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Association of oxidative stress with the secretion of prostate, seminal vesicles, and epididymis among Libyan infertile men

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Declaration

I, Awatef Almabruk Saleh Abukliesh, the undersigned hereby confirm that the work contained in this thesis, unless otherwise referenced is the researcher's own work, and has not been previously submitted to meet requirements of an award at this University or any other higher education or research institution, I furthermore, cede copyright of this thesis in favour of University of Tripoli.

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Abstract

Background: Oxidative stress (OS) is a hazardous process that can damage and deform sperm, leading to male infertility. This process occurs when the generation of free radicals from reactive oxygen species (ROS) surpasses the total antioxidant activity in semen. Since seminal plasma creates the environment for the proper functioning of spermatozoa, many substances secreted by male accessory glands (MAGs) have a role in sperm physiology. Thus, a change in the biochemical composition of the seminal plasma could be directly related to infertility. **Aim**: This study aimed to investigate the association of OS with secretion of the prostate, seminal vesicles, and epididymis in infertile Libyan men. **Materials and methods**: This study included 79 infertile men and 25 fertile men, and the samples were collected from the fertility lab of the Yashfeen clinic and the Al Shark Lab in Tripoli, Libya. Firstly, a basic semen analysis was performed according to WHO (2021) guidelines, and then ROS levels in semen were measured using the Nitroblue tetrazolium (NBT) method. Subsequently, the performance of MAGs was assessed by selecting one biomarker for each gland, such as citric acid (prostate), fructose (seminal vesicles), and neutral α-glucosidase (NAG) (epididymis). Finally, statistical analysis was performed using the nonparametric Mann-Whitney U test to compare the two study groups and the Spearman correlation coefficient to determine the association between two separate variables. **Results:** The results showed a decrease in both levels of citric acid (mg/mL) and NAG (mIU/mL) in infertile men compared to fertile men ($P < 0.001$), while the fructose levels remained convergent in both study groups ($P > 0.05$). In contrast, infertile men had significantly higher seminal ROS levels than fertile men ($p < 0.001$). Citric acid, fructose, and NAG levels were positively correlated with semen volume and progressive motility. There were also positive associations among citric acid, NAG, sperm concentration, and normal morphology, as well as between total sperm count and total motility with NAG and citric acid, respectively. In addition, a significant negative correlation was found between seminal ROS levels and all normal sperm parameters, including sperm concentration, total sperm count, vitality, motility, progressive motility, and normal morphology. Besides, seminal ROS levels were negatively associated with citric acid levels and NAG activity. However, no correlation between seminal ROS levels and fructose levels was detected. **Conclusion:** This study provided new and specific insights into understanding the potential influence of OS on semen quality and secretion of the epididymis and prostate. Therefore, the evaluation of seminal ROS in conjunction with careful evaluation of the secretory products of MAGs may contribute to an accurate diagnosis of the pathophysiology of male infertility and, thus, the determination of appropriate therapy.

Keywords: Oxidative stress (OS), Infertile men, Reactive oxygen species (ROS), Male accessory glands (MAGs).

ارتباط الإجهاد التأكسدي بإفراز البروستاتا والحويصلات المنوية والبربخ بين الرجال الليبيين المصابين بالعقم

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المستخلص

خلفية البحث: الإجهاد التأكسدي هو عملية خطرة يمكن أن تلحق الضرر بالحيوانات المنوية وتشوهها مما تؤدي إلى عقم الذكور. تحدث هذه العملية عندما يتجاوز توليد الجذور الحرة من أنواع الأكسجين التفاعلية (ROS) إجمالي نشاط مضادات الأكسدة في السائل المنوي. نظرًا لأن البلازما المنوية تخلق البيئة اللازمة لعمل الحيوانات المنوية بشكل سليم، فإن العديد من المواد التي تفرزها الغدد الملحقة الذكرية لها دور في فسيولوجيا الحيوانات المنوية. وبالتالي ، يمكن أن يكون التغيير في التركيب الكيميائي الحيوي للبلازما المنوية مرتبطًا بشكل مباشر بالعقم.

الهدف من البحث: هدفت هذه الدراسة إلى التحقق من ارتباط الإجهاد التأكسدي بافرازغدة البروستاتا والحويصلات المنوية والبربخ لدى الرجال الليبيين المصابين بالعقم.

