The Republic of Iraq
Ministry of Higher Education and
Scientific Research
Mosul University / College of Science
Department of biology

Nitric Oxide Synthase Gene Changes in Youth Infertile Male in Mosul / Iraq

an M.Sc. Thesis

Presented by:

Zaid Hashim Ilias Sultan Aljammas

In Partial Fulfillment of the Requirements for the Degree of Master

In

Biology / Zoology

Supervised By

Lecturer

Dr. Mowafak Khalil Hasan Ali

1444 A.H. **2023 A.D.**

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Summary

Infertility refers to the inability to conceive within a year of marriage .. The causes that lead to male infertility have increased, including the increased levels of free radicals resulting from oxidative stress. Mitochondria also have an important role in the movement of sperms by providing the energy needed for the movement of the tail of the sperms.

Eighty samples of semen fluid were collected from private fertility clinics in Mosul by a sterile specimen cup after a recommended 2–3 days of ejuaculated.

The samples were split into two groups: Acording Oligospermia group (40 sample), and Healthy control group (40 sample). The results were listed as mean± SD . The results of this work showed that sperm count (10.54±6.47) and percentage of active sperm (18.36±16.1) was significantly lower than control group in infertile group ($p<0.000$). While the non-motile sperm (61.31±27.2) was significantly higher in infertile group in comparison to control group ($p<0.05$). There is significantly decrease in seminal plasma hormones parameters Testosterone & Estradiol between oligospermia and control group.

The mitochondrial function parameters showed significant elevation in seminal plasma lactate in infertile group (5.44±0.76 μM) in comparison to control group and plasma lactate/ pyruvate molar ratio that showed significant elevation in infertile group (22.99±2.6) in comparison to control group. According the Total antioxidant capacity, it was significantly elevated in seminal plasma of infertile male in comparison to control group ($p<0.001$) and Seminal NOS qPCR data showed that there was significant elevation in NOS expression in infertile group in comparison to control group ($p<0.0001$) and significant positive correlation between NOS expression and seminal total antioxidant capacity ($p<0.001$).

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List of Abbreviation

ATP	Adinosen triphosphate
Ca+2	calcium ion
cAMP	cyclic Adinosin monophosphate
eNOS	endothelial NOS
ETC	electron transport chain
FMN	flavin mononucleotide
FSH	folicle-stimulating hormone
FSHR	folicle-stimulating hormone
HH	hypogonadotropic hypogonadism
IMM	inner mitochondrial membrane
iNOS	inducible NOS
LDH	Lactate dehydrogenase
LH	luteinizing hormone
MMP	Mitochondrial membrane potential
MPC	mitochondrial pyruvate carrier
Mt-DNA	mitochondrial DNA
NADPH	nicotinamide adenine dinucleotide phosphate
nNOS	neuronal nitric oxide
NO	Nitric oxide
NOA	Non-obstructive azoospermia
NOA	Non-obstructive azoospermia
NOS	nitric oxide
OS	oxidative stress
OXPHOS	oxidative phosphorylation
PDH	Pyruvate Dehydrogenase
RNS	reactive nitrogen substance
ROS	Reactive oxygen substance
WHO	world health organization
Ct_o	Control threshold
Ct	Cycle threshold

Chapter One

INTRODUCTION

Chapter One

INTRODUCTION

Infertility is a global disease that affects roughly 15% of couples; it is characterized by the inability to conceive despite unprotected sexual activity (Deshpande & Gupta, 2019)(Mowafak,2015). In nearly half of infertile cases, a male factor deficiency is present (Babakhanzadeh *et al*., 2020).

2.0 ml or more of semen should be ejaculated during each coitus, with a typical sperm count of more than 20×10^6 sperm in each ml. Thus, despite just one sperm fertilizing the ovum, the ejaculation should include a large number of sperms (Sunder & Leslie, 2021).

When compared to other prevalent ailments, male infertility affects a substantial number of the population, making it a huge global concern. Worrying statistics indicate that it is becoming more common (Leslie *et al*., 2022). Trauma, iatrogenic injury, gonadal toxicity, anatomical or genetic defects, systemic or neurological illnesses, antibodies against sperm. In addition, 30-40% of male infertility is unidentified (idiopathic male infertility) (Andrade *et al*., 2021).

The mitochondria (typically 10-12 per gamete) in human spermatozoa are clustered and helically structured around the neck and amid piece region of the sperm all over the outer dense fibers and axoneme (Gu *et al*., 2019). Mammalian sperm's energy is generated by mitochondria in the sperm mid-piece. Sperms need a steady supply of adenosine triphosphate from mitochondrial oxidative phosphorylation to operate properly, especially when it comes to motility (Escada-Rebelo *et al*., 2022).

The mitochondria have their own genetic material. Mitochondrial DNA (mt-DNA) of human is a circular, dual-stranded molecule that is found in

mitochondrial matrix. Two rRNAs, 22 tRNA, in addition to a slew of particular polypeptides are encoded by mt-DNA, which are all involved in normal cell development (Deshpande & Gupta, 2019) (Escada-Rebelo *et al*., 2022).

The past 24 years, many of clinical and model species have demonstrated that mitochondrial dysfunction can cause male infertility. Mitochondrial DNA mutations are major cause of mitochondrial illness, significant phenotypic variables are maternal transmission, high copy number (polyploidy), and mitochondrial DNA. (Ryzhkova *et al*., 2018).

Recently, the mitochondria-only substance known as mitochondrial NOS (nitric oxide) is found. Additionally, theca, granulosa, and oocyte cytoplasm are reproductive tissues that include iNOS (inducible) and eNOS (endothelial). Reactive nitrogen and oxygen species contribute to the bulk of apoptotic cascades. As co-substrates, all NOS isoforms use arginine, molecular oxygen, and reduced phosphate nicotinamide-adenine-dinucleotide (NADPH) (Woo *et al*., 2019; Rostamzadeh *et al*., 2020). A functioning NOS moves one electron from NADPH to the heme in the amino-terminal oxygenase domain. Utilizing the electron on the hem site, L-arginine is converted into L-citrulline and NO as well as reduced and activated oxygen (O2) (Stuehr & Haque, 2019). The NOS enzyme must convert hydroxylated l-arginine to n-hydroxy-l-arginine before oxidizing l-citrulline and not to create n-hydroxy-l-arginine in order to make NO(Stuehr & Haque, 2019).

(Neuronal Nitric Oxide) nNOS and (endothilial nitric oxide synthase)eNOS need an increase in intracellular calcium ion (Ca^{+2}) to bind to calcium through calmodulin. As calmodulin affinity to NOS rises, electrons are transported from NADPH to the heme in the oxygenase domain of the reductase domain. low Ca^{+2} intracellular concentrations of about 40 nm extremely binds to Calmodulin due to the amino acids presence structure of the iNOS (Pourbagher-Shahri *et al*., 2021).

The aim of study

1. General seminal fluid analysis from male oligospermia infertility and control group.
2. Determination of Nitric Oxide synthase ad it's gene change.
3. Estimation of semen plasma hormones as Testosterone, Estradiol, seminal plasma Pyruvate and L-Lactate.
4. Mt-DNA extraction, purity and Quantitative PCR assay for copy No. Asthenospermia < 40% sperm motility Teratospermia Abnormal Morphology.

Chapter Two

LITERATURE REVIEW

CHAPTER TWO

Literature Review

2-1 Male Reproductive System

The process of human reproduction is crucial to the survival of the human species. The male genital system in humans consists of the testes as the main organ and the epididymis, vas efferentia urethers, and vas deferentia as some of the subordinate organs. Auxiliary glands include, for instance, the seminal vesicles, the prostate gland, and the cowpers gland (Talwar, 2016).

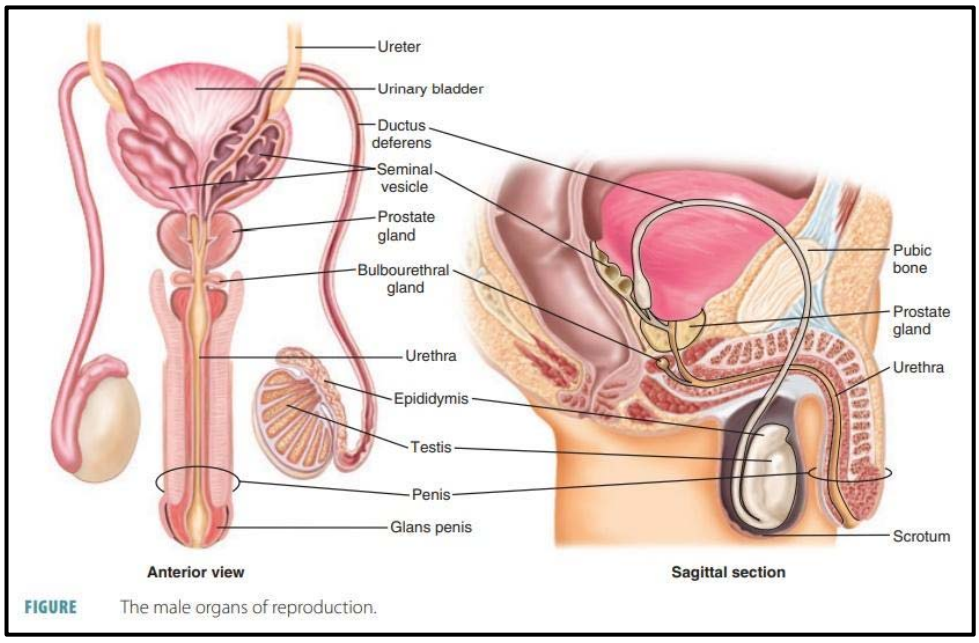


Figure (1): Male Reproductive System (Steger & Weidner, 2011)**.**

Male Reproductive System Consists of:

2-1-1 Testis

Two tiny, oval organs in the scrotum form the human testis. Each one measures 2.5 x 4 cm. There are around 370 seminiferous lobules in each testis, with a diameter of 180 micrometers (Suede *et al*., 2021). During the first stage of early embryonic development, the testes are located within the abdominal cavity; during the second stage, which starts at 17 weeks of pregnancy and lasts

until 28 weeks, they move to the scrotum. Hormons control the testicular migration, especially androgen and gonadotropin(Kuiri-Hänninen *et al*., 2019).

These lobules are located in the fibrous septa that divide the tunica albuginea from the mediastinum testis. Connective tissue encloses lymphatics, Leydig cells, blood vessels, and nerves. The testicular parenchyma is encased in a capsule made up of blood vessels, pressure-sensitive nerve fibers, and smooth muscle fibers. The Tunica albuginea called also capsule consist of houses fibroblasts, smooth muscle cells and collagen fibers (Suede *et al*., 2022).

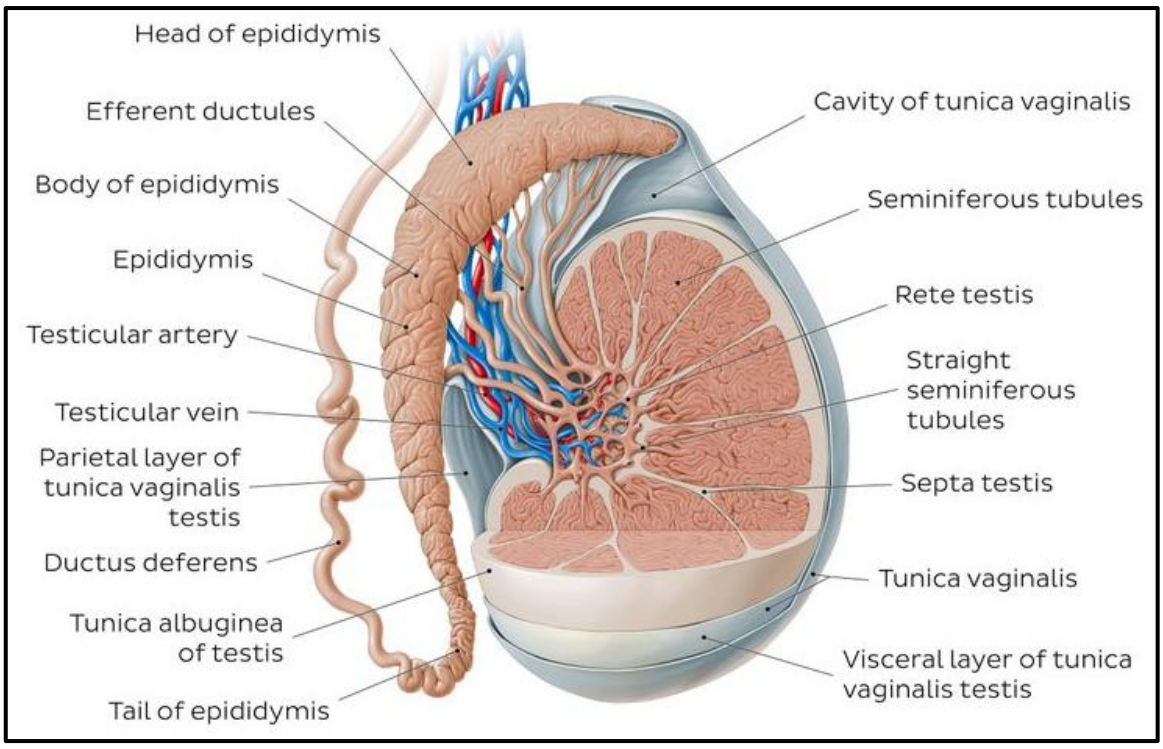


Figure (2): Human testis (Paul Kim *et al*., 2022)

2-1-2 Leydig Cells

These granulated cytoplasm cells are grouped together in the interstitium of the testis within connective tissue. The pituitary releases luteinizing hormone (LH), stimulates the Leydig cells to create testosterone, and is subsequently stored in the interstitium seminiferous tubules, making Leydig cells the principal site of androgen synthesis and secretion (Aladamat & Tadi, 2021).

2-1-3 Sertoli cells

The primary somatic component of the seminiferous tubules is made up of sertoli cells. They possess a multilobed nucleus, endoplasmic reticulum-rich cytoplasm, glycoproteins, and cytoplasmic droplets, the number of which is negatively connected with the expansion of spermatogenic cells (Staub & Johnson, 2018). These cells include spermatogonia, main and secondary spermatocytes, and spermatids at different embryonic stages. These cells govern spermatogenesis, release spermatids, and perform spermiation, among other vital functions (Maynard & Downes, 2019). By triggering trophic effects in gonadal Sertoli cells through this receptor (FSHR), which is present on Sertoli cells, folicle-stimulating hormone (FSH), and enhances spermatogenesis (Wang *et al*., 2022).

