes against oxidative stress in term of spermatogenesis. Histopathological examination of the diabetic rat testes showed that vitamins supplementation (C and E) significantly protected the testes structure as evidenced by a significantly higher Johnsen score, compared to the diabetic rats not supplmented with vitamins. **Kaplanoglu *et al.* (2013)** demonstrated that vitamin E- administered diabetic rats had no significant changes of the johnsen score. On the other hand, **Aybek *et al.*, (2008),** found that the Johnsen testicular biopsy score was not significantly different in the hyperglycemic group supplemented with vitamin C when compared to the non-supplemented one.

Chapter 6

Conclusion

The aim of the current study was to assess the efficacy of supplementation with vitamin C and E, either combined or separated, on the infertility of male diabetes patients.

The diabetic animal model was successfully created, after a single intraperitoneal injection of STZ in adult male Wistar rats.

Referring to our results, diabetes mellitus induces adverse effects on body and reproductive weights, sperm count, hormone levels, and histopathological characteristics. Hyperglycemic rats developed a decrease in epididymal and testicular weight. A significant decrease in testosterone, FSH, and LH levels was observed, in the hyperglycemic control group, compared to the normal one. Non-significant decrease, in epididymal sperm count of STZ induced diabetic rats, was observed too.

Treatment with a combined or separated supplementation of vitamin E and C caused a significant increase in final body weight, hormonal levels, sperm count, testis, and epididymal weight. The final body weight of hyperglycemic treated groups significantly increased, as compared to hyperglycemic and normoglycemic control ones. Epididymal weight significantly increased in hyperglycemic animals, supplemented with vitamin C, and those supplemented with vitamin C+E, as compared to the hyperglycemic control group.

Diabetes mellitus appears to be one of the major causes of spermatogenesis dysfunction and is related to a decreased sperm count by disturbing hormonal mechanisms. The increased level of testosterone, FSH, and LH was observed among vitamin C, E, and C+E supplemented animals.

Administration of vitamins C, and E significantly improved sperm quality and increased hormonal levels to the control despite presence of diabetes. The epididymal sperm

concentration, obtained in the vitamin C supplemented rats, was significantly higher than that of rats treated with vitamin E or a combination of vitamin C+E.

Histopathological examination of the testes of normoglycemic rats, showed normal histological structure of active mature functioning seminiferous tubules associated with complete spermatogenic series. The testes of hyperglycemic control rats revealed marked degeneration of most seminiferous tubules with absence of spermatogenic series in tubular lumen. Microscopic examination of the rats' testes, supplemented with vitamins, revealed normal histological structure of most seminiferous tubules with normal spermatogenic series.

Histomorphologic changes, observed in diabetic rat testes, included degenerated cells with reduced spermatogenesis. The mean Johnsen score value was reduced in the hyperglycemic rats and it significantly improved in the hyperglycemic + vitamins groups.

Vitamin E reduces OS by scavenging free radicals. The ability of this vitamin to maintain a steady state rate of free radical reduction, in the plasma membrane, depends on its recycling by external reducing agents such as ascorbate or thiols. In this way, vitamin E is able to function again as a free radical chain breaking antioxidant, even though its concentration is low. Vitamin C co-administration has a protective effect on testicular oxidative stress and steroidogenic dysfunction in diabetic treated rats. We conclude that DM does not affect the effectiveness of the treatment with vitamin C and E.

Our results show that vitamins C and E possess a beneficial effect in preventing histological changes observed in rats' testes as well as their serum testosterone levels. Since those vitamins are easily available and cheap, they could be employed to reduce oxidative stress in the body produced by DM.

Further research is recommended to assess the concentration of reactive oxygen species (ROS) and total antioxidant capacity (TAC) in seminal plasma of diabetes patients treated with antioxidant vitamins to establish their effects on sperm quality.

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Appendices