المواد وطرق العمل: اشتملت هذه الدراسة على 79 رجلاً يعانون من العقم و 25 رجلاً سليماً, حيث تم جمع العينات من معمل الخصوبة في عيادة يشفين ومعمل الشرق في طرابلس، ليبيا. أولاً ، تم إجراء تحليل الأساسي للسائل المنوي وفقًا لإرشادات منظمة الصحة العالمية (2021)، ومن ثم تم قياس مستويات ROS في السائل المنوي باستخدام صبغة نيتروبلوتترازوليوم (NBT). بعد ذلك ، تم تقييم أداء الغدد الملحقة الذكرية عن طريق اختيار علامة حيوية واحدة لكل غدة، مثل حامض الستريك (البروستاتا) والفركتوز (الحويصلات المنوية) وإنزيم ألفا جلوكوز المحايد NAG (البربخ). أخيرًا، تم إجراء التحليل الإحصائي باستخدام اختبار مان وتني اللامعلمي (Mann-Whitney U) لمقارنة مجموعتي الدراسة؛ ومعامل ارتباط سبيرمان (Spearman) لتحديد الارتباط بين متغيرين منفصلين.

النتائج: أظهرت النتائج انخفاضًا في كلا مستويين: حامض الستريك (مجم/ مل) و NAG (ميكرو/ مل) في الرجال المصابين بالعقم مقارنة بالرجال الذين يتمتعون بالخصوبة ($P< 0.001$)، بينما ظلت مستويات الفركتوز متقاربة في مجموعتي الدراسة ($P> 0.05$). في المقابل، كان لدى الرجال المصابين بالعقم مستويات أعلى بكثير من أنواع الأكسجين التفاعلية مقارنة بالرجال الأصحاء ($P< 0.001$). ارتبطت مستويات حامض الستريك والفركتوز وإنزيم NAG بشكل إيجابي مع حجم السائل المنوي والحركة التقدمية. كما كانت هناك ارتباطات إيجابية بين حامض الستريك، وإنزيم NAG مع تركيز الحيوانات المنوية والتشكل الطبيعي، وكذلك بين إجمالي عدد الحيوانات المنوية والحركة الكلية مع إنزيم NAG وحمض الستريك على التوالي. بالإضافة إلى ذلك، تم العثور على ارتباط سلبي معنوي بين مستويات ROS المنوية وجميع المعلمات الطبيعية للحيوانات المنوية، بما في ذلك تركيز الحيوانات المنوية والعدد الكلي للحيوانات المنوية والحيوية والحركة الكلية والحركة التقدمية والتشكل الطبيعي. إلى جانب ذلك، ارتبطت مستويات ROS المنوية سلبًا مع مستوى حامض الستريك ونشاط إنزيم NAG. ومع ذلك، لم يتم العثور على أي ارتباط بين مستويات ROS المنوية ومستويات الفركتوز.

الاستنتاج: قدمت الدراسة رؤى جديدة ومحددة لفهم التأثير المحتمل للإجهاد التأكسدي على جودة السائل المنوي ووظيفة البربخ والبروستات. لذلك ، فإن تقييم ROS في السائل المنوي مع التقييم الدقيق للمنتجات الإفرازية للغدد الملحقة الذكرية قد يساهم في التشخيص الدقيق للفيزيولوجيا المرضية لعقم الذكور، وبالتالي تحديد العلاج المناسب.

الكلمات المفتاحية: الإجهاد التأكسدي، الرجال المصابين بالعقم، أنواع الأكسجين التفاعلية، الغدد الملحقة الذكرية.

Dedication

To my father “Almabruk”, and my mother “Saida”, for their endless support and love, without which I would not have been able to achieve what I have.