2-1-4 Epididymis

It is a long, tortuous tube-like structure connected to the dorsal surface of each testis and acts as a conduit between the efferent and vas deferential ducts. (Maynard & Downes, 2019). Androgens control the endocytic, metabolic, also secretory activity of epididymal cells. Since principal cells play a major role in material absorption and secretion inside the epididymal lumen, they exhibit significant endocytic activity and secretory as well as sperm storage and maturation(James *et al*., 2020).

2-1-5 Seminal Vesicles

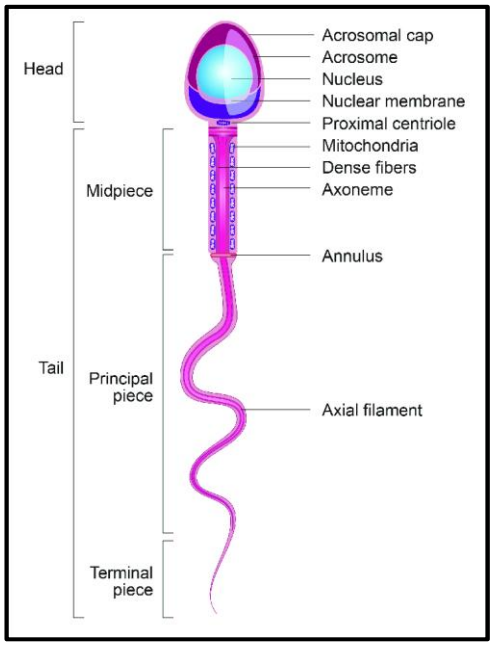
Are the site where two seminal fluid and nutritional ingredients of semen are produced (Lee *et al*., 2020). Seminal vesicles can be detected in early phase pregnancy. Human seminal vesicles have pseudostratified epithelium that is divided into two cell groups: main cells and basal cells. The main cells account as the bulk of cell population. At birth, these two cell groups begin to differentiate (Gonzales, 1989).

Seminal vesicles are coiling, lobulated tubes covered with secretory epithelium and produce a variety of proteins, enzymes, mucus, vitamins, amino acids, ions, minerals, fibrinogen, and hormones. Additionally, the seminal vesicles contain a high quantity of fructose, which is the primary source of energy for sperm in the ejaculate (Toragall *et al*., 2019). Other carbohydrates such as inositol, glucose, ribose, and sorbitol were also detected in seminal vesicle gland discharge, but in smaller amounts. All accessory sex glands contain the lipid hormone prostaglandin, and the seminal vesicles glands having the greatest concentration (Sadler-Riggleman & Skinner, 2018).

Sperms are suspended in seminal plasma, a liquid media, called the semen. Seminal plasma is released both before and after ejaculation by the accessory glands and the epididymis. The complex fluid known as seminal plasma is responsible for carrying ejaculated sperms from the testis to the intended egg cell. As the sperms travel through the female reproductive canal, it does not only transport them but also nourishes and protects them (Talluri *et al*., 2017).

Sperms are much smaller than the majority of other body cells, and their volume is much smaller than female germ cells. Each day sperms are synthesized in millions of numbers, although the majority of women only ovulate one egg every month. Like the majority of other cells in the body, the structure of sperms indicates how they work. In sperms, there are definite head, mid, and tail region. The sperm head contains a haploid cell with very small amount of cytoplasm. These features lead to tinier sperm in general (Talluri *et al*.,2017)

The bulk of the sperm cell's head is covered by the acrosome, which serves as a "cap" loaded with lysosomal enzymes and is crucial for sperm preparation and fertilization. Mitochondria are very concentrated in the center of sperm. The sperm cell as a whole will be propelled by the flagella, which extends from the neck and center of the sperm to the tail. In mature sperm cells, centrioles fabricate the axon, the main chain of the flagellum (Rothmann & Bort, 2018).



Figure(3): The sperm (Alves *et al*., 2020)**.**

Sperms lose the majority of their cytoplasm when developing, suggesting that mitochondrial preservation serves an important biological function. Given the significance of the mitochondria for reproduction, it has been hypothesized that sperm physiological processes, particularly motility, may be impacted by genetic abnormalities in the mitochondria. Even though mitochondrial DNA has been broadly researched in recent decades, the effects of mutations or polymorphisms in this gene on male infertility have only been the subject of a small number of investigations (Hammadeh *et al*., 2022). While sperm may use glycolytic energy for metabolic activity, oxidative metabolism is necessary for their ideal physiology and function (Rajender *et al*., 2010).

Male gametes must go through capacitation after ejaculation in order to fertilize the egg cells. According to (Tourmente *et al*., 2022), the two major metabolic processes that produce ATP, the principal energy source for sperm, are glycolysis and mitochondrial oxidative phosphorylation (OXPHOS).

Condition that effects on normal sperm shape or motility may reduce male fertility. For instance, anabolic steroids might cause abnormal sperm count and form in semen. The impact of smoking, excessive alcohol use, obesity, environmental factors, and lifestyle choices on human fertility was discussed by Punab and colleagues (Punab *et al*., 2017). Additionally, environmental toxins and poisons may cause gametes' direct toxicity. This affects both the amount and quality of them, leading to infertility (Punab *et al*., 2017; Tüttelmann *et al*., 2018).

2-2 Infertility

A one year of unprotected sexual activity without pregnancy can be defined as infertility or failure to conceive (WHO, 2021). Infertility affected around 15% wolrdwide (Jungwirth *et al*., 2017). The male element represents around 50% of cases, with sole culpability in 30% of those cases (Cannarella *et al*., 2021).

Those with secondary infertility have already had at least one pregnancy, while a primary infertility sufferer has never gotten pregnant. Recently, genetic causes have come into focus.

2-2-1 Causes of male Infertility

2-2-1-1 Anomaly of Anatomy

The lack of sperm production due to obstruction of the reproductive organs, the sperm canals (such as the seminal vesicles and ejaculation ducts). A wound or infection is typically the root of a blockage in the reproductive system. A hormone that regulates sperm production is testosterone. When pituitary gland, hypothalamus, produce abnormal hormones, a hormonal imbalance results. Cancers of the pituitary and testicles are two instances of conditions brought on by hormonal imbalance. The testicle's ability to produce sperm may be hindered

by a varicocele or medications (such as chemotherapy) that change the spermatogonia making cells (Cannarella *et al*., 2021).

2-2-1-2 Abnormality of sperm's quality and functions

Since the early 1930s, sperm analysis has been crucial for the assessment of infertility. Men's reproductive capacity may be severely impacted by conditions including varicocele, infections, and hormonal imbalances that affect the male genital system and can be evaluated using seminal fluid (Milachich & Dyulgerova-Nikolova, 2020).

Semen analysis is considered as corner stone in male infertility. Epididymal infection could be the cause of a significant reduction in sperm count, immotile and non-vital sperm. A high proportion of immotile sperm may result from structural problems with the flagellum, aberrant sperm morphology-related spermatogenesis diseases (Sunder and Leslie, 2021).

Semen analysis examines a variety of variables that are assumed to be crucial clinical information on the activities of the spermatozoa, sperm quality, and spermatogenesis. The initial step toward assessing the effect of genital pathophysiology on a man's reproductive capacity, even though the parameter may not be of clinical consequence, is to do a routine sperm analysis (WHO, 2021).

The number of sperms in a healthy man's sperm per millimeter varies from 15 million to 200 million. Oligospermia is defined as a decrease in number smaller than 15 million/ml that lowers a man's chances of induce pregnant without making him infertile, which impairs sperm production and health, may result from varicocele, or a vein swelling emptying the testis (Ling *et al*., 2020). Oligozoospermia is characterized by poor semen quality and low fertilization potential, as well as faulty sperm morphology and reduced sperm motility (WHO, 2021).

Azoospermia are divided into obstructive and nonobstructive types. Because it influences the affected person's control and treatment outcomes, this difference is clinically significant. Non-Obstructive Azoospermia (NOA) is a testicular condition that is intrinsic and brought on by a number of circumstances that, over time, significantly reduce sperm production. Severe spermatogenic insufficiency is typically caused by Hypogonadotropic Hypogonadism (HH), a disorder of the hypothalamus-pituitary-gonadal axis, or spermatogenic failure STF, which mostly affects spermatogenic cells in the first state (Andrade-Rocha, 2017).

One of the causes of infertility is a lack of mitochondria in the sperm. Sperm mitochondria are involved in critical and mobile processes, activation, acrosome response, and ova fertilization (Moraes & Meyers, 2018). Sperm motility declines are the second-most common cause of male infertility after sperm quantity declines (Piomboni *et al*., 2012).

2-3 Nitric oxide (NO)

Nitric oxide (NO), which has a short half-life few seconds, has a variety of biochemical and physiological potentials. This molecule, which was first identified in 1978, was proposed as the 1992 Molecule of the Year (Ricciardolo *et al*., 2020). It is a cellular messenger that is both internal and intracellular and is crucial for preserving the body's equilibrium (Dutta & Sengupta, 2022a). In most cases, NO fulfills its purpose by synthesizing cyclic guanosine monophosphate. The NO synthase mediates the production of the NO from L-arginine (NOS). There are three different isoforms of this enzyme: nervous, endothelial, and induction (Ricciardolo *et al*., 2020). The NO cascade activates a variety of pathways in the various tissues. For example, in endothelium as a vasodilator factor and a recognized relaxing factor in the cardiovascular system (Dutta & Sengupta, 2022a).

The neurological system consider NO as neurotransmitter. However; in certain instances, it also affects synaptic transmission, blood flow, platelet aggregation, neutrophil-induced cell toxicity, and long-term memory loss (Picón-Pagès *et al*., 2019).

In addition to the aforementioned roles, NO also affects sperm motility and capacity, ovulation, and menstruation(Dutta & Sengupta, 2022b). NO is a crucial paracrine messenger that plays a role in a number of physiological and pathological (Mohammed & Zuckerbraun, 2022).

2-3-1 Nitric Physiological Sources of Nitric Oxide

Mammals may synthesized NOs using three different NOS isoforms: (eNOS) endothelial NOS, (nNOS) neural NOS, and (iNOS) inducible NOS. Recently, the mitochondria-only substance known as mitochondrial NOS was found (Gantner *et al*., 2020). Additionally, theca, granulosa, and oocyte cytoplasm are reproductive tissues that include iNOS and eNOS. Reactive nitrogen and oxygen species contribute to the bulk of apoptotic cascades (Evans *et al*., 2021).

2-3-2 Nitric Biosynthesis of Nitric Oxide

L-arginine, molecular oxygen, and reduced phosphate nicotinamide-adenine dinucleotide (NADPH) serve as co-substrates for all NOS isoforms. A functioning NOS moves one electron from NADPH to the heme in the amino-terminal oxygenase domain (Bahadoran *et al*., 2021). Utilizing the electron on the hem site, L-arginine is converted into L-citrulline and NO as well as reduced and activated oxygen (O_2) (Gantner *et al*., 2020). The NOS enzyme must convert hydroxylated l-arginine to n-hydroxy-l-arginine before oxidizing l-citrulline and NO to create n-hydroxy-l-arginine in order to make NO (Bahadoran *et al*., 2021).

nNOS and eNOS employed an increase in intracellular calcium ion (Ca^{+2}) to bind to calcium via calmodulin. When calmodulin affinity to NOS rises,

electrons are moved from NADPH to the heme in the oxygenase domain of the reductase domain. Due to the inclusion of several amino acids in the structure of the iNOS, calmodulin binds to very low Ca^{+2} intracellular concentrations of around 40 nM (Yang & Tsai, 2022).

At this time, the luteum, which points to a reduction in cell activity. increased the generation of reactive oxygen species (ROS), including O_2 and hydrogen peroxide, and is seen in healthy ovaries and correlates with the analysis of the corpus luteum (H_2O_2). The loss of gonadotropin receptors, a reduction in the generation of cyclic adenosine monophosphate (cAMP), and finally a reduction in the steroidization of the corpus luteum after it has been destroyed are all consequences of free radical formation in ovarian tissue (Eitner *et al*., 2021).

2-3-3 Nitric Oxide and the Male Testicular Functions

NO has been found and its mechanism of action has been partly described in the testicular vascular endothelium. Therefore, it can be shown that NO is efficient in gonadotropin activation, androgen displacement and its migratory route to the Leydig cells (Dutta & Sengupta, 2022b). NO has a role in the control of steroid production as well as the regulation of blood flow, myofibroblast contractile activity and cell permeability in the testes. It also regulates sperm motility, since NO promotes sperm motility in low concentrations and reduces sperm motility in moderate to high quantities. In human semen (seminal fluid), a significant correlation is seen between the content of NO and the proportion of sperm lacking motility (Staicu *et al*., 2021).

Under physiological circumstances, a little amount of NO is produced, allowing the free radicals to scavenge. In contrast, excessive NO production may result under sperm toxicity and reduce sperm motility via the generation of peroxynitrite in pathological circumstances such infection, varicocele, or diabetes mellitus (Staicu *et al*., 2021). It's likely that the sperms released into the

female reproductive system will cause an immune reaction that encourages iNOS activity and generates a lot of NO (Staicu *et al*., 2021).

2-4 Penile Erection

Humans' dorsal branches of the penis, deep arteries of the cavernous sinus, cavernous sinus nerves in penile tissue, and pelvic mesh all exhibit NOS activity (Picón-Pagès *et al*., 2019). The adventitia layer of the penile vasculature, which innervated the corpus cavernosum and connected to the glandular cavernous tissue, shows NOS activity, which implies that NO is a physiological enhancer of erectile performance in this network. In addition to being expressed throughout the nerves, eNOS is widely dispersed in the corpus cavernosum of the endothelium sinusoidum and the penile endothelium (Melis & Argiolas, 2021).

2-4-1 Fertility and Nitric Oxide

Nitric oxide is a crucial modulator of sperm activities, as was previously highlighted (Buzadzic *et al*., 2015). However, in addition to its beneficial role in mediating crucial sperm activities, when its level rises over the physiological level in response to certain infections. It acts as a proinflammatory mediator, which has a negative influence on sperm functions, including motility and viability (Dcunha *et al*., 2022; Pezo *et al*., 2021).

Male infertility is primarily caused by malfunctioning sperm, and since sperm membranes are so rich in polyunsaturated fatty acids, they are very vulnerable to lipid peroxidation. Since NO is a potent nitrogen-derived free radical, it may result in lipid peroxidation in the sperm membrane, compromising the chromatin integrity and degrading sperm activities. However, despite significant advancements in studies examining of ROS role in male infertility, the damaging effects of reactive nitrogen species on the male reproductive system are currently poorly understood (Dutta & Sengupta, 2022b).

Despite the physiological significance of NO being supported by data in facilitating regular male sexual activity, excessive NO generation is said to have negative effects on semen parameters (Mohammed & Zuckerbraun, 2022 ;Buzadzic *et al*., 2015). The number of sperms attached to the zona pellucida observed to be significantly lower in the presence of 10-4 mol sodium nitroprusside than in the control group that received no NO treatment. The same sodium nitroprusside concentration resulted in a substantial reduction in sperm viability (Khodaei *et al*., 2016).

Additionally, studies have shown that NO toxicity may change the normal morphology of sperms (WU *et al*., 2004). Furthermore, infertile men had seminal plasma NO concentrations (5.74 1.01 mol/L) that are more likely to cause capacitation inhibition than normal, fertile males (3.88 0.53 mol/L).In case of infertile males, decreased sperm metabolism was linked to elevate NO levels. In general, it is crucial that the body's natural defense adequately control NO levels to minimize negative effects of NO on sperm activities (Martins *et al*., 2019).

Oxidative stress (OS) and an excess of white blood cells in the seminal ejaculation are two symptoms of leukocytospermiam, which have been associated to NO toxicity. Leukocytospermia impairs sperm motility and causes spermatozoa to clump together, which may lead to infertility (Sharma *et al*., 2022). Additionally, it causes the production of cytotoxic cytokines, which might be a sign that an infectious sickness condition is present at the root(Sharma *et al*., 2022).

According to some theories, leukocytospermia is the primary cause of OS and the most visible process that results in an excessive generation of NO (Sharma *et al*., 2022). High NO levels have also been linked to varicocele, one of the most prominent causes of male infertility. varicocele causes the veins in the pampiniform plexus to enlarge and cross the cord, lead to obstruct blood flow there. There are several varicocele symptoms, such as testicular hypoxia

and germ cell failure brought on by a blocked tiny artery, a reduction in the generation of gonadotropin, an increase in scrotal temperature and testicular dysfunction, have been linked to NO, notably that generated by iNOS. Although there may be disturbances caused by varicocele, the precise mechanism by which NO contributes to its pathogenesis is yet unknown (Shiraishi & Naito, 2007 ; Ozbek *et al*., 2000).

NO also has an impact on the erectile dysfunction (ED) mechanism. ED is the inability to reach or maintain erections that are adequate for a satisfying sexual encounter. The soluble guanyl cyclase/cGMP/cGMP pathway, which is induced by NO, mediates erection. Several proteins are phosphorylated during this process, which causes smooth muscle to relax and blood to fill the sinusoidal gaps of the penis (Melis & Argiolas, 2021). However, since NO competes with oxyhemoglobin or superoxide anion to produce harmful peroxynitrite in ED, there may not be enough NO to activate this pathway (Radi, 2018).

2-5 Mitochondria

Circular intracellular structures that might be mitochondria were first described in the 1840s. Altmann, however, was the first who identify the pervasiveness of those forms in 1890 and referred them as "biological mother cells." He determined they are "basic animals," existing in the cells and carrying out essential functions. The name "mitochondria" was subsequently created by Benda in 1898. Greek language for "line" and "particle," respectively, refer to the process through which these structures are created during spermatogenesis (Mendes *et al*., 2022).

As seen in the diagram, mitochondria constitute the cell's "power house" (Fig. 4). Without them, cells wouldn't be able to obtain enough energy from nutrition and their many tasks would cease. Every area of a cell's cytoplasm may

include mitochondria, albeit the total number of them varies from a few hundred to several thousand, depending on how much energy the cell needs. The outer mitochondria membrane (OMM) and the inner mitochondria membrane (IMM), two membranes found in mitochondria, differ in size and shape (Barbagallo *et al*., 2020).

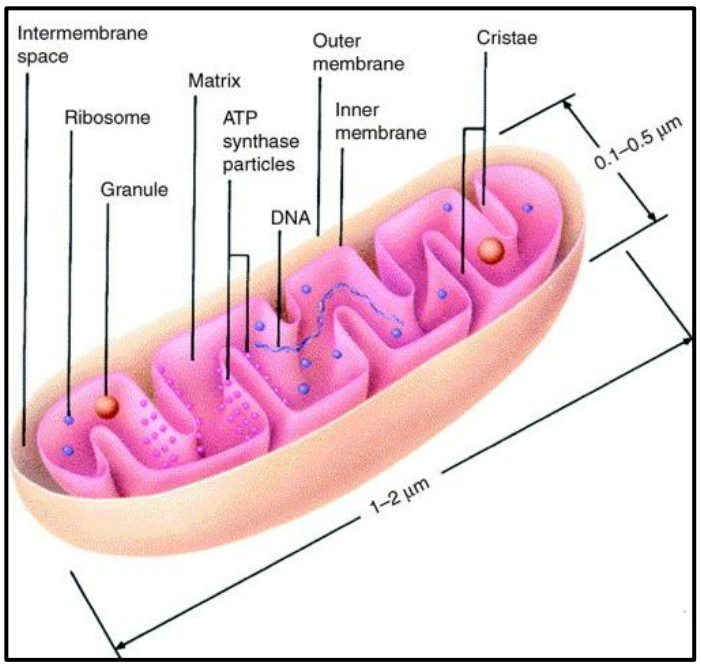


Figure (4) Mitochondrial structure (Freya & Mannellab, 2000)

The inner mitochondrial membrane is where the respiratory chain and ATP synthase are located (Ahmad *et al*., 2021). Both the mitochondrial DNA and the primary nuclear DNA include genes that code for the roughly 1500 structural and functional proteins that make up mitochondrial DNA. Mt-DNA is 16.5 kb circular DNA molecule which has 37 gene. IMM controls ion and metabolite transport as well as the oxidation state of cells through transporters in addition to producing ATP via oxidative phosphorylation (OXPHOS) (Basu *et al*., 2020).

Mitochondrial Membrane Potential (MMP) defined as the proton gradient created by the movement of electrons through the Electron Transport Chain (ETC), it is utilized by ATP synthase enzyme to make ATP (Neupane *et al*., 2019). Four multi-subunit complexes (complexes I, II, III, and IV) and two

electron shuttle molecules (cytochrome and ubiquinone) work together in the mitochondrial respiratory chain to transfer reducing equivalents from electron sources to oxygen molecules (Fig. 5) (Genova and Lenaz, 2014).

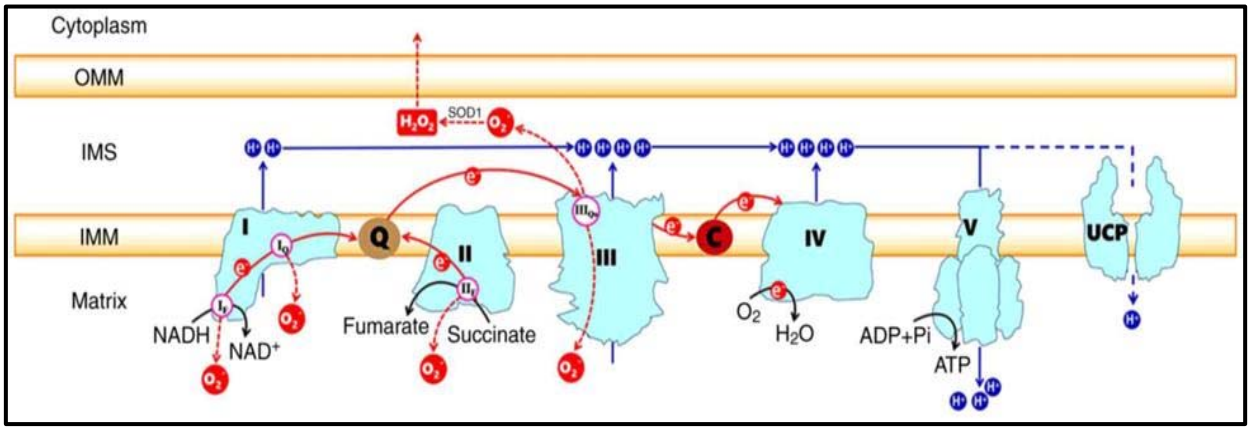


Figure (5): The Four Complexes of Mitochondrial Respiratory Chain (uncoupling protein)UCP, (ubiquinone) Q, (cytochrome c) C (superoxide dismutase) SOD (Zhao *et al*., 2019)

Proton motility in mitochondria produced by nearly all respiratory complexes actively pump protons into the membrane space from the matrix. Numerous experimental findings support the idea which include that In the inner mitochondrial membrane, these complexes join to produce supramolecular or supercomplexes. (Wittig *et al*.,2006; Genova *et al*.,2008). Complex I, also known as NADH dehydrogenase, is the most important and initial link in the mitochondrial respiratory chain (Koopman *etal*., 2010). The intracellular oxidation of NADH is catalyzed by complex I., which is regarded as the primary source of free radical production by electron leakage (Piomboni *et al*., 2012).

2-5-1 Sperm Mitochondrial DNA (mtDNA)

Mt-DNA genes produce 24 ribonucleic acid (RNA) molecules and 13 mitochondrial proteins.Mt-DNA was initially found in chick embryos in the 1960s using electron microscopy (Davison *et al*.,2019; Popova *et al*., 2022).

Mitochondrial DNA copy number is crucial for controlling spermatogenesis. For proper sperm activity, spermatozoa must have a certain number of copies of the mt-DNA. It has been proposed that sperm function is dependent on a certain mt-DNA threshold (Wu *et al*., 2019).

Although sperms get their energy from glycolysis, oxidative cell metabolism is also essential for sperm to operate normally physiologically. Energy production (through ATP) and the generation of free radicals (ROS) are the two most significant biological processes that take place in sperm mitochondria (Chianese & Pierantoni, 2021).

The process of glycolysis involves continuously delivering carbon atoms to the mitochondria, which are subsequently fully oxidized. Only two molecules of ATP are produced when glucose is transformed by glycolysis into two molecules of pyruvate. On the other hand, unless it is used in anabolic pathways, pyruvate will continue to deteriorate. However, since they are terminal cells, these paths are seldom seen in sperm. In the majority of studies, the assumption that sperm has a broad spectrum of metabolism has emerged as a common and reasonable one (Reynolds *et al*., 2017).

The mitochondrial Pyruvate Carrier (MPC) is responsible for delivering pyruvate, a byproduct of cytosolic glycolysis, to the mitochondria(MPC) (Zangari *et al*., 2020). Acetyl-CoA, the main fuel for the citric acid cycle, is produced when Pyruvate Dehydrogenase (PDH) decarboxylates pyruvate in the matrix. On the other hand, pyruvate metabolism and lactic acid metabolism have a close relationship. Pyruvate is reduced by enzymes to lactic acid, a process that mostly takes place under anaerobic conditions. The cytosolic NAD^+ needed for glycolysis is regenerated during this process. Sperm may transport lactate produced in the cytoplasm to mitochondria for further metabolism (Naifeh *et al*., 2022).

The predominant (if not the only) source of ATP in human sperm, which powers energy-demanding movement and ability tasks, seems to be glycolysis. Hereng observed that exogenous pyruvate, which indirectly transports NAD^+ via the conversion of pyruvate to lactate, which is mediated by LDH, is necessary for effective glycolysis (Tourmente *et al*., 2022).

2-6 Oxidative phosphorylation

On the other hand, oxidative phosphorylation is a highly coordinated process that includes the respiratory chain and ATP synthase, two mitochondrial inner membrane components (Du Plessis *et al*., 2015). One of the seven subunits of respiratory complex 1, which is involved in the initial stage of the mitochondrial energy production pathway for the electron transport chain of OXPHOS, is encoded by the mt-ND1 gene (Marra *et al*., 2021).

The NADH molecule's electrons are transferred into the ubiquinone molecule. The energy for the production of ATP is then provided by the transfer of the electrons from ubiquinone to a number of different enzyme complexes (Deshpande and Mohiuddin, 2021). On the other side, the increase in mitochondrial oxidative metabolism has an impact on sperm volume. In addition to varying across mammalian species, glycolysis, or mitochondrial OXPHOS, may also depend on the amount of oxygen and substrate that are present in the same species as humans.To put it another way, sperm adjusts glycolytic flow or mitochondrial respiration depending on the energy requirement in response to changes in metabolic status (Du Plessis *et al*.,2015).

Different substrates may effectively increase sperm motility, mitochondria can work at different degrees. The glycolytic pathway, which contains glycerol, lactate, pyruvate, and acetic acid, is used by sperms to metabolize a variety of monosaccharides, including glucose, fructose, and mannose. Numerous diverse

substrates are capable of supporting glycolysis. For every mole of glucose that is converted into glyceraldehyde 3-phosphate, 2 moles of ATP are needed. Pyruvate is synthesized from glyceraldehyde 3-phosphate, This causes 1 mole of glucose to be oxidized in order to create 2 moles of ATP. The TCA cycle start with pyruvate, which generates the molecules FADH2 and NADH. In the electron transport chain ATP molecules areproduced by oxidizing NADH and FADH2 through oxidative phosphorylation (Deshpande & Mohiuddin, 2021). Considering that sperms are terminal cells whose primary job is to give the egg paternal DNA during fertilization, it is not unexpected that they have a variety of metabolic mechanisms to preserve mobility. These strategies rely on the substrates that are available (Qi *et al*., 2022).

2-6-1 Oxidative stress

The term "oxidative stress" (OS) refers to an imbalance between the cell's production of oxidants and its defense mechanisms, or antioxidant activities. OS have a crucial role in male infertility. Reactive oxygen substance (ROS), a subclass of free oxygen radicals that manifest themselves inside of cells, including reactive nitrogen substance (RNS) and (ROS). This study will concentrate on the role of free nitrogen radicals in male infertility. Nitric oxide, peroxynitrite anion, nitroxyl ion, and compounds containing nitrosyl are a few examples of RNS (Pérez-Torres *et al*., 2020). RNS is required for many physiological functions, nonetheless, in excess, which result in nitrosative stress. It might be damaging to the male reproductive system. Poor sperm function and a poor condition of fertilization are both strongly influenced by nitrosative stress. Smooth muscle cells, mesangial cells, platelets, and hepatocytes are a few of the sources in the human body that create RNS (Fatima, 2018). Several parts of the male reproductive system, including the accessory glands, seminal ejaculate, testes, epididymis, ducts and penis, are especially rich in RNS. These sources may be categorized depending on their structure and type of cell.