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List of Abbreviations

AcCoA	Acetyl CoA
AR	Androgen Receptor
ATP	Adenosine Triphosphate
cAMP	Cyclic Adenosine Monophosphate
CAT	Catalase
Ca^{+2}	Calcium
Cu^{+2}	Copper
DHT	Dihydrotestosterone
ER	Estrogen Receptors
FSH	Follicle-Stimulating Hormone
G6PD	Glucose-6-Phosphate Dehydrogenase
GnRH	Gonadotropin-Releasing Hormone
GPx	Glutathione Peroxidase
GR	Glutathione Reductase
GSH	Reduced Glutathione
GSSG	Oxidized Glutathione
H_2O_2	Hydrogen peroxide
HMPS	Hexose Monophosphate Shunt
IL	Interlukin
K^+	Potassium
LH	Luteinizing Hormone
LPO	Lipid Peroxidation
MAGI	Male Accessory Gland Infections
MAGs	Male Accessory Glands
MDA	Malondialdehyde

MFI	Male-Factor Infertility
Mg^{+2}	Magnesium
MGT	Male Genital Tract
mtDNA	Mitochondrial DNA
Na^{+}	Sodium
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NAG	Natural Alpha Glucosidase
NaOH	Sodium Hydroxide
NBT	Nitro Blue Tetrazolium
NO	Nitric Oxide
NOX	NADPH Oxidase
$O_2^{\bullet-}$	Superoxide anion
OAA	Oxalacetate
OD	Optical Density
$^{\bullet}OH$	Hydroxyl radicals
$ONOO^{-}$	Peroxynitrite
OS	Oxidative Stress
PKA	Protein Kinase A
PMN	Polymorphonuclear
PRRs	Pattern Recognition Receptors
PUFAs	Polyunsaturated Fatty Acids
RNS	Reactive Nitrogen Species
$ROO^{\bullet}$	Peroxyl
ROS	Reactive Oxygen Species
SDF	Sperm DNA Fragmentation
SOD	Superoxide Dismutase

SP	Seminal Plasma
T	Testosterone
TNF-α	Tumor Necrosis Factor-alpha
WBCs	White Blood cells
WHO	World Health Organization
Zn^{+2}	Zinc

1. Introduction

Over the past few years, infertility has acquired more popularity and attention as a serious public health problem, affecting about 15-20% of married couples globally (Szczykutowicz *et al.*, 2019). However, infertility rates are substantially higher in other parts of the world, reaching above 30% in North Africa, Sub-Saharan Africa, the Middle East, Central and Eastern Europe, and South and Central Asia (Mascarenhas *et al.*, 2012). About 50% of all infertility cases are due to male-factor infertility (MFI) (Oliva *et al.*, 2001). In Libya, however, MFI accounts for about 70% of infertility cases, which is very high compared to other regions of the globe (Eldib and Tashani, 2021).

Fertile men's sperm production has decreased globally during the last 50 years, from a mean concentration of 133 million/ml to 66 million/ml. These statistics also showed that the sperm count of fertile Libyan men has reduced to a mean of 65.0 million/ml (Carlsen *et al.*, 1992). Although the majority of male infertility cases are idiopathic, this drop is likely due to the detrimental impact of oxidative stress (OS) on sperm quality (Agarwal and Prabakaran, 2005).

Infertility affects both men and women and is defined as a person's inability to contribute to conception after 12 months or more of regular, unprotected sexual relations with the same partner (Agarwal *et al.*, 2019). At puberty, spermatozoa are created in the testis via an intricate process known as spermatogenesis (Durairajanayagam *et al.*, 2015). Subsequently, spermatozoa are suspended in seminal plasma (SP), a fluid secreted by male accessory glands (MAGs) (Ramzan *et al.*, 2015).

The SP contains many organic and inorganic compounds that play a significant role in supporting and protecting spermatozoa from infections and oxidative damage during their passage through the male and female reproductive tracts (Juyena and Stelletta, 2012). Therefore, recent research has focused on the role of these compounds on sperm function and in evaluating the secretory functions of MAGs (De Jonge and Barratt, 2006). According to the World Health Organization (WHO), citric acid, fructose, and neutral alpha-glucosidase (NAG) are the most diagnostic biomarkers for the functions of the prostate, seminal vesicles, and epididymis, respectively (WHO, 1999; WHO, 2010).

In addition, several previous studies suggest an association between these biomarkers and most sperm characteristics, such as sperm count, morphology, motility, and progressive motility (Sundaram *et al.*, 2016). The principal function of citric acid is to maintain the pH and osmotic balance of semen, which maintains membrane function and sperm shape (Owen and Katz, 2005). On the other hand, fructose provides energy for sperm motility and vitality under anaerobic conditions via fructolysis to lactate (Kumar and Sharma, 2017).