2-6-2 Nitric Oxide Production and Reactive Nitrogen Species Formation

Nitric oxide (NO) is created by the enzyme Nitric Oxide Synthase (NOS) from L-arginine. To create NO and a byproduct called L-citrulline, it requires oxygen as well as a number of cofactors, such as Nicotinamide adenine Dinucleotide Phosphate (NADPH), Flavin MonoNucleotide (FMN), Flavin adenine dinucleotide (FAD), calmodulin, and calcium (Juan *et al*., 2022). There are three types of NOS which are neuronal (nNOS), inducible (iNOS), and endothelial (eNOS). Tetrahydro-biopterin, which is necessary for efficient NO production, is present in the reductase domain of every isoform(Poulos & Li, 2017). Additionally, a testis-specific subtype of NOS has only recently been shown to play a significant role in NO generation (Vermeulen *et al*., 2019). This subtype is exclusively found in the testis' Leydig cells, which suggests that it is involved in steroidogenesis. In the seminiferous epithelium, iNOS has been linked to the preservation of germ cell populations. According to studies, iNOS alone may partly trigger alpha-fodrin proteolysis, which heightens germ cell necrosis(Doshi *et al*., 2012).

Most cells don't naturally produce iNOS; instead, they only do so in response to inflammatory or immune stimuli, such peritoneal macrophages, as well as interleukin-1 and tumor necrosis factor-alpha, are macrophage products (Ross *et al*., 2021).

The testis contains eNOS, iNOS, and nNOS, which shows that NOS are crucial to spermatogenesis. In addition to NOS, the body generates NO via a variety of other chemicals and biological processes (Yu *et al*., 2017).

Numerous studies have linked the pentose phosphate pathway, which generates NADPH, and the rate-limiting enzyme mitochondrial glucose-6-phosphate dehydrogenase to the stimulation of L-arginine to L-citrulline conversion, which results in the synthesis of NO. Peroxynitrous acid (HONOO),

a breakdown product of peroxynitrite, may result in tyrosine nitrosation, which aids in signal transmission, and peroxidative damage, and is known to be harmful to reproductive organs, notably sperms (Cruz & Fardilha, 2016). Peroxynitrite and HONOO often interact with the spermatozoa's nucleic acids, proteins, and lipids either directly or indirectly via radical-mediated pathways to produce these detrimental effects (Serrano *et al*., 2020).

Chapter Three

MATERIALS AND METHODS

CHAPTER THREE

3 - Materials and Methods

3-1 Samples collection

Following (2-3) days of abstinence, samples of human seminal fluid were taken in accordance with WHO guidelines and placed in a sterile specimen cup.

Eighty seminal fluid samples were obtained from volunteers in Mosul, with the control group consisting of healthy males from August to May 2022.

The samples were split into two groups:

Group 2 includes 40 samples (from healthy volunteers as control)

Group 1 includes 40 samples oligospermia males

Samples from those who smoke, drink excessively, have obstructive azoospermia, and are chronic illness patient. Therefore, they were not included in the research.

Men were collected a sample of semens in a sterile cup in order to undertake general semen analysis. The typical method for doing this is by masturbation, although occasionally, with the use of a unique condom, A semen sample may be taken during sexual contact. For many years, laboratory workers have evaluated semens by the microscope and manually counting the sperms (WHO 2021).

Table (1) The Typical Range of General seminal fluid analysis (WHO, 2010).

Parameters	Normal range according to WHO
Liquefaction time (min.)	shorter than 60 min.
Volume (ml)	2 ml
% ofactive motility	50% Forward
Total number (million/ml)	20 million/ ml
% Typical morphology	More than 4%
% Sluggish & immotile	Below 60%
PH	7.2-7.8
WBC	Fewer than 1 Million

Table (2): The study's utilized kits under study

Kit	Origin
Testosterone kit	Tosoh / Japan
Estradiol	
L-Lactate	Cayman chemical /USA
Pyruvate	
DNA extraction	Addpreb genomic extraction Kit / Korea
PCR	Promega corporation /USA

Table (3): Represents The instruments used under study and their origin

Devices	Origin	Origin
CASA (computer-assissted-semen analysis)	Eclipse E200/ Japan	Stone m Staffordshire, ST15 0SA, UK
Tosoh hormones system	Japan	Multi plate Fluorescence analyzer
Pyruvate and L_lactate analyzer	Multi plate Fluorescence analyzer	IMPLEN GMBH /Germany
R.T PCR max	Stone m Staffordshire, ST15 0SA, UK	JRAD / Syria
Nanophotometer N50 touch	IMPLEN GMBH /Germany	Eclipse E200/ Japan
Incubatore	JRAD / Syria	Whirlpool/ USA
Water-Bathing Boiler HH-2 XMTD-204	Nuohai/ China	MPW /Poland
Vortex mixer	Gemmy industrial corp./Talwan	Gemmy industrial corp./Talwan
Centrifuge	MPW /Poland	Nuohai/ China
Refrigerator	Whirlpool/ USA	

3-2 General Seminal Fluid Examination:

Semen is generally a semisolid coagulated substance immediately after ejaculation into the collection vessel. At 37 ℃, when the semen typically starts to liquify (thin), lumps of varying sizes may be seen in the liquid. As the liquefaction process proceeds, the semens turn into homogeneous and watery, ultimately left only as tiny clots. At room temperature, the entire sample normally liquifies after a quarter of an hour, though it rarely takes an hour or longer. At room temperature, the sample was liquify in 60 minutes, however it normally happens in less than 30 minutes (WHO 2010). At room temperature, a typical sperm sample will liquify in 60 minutes. However this frequently happens in shorter than 30 minutes (WHO 2021).

3-2-1 Fluid Volume

It's crucial to measure volumes accurately for any sperm evaluation since it allows the total quantity of sperm in ejaculation to be calculated. A graduated cylinder should be used to measure the volume of ejaculation. The typical volume should be 2 ml or more (WHO 2010).

3-2-2 examination by Microscope

As previously stated, the microscopic investigation was performed utilizing the CASA approach. The sperm motility and morphology are assessed during the microscopic inspection of the sample.

3-2-3 Motility

This test determines the progressive motile sperm swimming in a straight line or large circles, and then immobile and slow sperm counts were performed in the same area. Free-tailed sperm should not be counted (WHO 2021).

3-2-4 Morphology

The acrosome occupies at (40–70%) of the head's surface area in normal sperm, which also have an oval head and a pale front and a darker rear. The head aspect ratio should be between 1.50 and 1.75. Only one tail should be affixed to the underside of the head's symmetrically positioned socket. Teratozoospermia are defined as sperm samples that include fewer than 30% of normal total sperm (WHO 2021).

3-3 Hormonal Measurements in Semen Fluid

The testosterone and estradiol hormones were measured in the semen of both oligospermia and the control group by using the automated immunoassay analyzer technique from the Japanese company TOSOH Enzyme Linked Fluorescent Assay.

3-3-1 TOSOH Automated Immunoassay Analyzer (AIA-360)

It is a modern, sensitive, specialized, and high-accuracy immunoassay technique that relies on a fluorescent immune enzyme and the interaction between antibodies and antigens. In each session, 25 blood serum samples are measured in the twenty-five cups, all marked with a code, and each containing a ready-made lyophilized reagent. It takes about ten minutes. This technique consists of four main steps, which are adding a blood serum sample, then incubating, followed by washing, and finally adding the substrate. The device consists of the standard kit AIA-360 Standereds, the analyzer and the energy conditioner, in addition to the general reagents:

AIA-PACK Substrate II

AIA-PACK Wash Concentrate

AIA-PACK Dilute Concentrate

A - Preparation of reagents:

The basic material, which consists of:

- The AIA-PACK Substrate reagent II is 4- Methyl Umbelliferyl phosphate (4MUP) with a lyophilized preservative, Sodium azide.
- AIA-PACK Substrate reconstituent II buffer with Sodium azide liquid preservative

Reagent prepare:

All reagents were placed at a temperature of (18-25) °C before preparing the reaction solutions, then the contents of one vial of AIA-PACK Substrate

reconstituent II were added to the lyophilized AIA-PACK Substrate reagent II and were mixed well to dissolve all solid substances

B- Concentrated washing solution consisted of:

1- AIA-PACK Wash Concentrate
2- Buffer solution (basic phosphatase)

Reagent prepare:

The entire contents of the washing solution (25 ml) were added to 500 ml of distilled water and mixed together;

C- dilution solution consisted of:

1- AIA-PACK Dilute Concentrate, and

2- Buffer solution (alkaline phosphatase) with antiseptic

Reagent prepare:

Twenty ml of the diluted solution was added to 800 ml of distilled water and mixed well before used.

The prucedure

The sample cups of the automatic immunoassay analyzer containing the solid phase and covered with lyophilized antibodies were taken and marked with special symbols and mixed with a specific amount of blood serum containing the specific hormone. These antibodies were linked with the marked bovine alkaline phosphatase enzyme, then washed to remove non-conjucated antibodies. Associated with the enzyme and incubated with the base substance 4MUP, the device was turned on, the rate of fluorescence intensity resulting from the reaction was read, and the fluorescence substance was 4-Methyumbelliferone, and the concentration of the specific hormone was calculated according to a computer program for the automated immunoassay analyzer device.

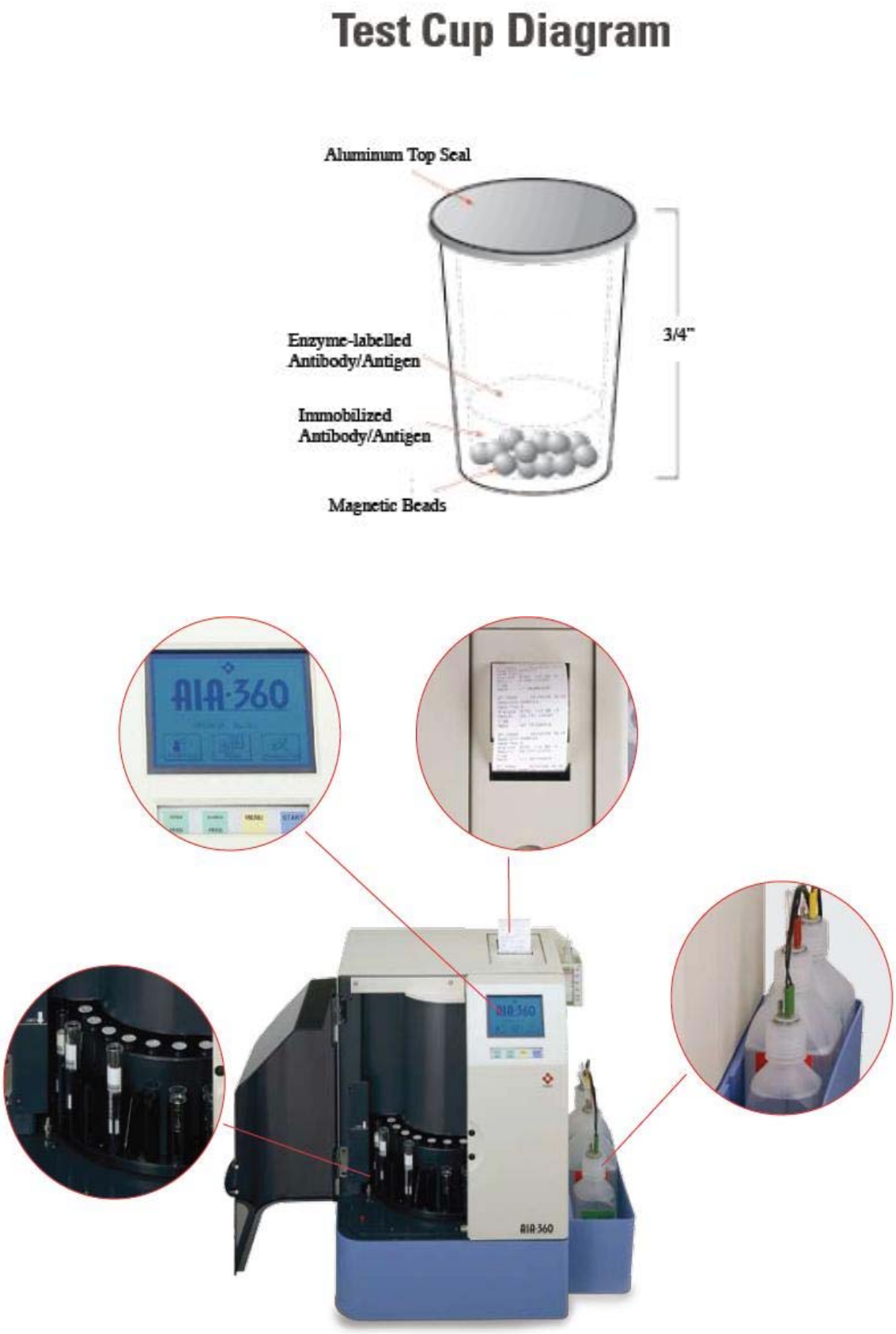


Figure (6): Automated immunoassay analyzer TOSOH

3-3-2 Testosterone concentration measurement

The concentration of seminal fluid testosterone in male was determined by (AIA-360) as a quantitative method and by using the prepared diagnostic kit from TOSOH company.

principle:

The principle of the method depends on the competition of the seminal fluid testosterone hormone present with the hormone associated with the enzyme marker on the antibodies released from the magnetic balls and then washing them to remove the residues of the hormone not bound to the enzyme and then incubating them with the base material (4MUP) where the amount of the hormone bound to the marker enzyme is inversely proportional to the testosterone concentration.

Procedure:

The sample cups of the automatic immunoassay analyzer device containing rat blood antibodies were taken and marked with special symbols and mixed with 45 microliters of seminal fluid and linked with bovine alkaline phosphatase enzyme with sodium azide as a preservative. The device was turned on. The resulting fluorescence rate was read Interaction and calculating testosterone hormone concentration was according to a computer program for the immunoassay analyzer device with unit nmol/L

3-3-3 Estradiol concentration measurement

The concentration of E2 hormone in seminal fluid was determined using (AIA-360) method as a quantitative method and by using the diagnostic kit prepared from the company TOSOH.

principle:

The principle of the method depends on the competition of the estradiol hormone with the enzyme marking this hormone for specific sites to bind to the

specialized anti-E2 antibodies to this hormone. The amount of the enzyme marking this hormone is inversely proportional to the concentration of the hormone E2 present in seminal fluid.

Procedure:

The sample cups of the automatic immunoassay analyzer device containing anti-E2 antibody in rabbit blood were taken and marked with special symbols and mixed with 50 microliters of E2 hormone in blood serum linked to the bovine alkaline phosphatase enzyme. E2 hormone according to a computer program for the **immunoassay analyzer device in** nmol/L

3-٤ Seminal Plasma Pyruvate

Pyruvate levels in seminal plasma were estimated by a specific kit from the Cayman Chemical Company/USA. It offers a method based on fluorescence for detecting pyruvate in plasma (Jankowska-Kulawy *et al*., 2010; Egras *et al*., 2011).

Test Principle

The balance between NAD^+ and NADH is shown by the lactate / pyruvate ratio, which also shows the redox status of the cell, that relies on lactate and pyruvate interconversion through lactate dehydrogenase.

In assay, pyruvate oxidase catalyzes conversion of pyruvate to acetyl phosphate, hydrogen peroxide (H_2O_2), and carbon dioxide. In the presence of horseradish peroxidase, H_2O_2 combines stoichiometrically with 10-acetyle-3,7, -dihydroxiphenoxazine (ADHP) to generate the extremely fluorescent compound resorufin.

Resorufin fluorescence is measured at excitation wavelengths of 530-540 nm and emission wavelengths of 585-595 nm.

$$\text{Pyruvate} + O_2 + H_2O \text{ phosphate } \xrightarrow{Pyruvate\ oxidase} \text{acetyle phosphate} + CO_2 + H_2O_2$$

$$H_2O_2 + \text{ADHP} \xrightarrow{Horseradish\ peroxidase} \text{Resorufin } (EX_{530}/EX_{590})$$

Plate set up:

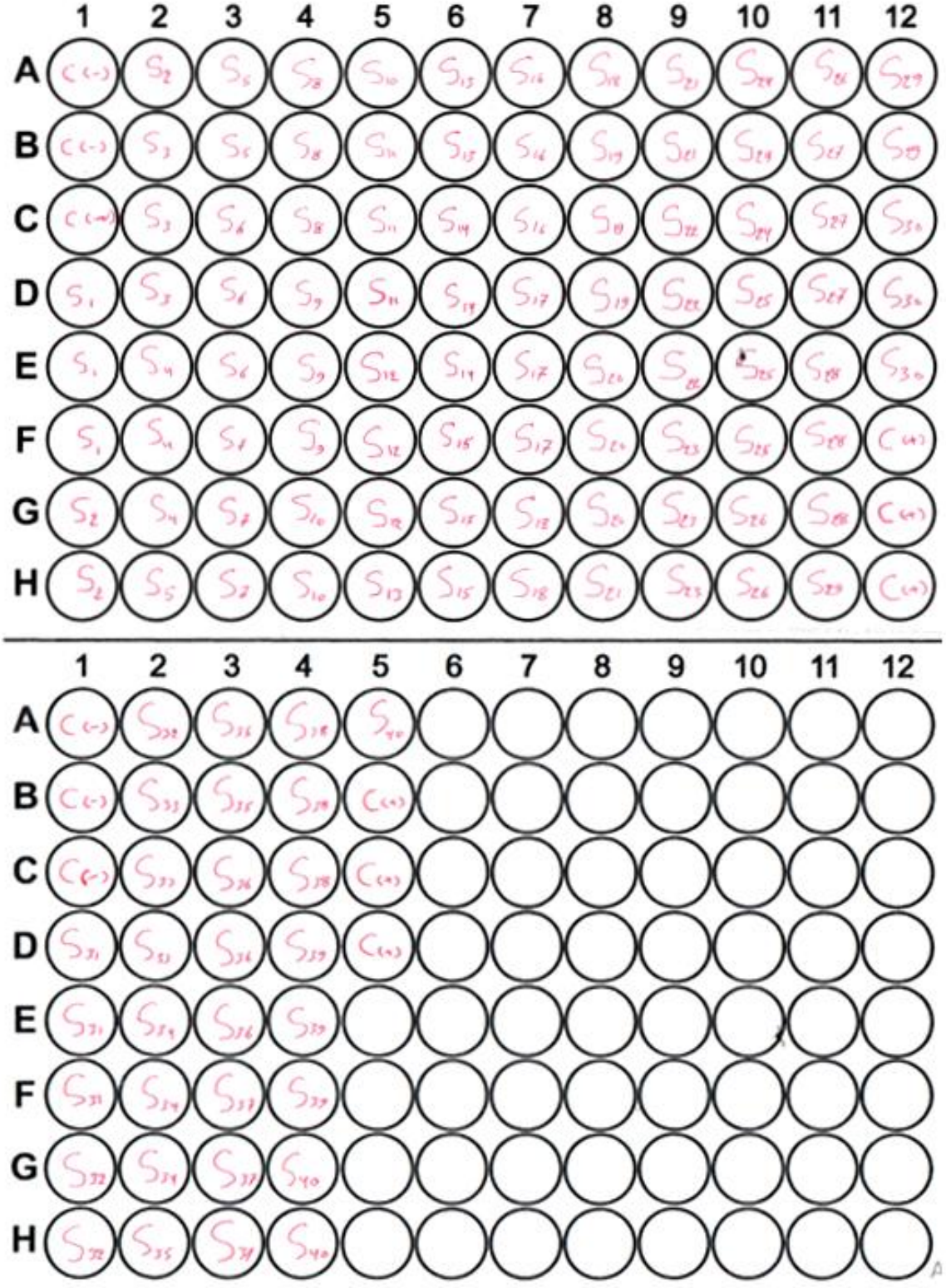


Figure (7): Pyruvate estimation kit Triplicate 96- well. (C-) represents negative control, (C+) represents positive control, (S) represents oligospermia samples.

Triplate's pyruvate standard curve has to be measured using the samples. A minimum of three tests have been performed on each sample

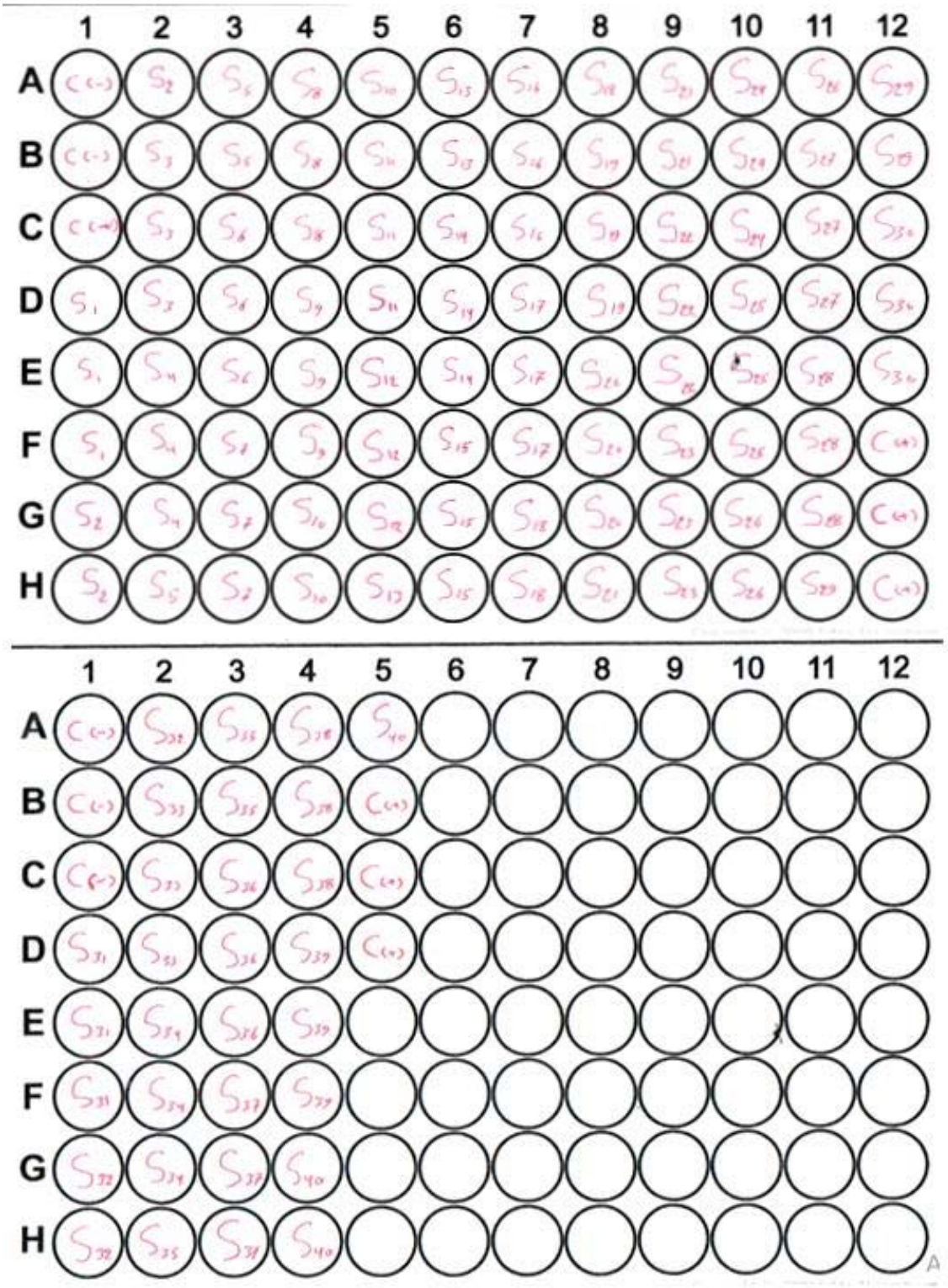


Figure (8): Pyruvate estimation kit Triplicate 96- well. (C-) represents negative control, (C+) represents positive control, (S) represents control samples.

Procedure

1. Standard well: fill the indicated wells on the plate with 50µl detection buffer, 50 µl cofactor mixture, 10µl fluorescence detector, and 20µl standards (tubes A to H).
2. Sample Wells to at least two wells, add 50µl Detection Buffer, 50µl Cofactor Mix, 10µl Fluorescence Detector, and 20µl Sample.
3. The plate should be incubated for 20 minutes at room temperature before reading with an excitation wavelength of 530-540 nm and an emission wavelength of 585-595 nm.

Calculations

The pyruvate concentration in the samples was calculated using the following equation:

Pyruvate (µM) =[CF-(y-intercept) / slope[×2*×sample dilution

* This is the dilution factor used to compensate for the deproteination of the samples with 0.5 M MPA.

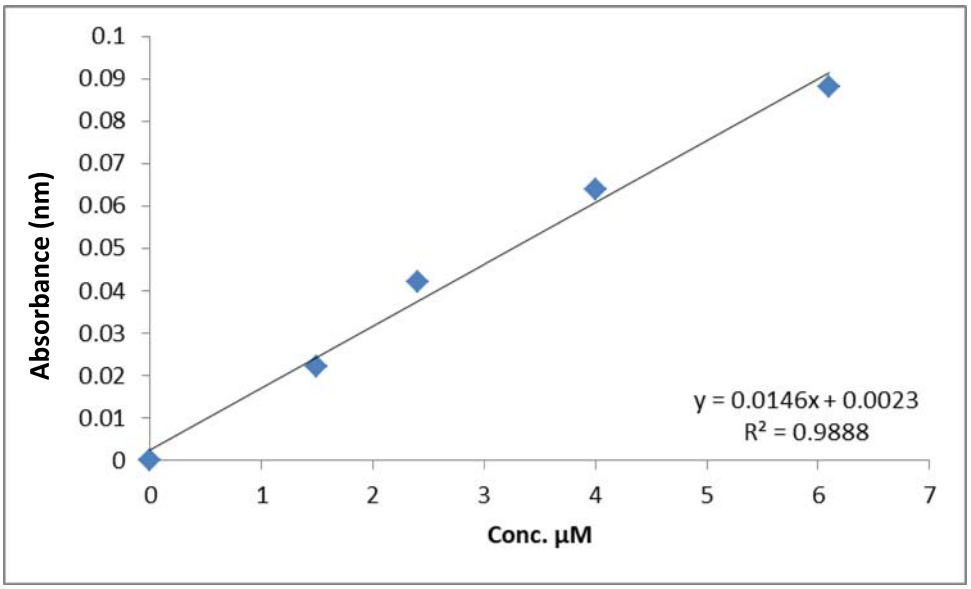


Figure (9): Standard Curve of Pyruvate

3-5 Seminal plasma L-lactate measurement

Lactate level in sperm plasma was determined using a kit of Cayman Chemical firm, which offers a fluorescence-based approach for detecting L-lactate in plasma (Du *et al*., 2010; Robergs *et al*., 2004).

The principle:

The lactate to pyruvate ratio represents the redox state of the cell and depicts the balance between NAD^+ and NADH, which is reliant on lactate and pyruvate interconversion via lactate dehydrogenase.

In the test, lactate dehydrogenase catalyzes the reduction of NAD^+ to NADH as well as the conversion of lactate to pyruvate. Excitation and emission wavelength measurements reveal that NADH reacts with the fluorescent substrate to create a highly fluorescent product with 530–540 nm excitation and 585–595 nm emission wavelengths.

Plate design

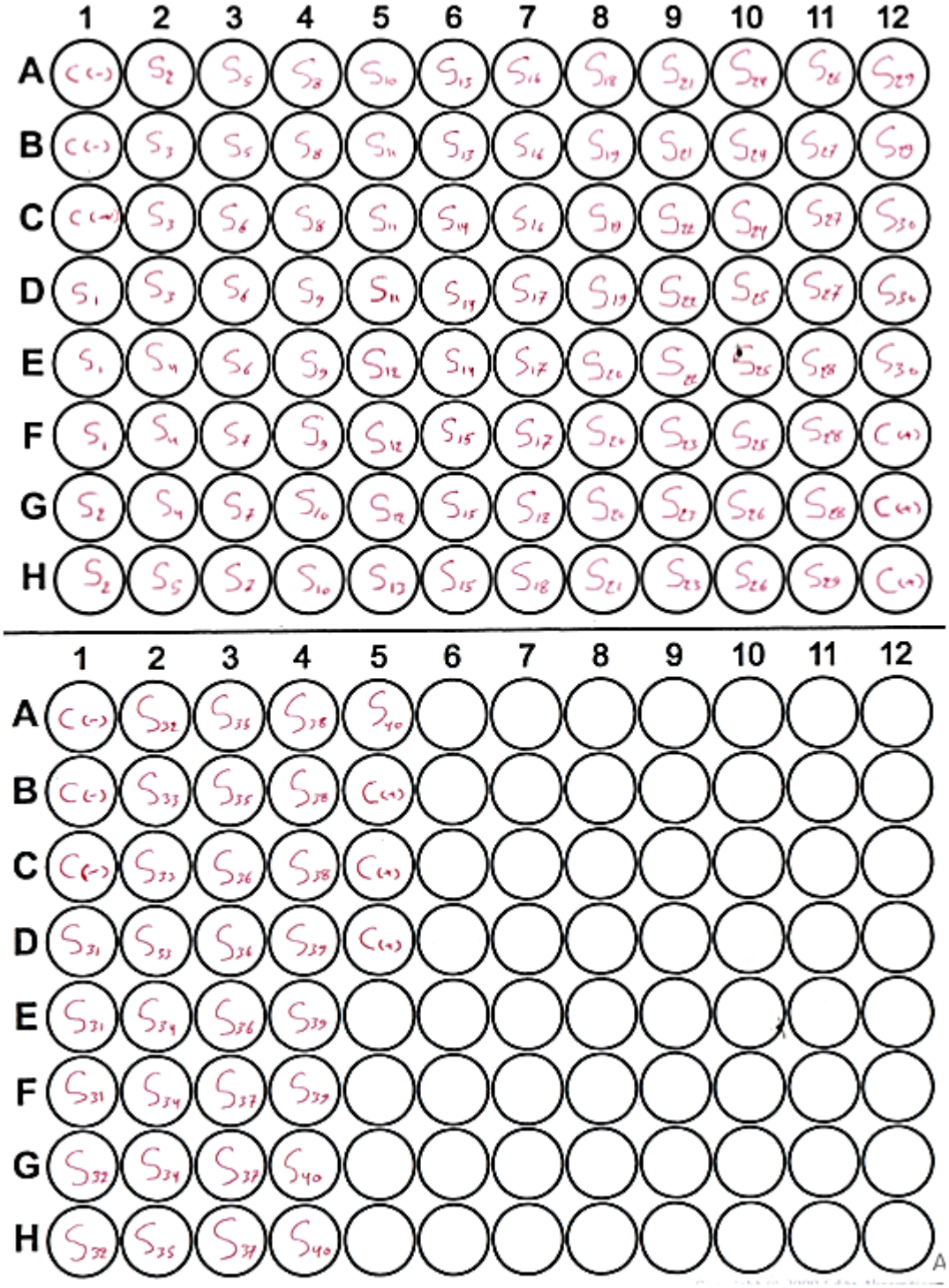


Figure (10): Triplicate 96- well of L-lactate estimation kit. (C-) represents negative control, (C+) represents positive control, (S) oligospermia samples

Each sample must be tested using the L-lactate standard curve in triplicate at least three times.

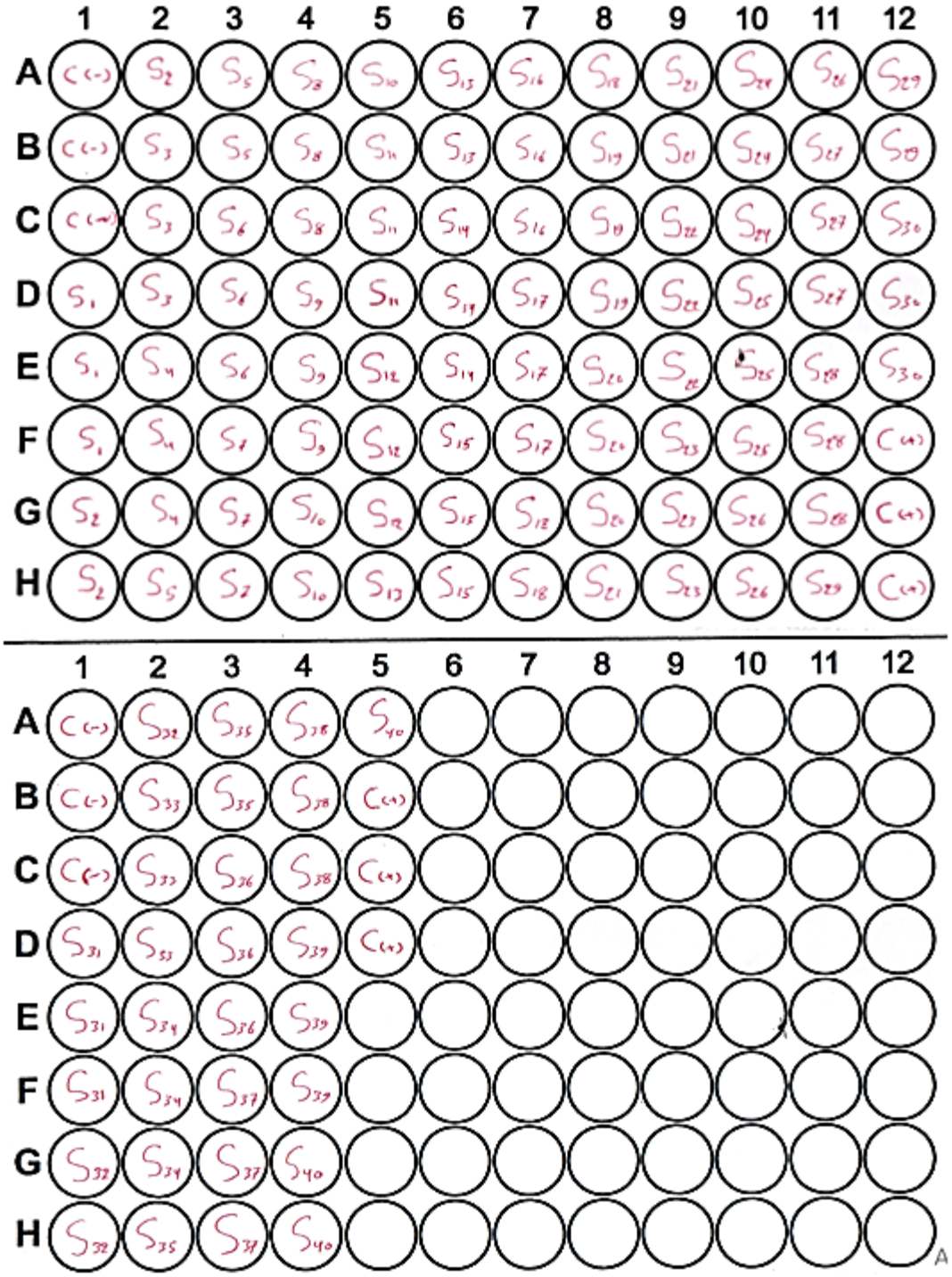


Figure (11): Pyruvate estimation kit Triplicate 96- well. (C-) represents negative control, (C+) represents positive control, (S) represents control samples.

Procedure

1. Standard wells: Fill the specified hole on the plate with 20µl of the standard (A-h test tube).
2. Sample wells: At least two wells should contain 20µl of each sample.
3. Add 100 µl Assay Buffer Cofactor Mix and 20 µl Fluorescent Substrate to all wells used.
4. To start the reaction,add 40 µl of enzyme mix to all the used wells
5. Use wavelengths of 530–540 nm for excitation and 585–595 nm for emission to read the fluorescence after incubating the plate at room temperature for 20 minutes. At up to 45 minutes, fluorescence could be stable.

Calculations:

The L-lactate concentration in the samples was determined using the formula:

L-lactate (µM)=] cF-(y-intercept) /slope[×2*×sample dilution

* the dilution factor to account for treating the samples with 0.5 M MPA to remove protein.

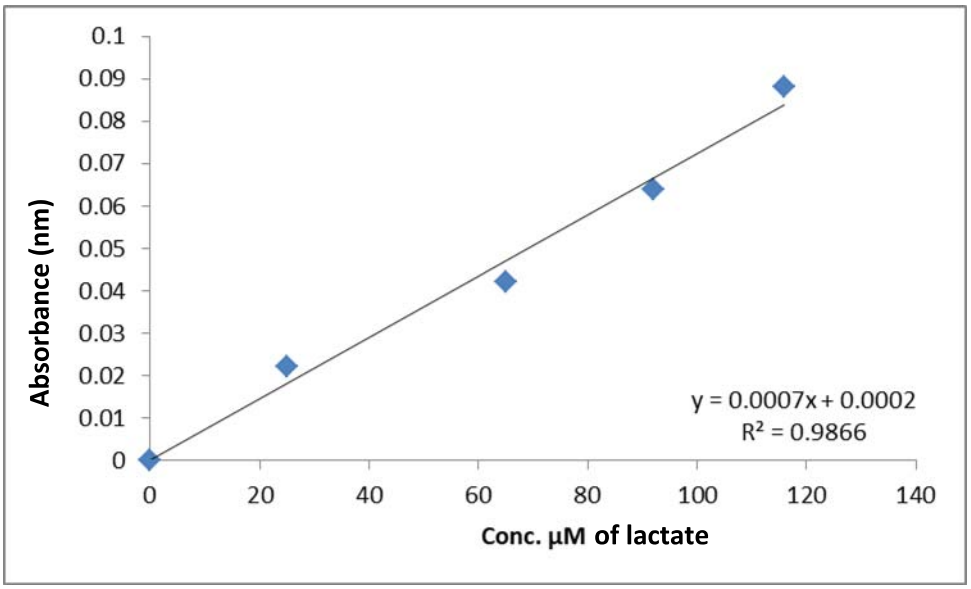


Figure (12): Standard Curve of L-lactate

3-6 Deoxyribonucleic acid extraction process:

Using the AddPrep Genomic DNA Extraction Kit, DNA was extracted as follows:

1. Twenty µl of proteinase K solution (20 mg/ml) will be added to micro centrifuge tube of 1.5 ml
2. Two hundred (200) µl of semen plasma is transferred to a 1.5 ml micro-centrifuge tube that contains Proteinase K.
3. Two hundred (200) µl Binding Solution is added to the sample tube and mixed thoroughly with a vortex for fifty seconds.
4. Incubate the sample tube at 56 ° C /10 minutes. There is no effect on the purified DNA if incubation for a long time.
5. Two hundred (200) µl absolute ethanol is added and mixed well with vortex for 15 seconds: then allow the droplets to adhere under the cap by briefly swirling down.
6. Use a 2.0 ml collection tube to carefully transfer the lysate to the top reservoir of the spin column without soaking the tube's edges.
7. One minute of centrifuging at 13,000 rpm: Pour the liquid into the spin column, attach it, and add the 2.0 ml collection tube.
8. Spin column with collecting tube filled with 500 µl of Wash 1 solution; centrifuge at 13,000 rpm for one minute. To attach the spin column with a 2.0 ml collecting tube, drain the effluent out.
9. Five hundred µl of Wash 2 solution should be added to the spin column using a 2.0 ml collecting tube. The spin column should then be centrifuged at 13,000 rpm for 1 minute, with the effluent being poured off before mount ing the spin column.
10. To dry the spin column, centrifuge at 13,000 rpm for one further minute to eliminate any remaining ethanol.

11. The spin column should be transferred to a fresh 1.5 ml microcentrifuge tube.
12. With a microcentrifuge tube, add 100 to 200µ l of the elution solution to the spin column and let it stand for at least one minute.
13. Elute genomic DNA by centrifuging at 13,000 rpm for 1 minute..

3-7 Assessment of DNA quality and quantity using Nano drop

IMPLEN GMBH's Nano drop (nanophotometer) equipment (Fig. 23) is used to measure the amount of DNA in each sample after DNA extraction.

Principle

In solution, nucleic acids absorb light with a peak at 260 nm in the UV spectrum. The Beer-Lambert law is used along with absorbance at 260 nm to calculate the concentration of nucleic acids in solution. By calculating the 260/280 nm and 260/230 nm ratios, absorbance data may also be utilized to determine nucleic acid quantities and estimate nucleic acid purity. Further, the level of nucleic acid tagging may be assessed using probes that contain fluorescent dyes.

Procedure

1. From the main panel, select the nucleic acid technique..
2. The correct volume of blank solution was pipetted onto the lighted sample window using a micropipette. When the arm is lowered, the illumination automatically goes off.
3. Lower the specimen arm and press the blank button to start a blank measurement.
4. With a slightly damp, lint-free tissue, the sample arm's measuring glass and mirror were cleaned. using water, isopropanol, or 70% ethanol.

5. The sample was mixed using the VM-300 vortex mixer to produce a homogeneous condition.
6. Pipette the proper quantity of sample solution onto the sample window that is lighted.
7. After the measurement is complete, lift the sample arm, wipe the surfaces, and then apply the next sample(((NanoPhotometer C40 /N50 /N60 /NP80 User Guide).

Figure (13): Nano drop IMPLEN GMBH Nano drop

3-8 Quantitative Polymerase Chain Reaction (qPCR)

The GoTaq qPCR master mix from Promega was used to measure the relative gene expression (A6000). Each sample underwent several reactions for each gene of interest and for the maintenance genes. Cts were generated to

compare variations in gene expression between our samples. The Ct value (30-35) is the difference between the interest gene's Ct and the internal gene determined for each sample. ΔΔCt is estimated as the variance between the ΔCt values of the test and control specimen, the fold change in gene expression is evaluate by 2 (ΔΔCt).

A refrigerated 96-well PCR plate (Sigma, UK) was used to hold the samples, which were made by setting the DNA concentration to 10 ng/ml in a final volume of 3.5µl of nuclease-free water. Prior to transferring the samples to the PCR machine, a master mix comprising 12.5µl of GoTaq qPCR master mix, 2.5µl of each related primer, and 4µl of DNAse ultrapure RNAse-free water in each well was added. The results were to analyses using Eco-study software (Fig. 13). Programed thermal circulator is seen in (Fig. 14).

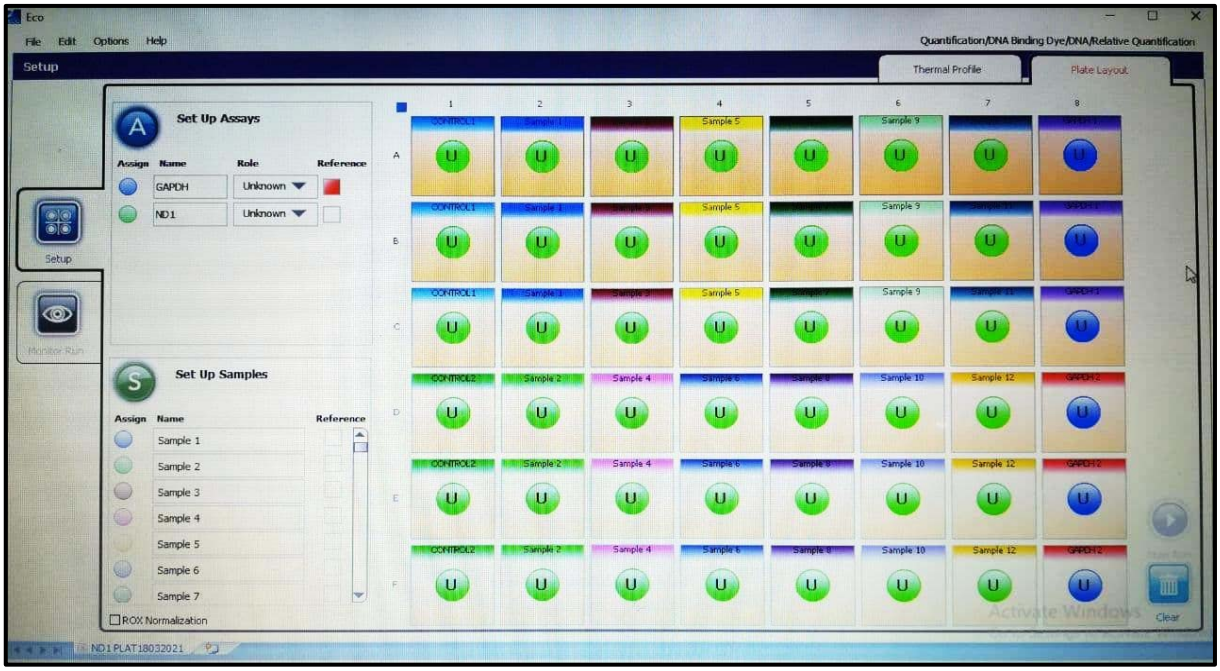


Figure (14): MxPro3005P qPCR software (Ecological research software).

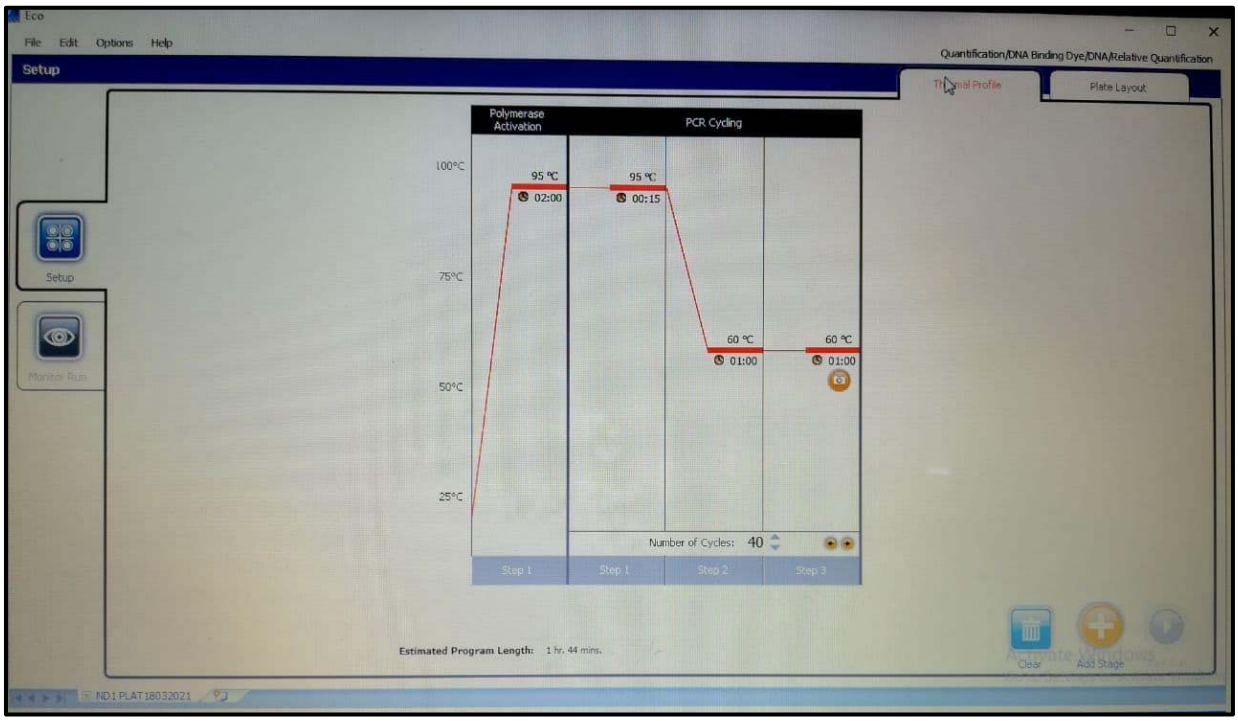


Figure (15): The predicted runtime for the thermal circulator is 1 hour and 44 minutes.

3-8-1 Quantitative PCR principle

A DNA fragment may be produced from an extract using PCR and replicated several times in vitro. According to RT-PCR results, matrix DNA can be either mitochondrial DNA, complementary DNA produced from a messenger RNA extract (poly-A RNA) or genomic DNA. It is a method for substantially eliminating a specific DNA sequence from a DNA sample. This amplification involves the replication of a double-stranded DNA template. Denaturation, primer hybridization, and elongation are the three phases that make up this process. Exponential amplification occurs as a result of each synthesis phase's products serving as templates for the next step (Kadri *et al*., 2019).

Table (4): Primers sequences for genes

Gene	Sequence	Size
Homo sapiens GAPDH	F CGGGTCTTTGCAGTCGTATG R CTGTTTCTGGGGACTAGGGG	168
Homo sapiens iNOS	F GAGTCCCCAGGAGTCAGGAT R CCGGGCTTACAGACTCACAG	189
Homo sapiens mt-ND1	F ATTATCGCCCCAACCCTCTC R GCTCGTAGGGCTCCGAATAG	191

Figure (16): A represent qPCR master mix, B represent promega kit.

3-9 Statistical analysis

The data were shown as mean ± SD. At a p-value of≤ 0.05, ANOVA and paired t-tests are employed to determine the level of significance. The correlation between the measured parameters was investigated using person correlation.

Chapter Four

RESULTS AND DISCUSSION

CHAPTER FOUR

Results and Discussions

4-1 Semen analysis

The results of this work showed that the sperm count and percentage of active sperms was significantly lower than the control group in the infertile group ($p<0.000$). While the non-motile sperm was significantly higher in infertile group in comparison to control group ($p<0.05$) (Haneen E & Mowafak k, 2022), percentage of sluggish sperms showed no significant change between the two groups. Infertile group shows significant elevation in WBC count in comparison to the control group which means the infectious diseases can affect male fertility in many ways. Possible consequences are the impairment of spermatogenesis, induction of autoimmune mechanisms, sperm, and inflammatory obstruction of the ejaculatory duct also decreased sperm motility was noted in semen samples as shown in Table 5. (Khan,S *et al* 2012)

Table (5): mean ± SD. of parameter of General seminal fluid analysis in both infertile males and control

Parameter	Control mean ± SD	Infertile mean ± SD	P- value
Count	70.9±28.2	10.54±6.47*	0.000
Active	35.9±16.5	18.36±16.1*	0.000
Sluggish	15.46±13.8	12.23±11.6	0.278
WBC Cell/µl	5.5×10^3	15.6×10^3	0.000
Non-motile	48.61±23.24	61.31±27.2*	0.034

*= significant less than P< 0.05

4-2 Seminal plasma hormones

Seminal plasma hormones showed that seminal plasma Testosterone, Estradiol and Testosterone/ Estradiol ratio showed no significant changes between the infertile groups in comparison to control group as shown in Table 6.

The relationship between sperm genes and Sperm function yielded conflicting results Bremner *et al*. that Testosterone deficiency in men are usually caused by symptoms of hypogonadism, including decreased erection and libido (Beyler and Zaneveld, 1982) . Seminal testosterone, estradiol levels and testosterone/estradiol levels ratios may be a good indicator for predicting normal spermatogenesis if the reason for the lack of sperms and its motility is due to a hormonal disorder (Zhang *et al*., 2010).

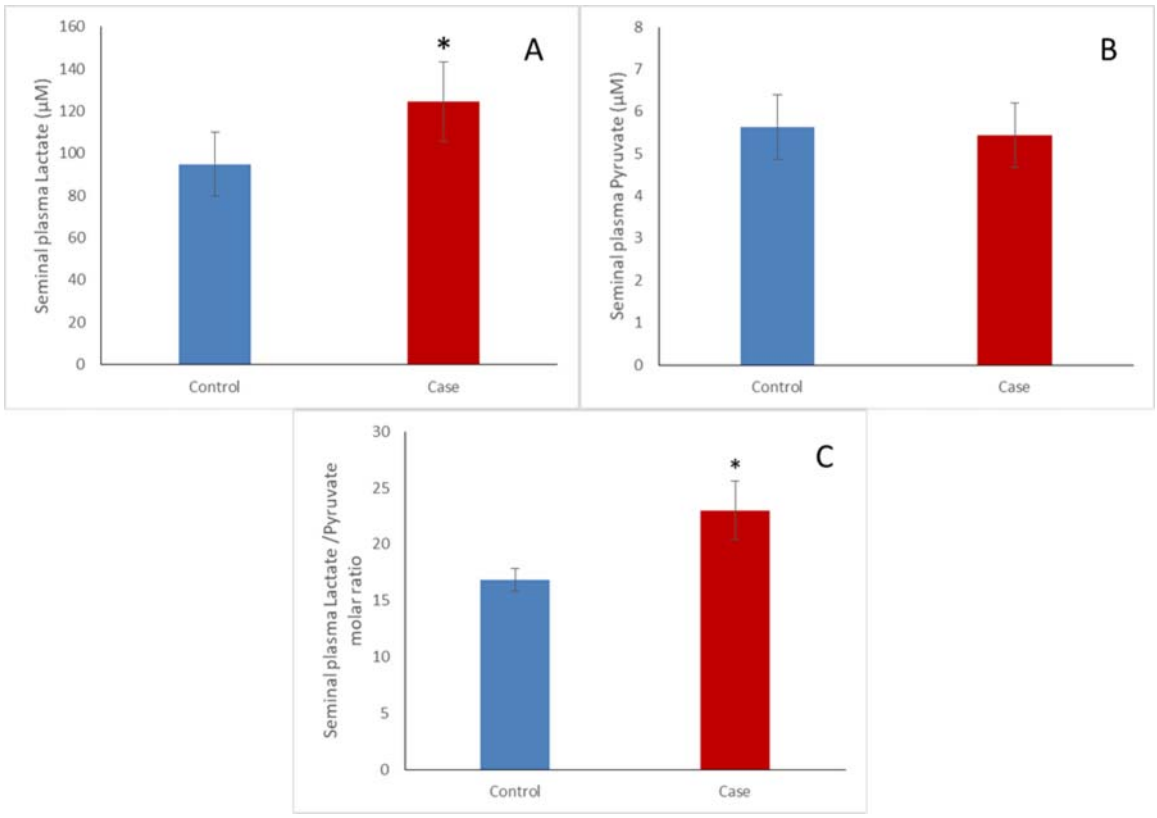
Table (6). mean ± SD. of Seminal Plasma Hormone concentration in infertile group and control group

Parameter	Control	Infertile	p-Value
Testosterone nmol/L	9.36±1.8	8.11±3.5	0.13
Estradiol pmol/L	129.4±60	113±52	0.1953
T/E_2 ratio	0.081±0.021	0.0768±0.03	0.4704

* p-value < 0.05 considered significance

4-3 Mitochondrial function

Indirect mitochondrial function parameters showed a significant elevation in seminal plasma lactate in infertile group (124.5±18.76) µm in comparison to the control group (94.8±15.13) at ($p<0.001$) as showed in Figure 1A. Seminal plasma pyruvate showed no significance in infertile group (5.44±0.76) µm in comparison to the control group (5.63±0.77) µm ($p>0.05$) as showed in Figure 1B. Seminal plasma lactate/ pyruvate molar ratio showed a significant elevation in infertile group (22.99±2.6) in comparison to control group (16.8±1.02) at ($p<0.001$) as showed in Figure1C.



Seminal plasma lactate and pyruvate

Fig-1- (mean±S.D) concentration of seminal plasma hormones in group infertile men and control group

1A: For lactate concentration in µm/ml

1B: For pyruvate concentration in µm/ml

1C: The Ratio between lactate/pyruvate in both group

Sperms mitochondrial copy number showed significant reduction in infertile group in comparison to control group ($p<0.0001$) as shown in Figure 2.

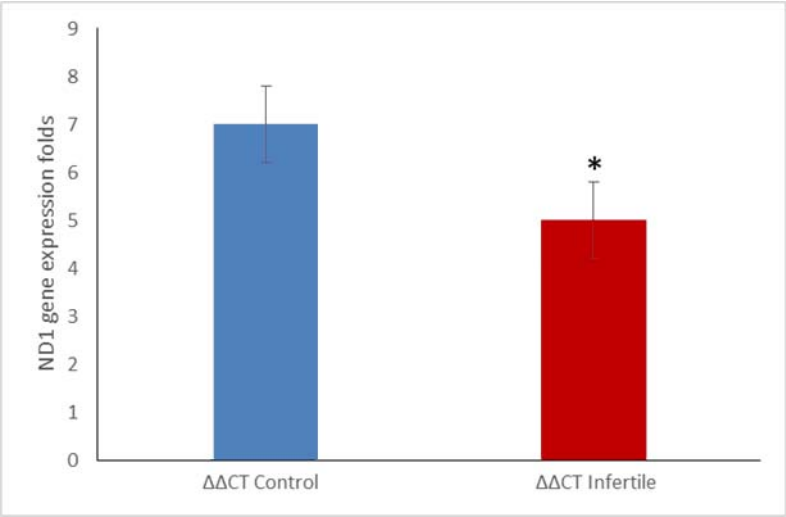


Fig-2- Sperm Mitochondrial copy No. in infertile Males comparison to control group (P≤0.001)

Total antioxidant capacity significantly elevated in seminal plasma of infertile male in comparison to control group ($p<0.001$) as shown in figure 1.

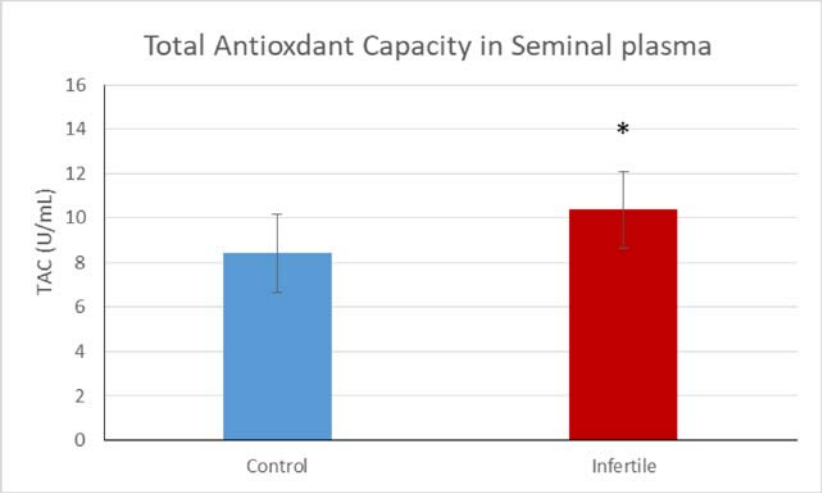


Fig-3- Total Antioxidant capacity (1 u/ml) in both infertile males and control group.

Seminal NOS qPCR data showed that there was a significant elevation in NOS expression in infertile group in comparison to control group ($p<0.0001$) as in figure 2.

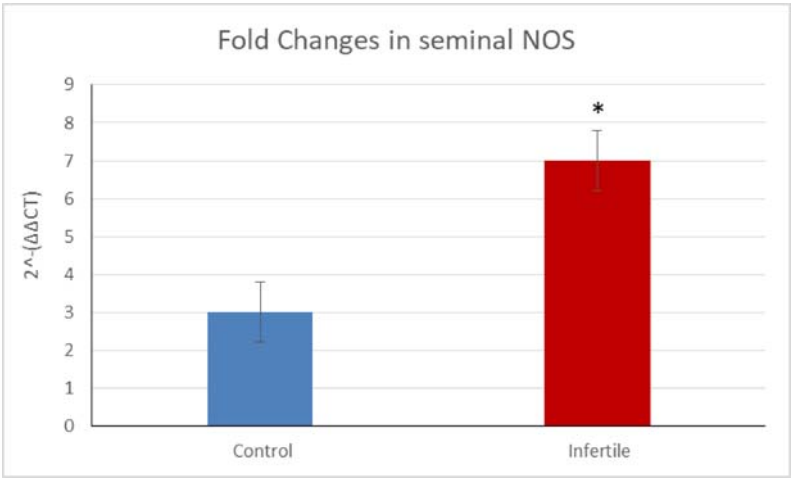


Fig-4- Seminal NOS qPCR data

Linear Regression analysis showed that there was no significant correlation between NOS expression and seminal total antioxidant capacity in healthy group ($p>0.05$, F value= 2.04, r2= 0.1019) as shown in Figure

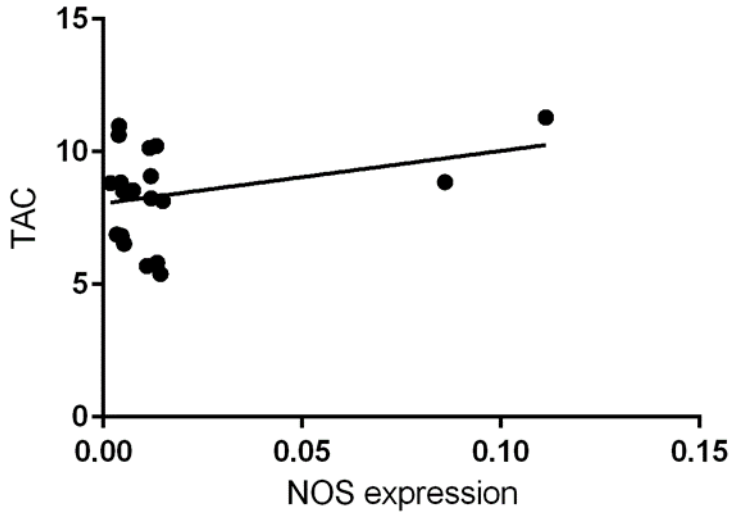


Fig-5- Linear Regression Analysis between NOS expression and TAC

In infertile group, Linear Regression analysis showed that there was a significant positive correlation between NOS expression and seminal total antioxidant capacity ($p<0.001$, F value= 74.52, r2= 0.8054) as shown in Figure

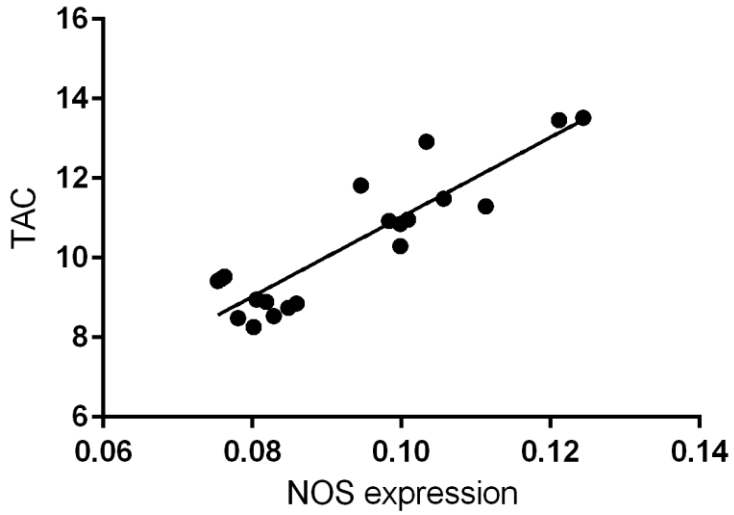


Fig-6- Linear Regression analysis between NOS expression and TAC

Infertility is a multi-factorial condition, including genetic, anatomical and environmental factors. NO represents the most potent nitrogen free radicals(Ribas-Maynou & Yeste, 2020), which instantly react with superoxide to form highly toxic peroxynitrite hydroxyl radical and singlet O2, both of which have direct and indirect distractive ability on sperms by producing aggressive environment that damage DNA, mitochondria, cell membrane and other ultra-cellular structures(Ighodaro & Akinloye, 2018). Up regulation of NOS leads to a significant increase in NO concentrations which significantly induce oxidative stress (Zhen *et al*., 2008). Oxidative stress significantly damage genetic material and reduce sperm formation, integrity, energy turnover, motility and morphology (Drevet *et al*., 2022) and this agrees with results of this work. It has been demonstrated that abnormal sperms and seminal leukocytosis are the primary source of ROS generation in human seminal fluid (Li *et al*., 2020). Activated leukocytes are capable of generating 100 times the quantity of reactive oxygen species and reactive nitrogen species (ROS and RNS) as non-activated leukocytes (Yánez-Ortiz *et al*., 2021). Leukocytes may be triggered in a number of ways including inflammation and infection (Khodamoradi *et al*., 2020) and this can explain the severe drop in seminal plasma's total antioxidant capacity.

Seminal plasma's total antioxidant capacity has significantly contributed in protecting the sperms from oxidative damage especially in the epididymis, which exhibits region–specific antioxidant activity. It may protect sperms from oxidative damage while being stored at this location (Gupta *et al*., 2021). According to Henkel *et al*. (1999), men who have undergone vasectomies have a reduced overall antioxidant capacity, lower thiol group concentrations, and greater levels of lipid peroxidation in their seminal plasma than men who have an intact reproductive system (Henkel *et al*., n.d.)

Additionally (Fafula *et al*., 2018) and his group described a significant elevation in NO synthase activity in infertile male due to a significant activation of the inducible isoform of NO-synthase showing that NO synthesis is disrupted (Fafula *et al*., 2018). The arginase/NOS ratio altered toward predominance of iNOS-derived NO generation in individuals with lower reproductive potential (Doshi *et al*., 2012). Gholinezhad *et al* described positive correlation between NOS expression and seminal total antioxidant capacity (Gholinezhad *et al*., 2020) and this agreed with results obtained in this work.

In this study's main results are that infertile men's semen samples included more damaged sperms and upregulation in nitric oxide synthase than normozoospermic fertile men's semen samples, with significantly lower TAC in infertile men' than fertile. In infertile group, there was positive correlation between NO synthase expression and TAC. The current findings indicate an excess of nitrogen free radicals.

This work described non-significant changes in seminal plasma testosterone, estradiol and testosterone / estradiol ratio and this agrees with Salim & Alkataan, (Salim & Alkataan, 2022) as well may be related to age of the infertile group in this work.

The significant elevation in seminal lactate is due to decreased activity of the lactate dehydrogenase (LDH) enzyme, which catalyzes the interconversion of lactate to pyruvate, implying a decrease in the conversion of NADH to NAD+

(Mumcu *et al*., 2020) and this agrees with results obtained by Junaid 2022 (Salim & Alkataan, 2022). The low LDH activity may be related to high free radical that led to induce LDH dysfunction. The oxygen free radical may be Extrinsic ROS (leukocytes) and intrinsic ROS (spermatozoa) which are the two primary sources of ROS in semen (Li *et al*., 2020).

The lactate/pyruvate shuttle system's interconversion of lactate to pyruvate is critical for increasing sperm motility as it provides the source of energy. The high seminal lactate is associated with low sperm count, and motility and this related to restricted energy supplied due to reducing the substrate that went to the tricarboxylic acid cycle. This initiates energy generation inside mitochondria by oxidative phosphorylation (Martínez-Reyes & Chandel, 2020)

There are mounting evidence suggesting and glycolysis is the predominant metabolic pathway for the energy support of sperm motility in many species (Peña *et al*., 2021). The assumption that mitochondria are the motor of mammalian spermatozoa is still accepted and low mitochondrial copy number is associated with reduced glycolysis activity and the low ATP synthesis modulating sperm activities based on the availability of energy substrates in the environment (Darehbagh *et al*., 2022). Defective mitochondria, as the primary cellular organelles for the interaction between ROS formation and intrinsic apoptosis-like processes, would have a critical role in determining sperm survival (Liu *et al*., 2019). Even with no significant changes in male sex hormones in young infertile male, the direct and indirect mitochondrial function showed significant reduction in comparison to control.

Conclusions and Recommendations

Conclusions

1. Infertile group shows significant elevation on WBC in control to control group.
2. No significant difference in percentage of sluggish sperms between the two group under study.
3. A significant reduction in sperms mitochondrial copy numbers in infertile group than in control group.
4. A significant elevation in total anti-oxidant capacity in seminal plasma of infertile male in comparison to control group.
5. A significant elevation in NOS expression in infertile group comparison to control group.

Recommendations

1- Geographic scope and potential for employing more samples.

2- Include the general semen analysis in the required examination before marriage.

3- Study the role of free radicals as factors of male infertility.

4- Study the relationship between mt-DNA and oligospermia infertility.

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الخلاصة

يشير العقم الى عدم القدرة على الانجاب خلال عام من الزواج مع تجنب موانع الحمل، زادت حالات العقم عند الذكور خلال العقود الثلاثة الماضية، لأسباب عديدة منها زيادة مستويات الجذور الحرة الناتجة عن الاجهاد التأكسدي. كما تلعب بيوت الطاقة دورا مهما في حركة الحيوانات المنوية بتوفير الطاقة اللازمة لحركة الحيوان المنوي.

جمعت (٨٠) عينة من سائل منوي من الذكور المتطوعين في مدينة الموصل/العراق، استخدمت حاويات نظيفة معقمة مخصصة لهذا الغرض وبعد النصح بفترة انقطاع عن ممارسة الاتصال الجنسي واخذ كامل القذفة لمدة (٢-٣) يوم وللفترة من آب ٢٠٢٢ ولغاية آيار ٢٠٢٣. قسمت العينات الى مجموعتين: الاولى تضمنت (٤٠) عينة لسائل منوي أشخاص يعانون من نقص عدد النطف Oligospermia وتضمنت المجموعة الثانية (٤٠) عينة أشخاص سليمين اعتبروا سيطرة . اخذ الوسط الحسابي ± الانحراف القياسي والنسب للعدد الكلي اظهرت النتائج وجود انخفاضاً معنوياً في كل من عدد الحيوانات المنوية (6.47±10.54) في قليلي النطف وبلغت النسبة المئوية لهم (16.1±18.36) مقارنة بمجموعة السيطرة كما لوحظ ارتفاعاً معنوياً في معدل النسب المئوية للحيوانات المنوية غير المتحركة بلغت (27.2±61.31) مقارنة بمجموعة السيطرة كما وجد انخفاضاً غير معنوياً في مستوى هورموني الذكورة Testosterone & Estradiol مقارنة بمجموعة السيطرة . وأظهرت نتائج وظائف المايتوكوندريا ارتفاعا معنويا في لاكتيت السائل المنوي في مجموعة قليلي النطف فضلا عن وجود ارتفاعا معنويا في نسبة اللاكتيت الى البايروفيت لمجموعة قليلي النطف عند مقارنتها بمجموعة السيطرة . واظهرت نتائج البحث ارتفاعا معنويا في سعة مضاد الاكسدة الكلي في السائل المنوي لمرضى قليلي النطف عند مقارنتها بمجموعة السيطرة . كما اظهرت نتائج qPCR وجود ارتفاعا معنويا في NOS لمجموعة نقص الحيوانات المنوية عند مقارنتها بمجموعة السيطرة .

﴿ لِّلَّهِ مُلْكُ ٱلسَّمَٰوَٰتِ وَٱلْأَرْضِ ۚ يَخْلُقُ مَا يَشَآءُ ۚ يَهَبُ لِمَن
يَشَآءُ إِنَٰثًا وَيَهَبُ لِمَن يَشَآءُ ٱلذُّكُورَ ٤٩ أَوْ يُزَوِّجُهُمْ
ذُكْرَانًا وَإِنَٰثًا ۖ وَيَجْعَلُ مَن يَشَآءُ عَقِيمًا ۚ إِنَّهُۥ عَلِيمٌ قَدِيرٌ ٥٠

﴾

صَدَقَ اللهُ العَظِيم

سورة الشورى، الآية (٤٩-٥٠)

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة الموصل / كلية العلوم

قسم علوم الحياة

التغير الجيني لأنزيم أوكسيد النتريك
لدى الشباب الذكور العقيم في الموصل/العراق

زيد هاشم الياس سلطان الجماس

رسالة ماجستير

علوم الحياة / علم الحيوان

باشراف

المدرس

الدكتور موفق خليل حسن علي

١٤٤٤هـ ٢٠٢٣م

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة الموصل / كلية العلوم

قسم علوم الحياة

التغير الجيني لأنزيم أوكسيد النتريك
لدى الشباب الذكور العقيم في الموصل/العراق

رسالة تقدم بها الطالب

زيد هاشم الياس سلطان الجماس

الى

مجلس كلية العلوم في جامعة الموصل وهي جزء من متطلبات

شهادة الماجستير في اختصاص

علوم الحياة / علم الحيوان

باشراف

المدرس

الدكتور موفق خليل حسن علي

١٤٤٤هـ **٢٠٢٣م**