Although the exact role of NAG in sperm function is unknown, some researchers suggest that it contributes to sperm maturation by modifying surface glycoproteins because NAG breaks down 1,4-linked oligosaccharides into glucose molecules (Ben Ali *et al.*, 1994; Dias *et al.*, 2004). The activity of NAG is measured to localize the blockage and differentiate between obstructive and non-obstructive azoospermia (Yassa *et al.*, 2001).

Testosterone (T) is a primary androgen in men, where it plays a critical role in spermatogenesis and regulates MAGs secretions in conjunction with prolactin (Mann and Lutwak-Mann, 1981). Numerous variables, however, may contribute to impaired spermatogenesis and MAGs secretion, such as OS, which is one of the reasons that affect male reproductive function, either directly via sperm damage or indirectly via disruption of endocrine hormones (Darbandi *et al.*, 2018; Leisegang *et al.*, 2021).

According to current guidelines, OS is responsible for 30–80% of infertility cases. OS occurs when the production of reactive oxygen species (ROS) exceeds the capacity of the antioxidant defense mechanisms (Tremellen, 2008). Thus, elevated ROS levels impair sperm function by degrading sperm biomolecules, including lipids, proteins, and nucleic acids. However, trace amounts of ROS are essential for spermatozoa activities, such as capacitation, hyperactivation, acrosome response, and sperm-oocyte fusion (Henkel, 2011).

The OS results not only from increased ROS production but also from decreased antioxidant capacity (Takeshima *et al.*, 2021). The SP contains endogenously produced antioxidants that protect the sperm from OS by neutralizing oxidizing factors. There are two types of seminal antioxidants: enzymatic, including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR), as well

as non-enzymatic, such as minerals and vitamins A and C (Lourenco and Fernandes, 2015).

The two principal ROS generators in the seminal fluid are leukocytes and immature spermatozoa (Takeshima *et al.*, 2021). Moreover, many exogenous ROS may be generated by a range of lifestyle and environmental factors, including unhealthy foods, excessive smoking, and alcohol use (Dutta *et al.*, 2021).

Unfortunately, due to the breakdown of the government, the incapacity of the institutions, and the fragility of the economy in Libya, there is the monitoring of the accumulation of food and beverages that do not meet quality standards and are unfit for human consumption, in addition to the use of plastic bags, containers, and bottles in refrigerators in homes, shops, restaurants, and cafes, as well as the spread of the widely practiced habit of smoking and hookah. All of this information supports the hypothesis that OS contributes to male infertility.

1.1. Aim of the study

This study aimed to investigate the association of OS with secretion of the prostate, seminal vesicles, and epididymis in infertile Libyan men by studying the following objectives:

1.1.1. Objectives

1. Evaluating the semen parameters (volume, viscosity, sperm count, motility, morphology, and vitality) for infertile and fertile subjects.
2. Estimating the presence of seminal ROS in the study population.
3. Measuring fructose, citric acid, and NAG in patients and controls as diagnostic markers for seminal vesicles, prostate, and epididymis, respectively.

2. Literature review

2.1. Male-factor infertility (MFI)

Almost 7% of men on the globe are infertile, i.e., MFI contributes to half of the infertile couples (Mehra *et al.*, 2018). Despite advances in reproductive biology technologies, the causes of male infertility remain unknown in 30% of cases. These cases are called "idiopathic" because the men involved have no prior history of reproductive problems and their sperm parameters are normal (Poongothai *et al.*, 2009).

MFI is related to poor semen quality, which can be measured by macroscopic evaluations, such as colour, pH, liquefaction, viscosity, and abnormally high/low semen volume (Hyperspermia/Hypospermia), as well as microscopic evaluation of sperm, such as low sperm concentration (Oligozoospermia), low motility (Asthenozoospermia), abnormal morphologies (Tretatozoospermia), a high percentage of dead sperm (Necrozoospermia), and absence of sperm in the semen (azoospermia) (Tournaye and Cohlen, 2012; Caraballo *et al.*, 2019). Regularly, the WHO publishes instructions for the examination and treatment of human sperm in laboratories (Khomami, 2018). The edition published in 2021 provides practical advances in semen analysis **(Table 2.1)**.

Table 2.1. The reference values for semen parameters according to WHO (2021).

Semen parameters	WHO 2021
Semen volume (ml)	1.4
Sperm concentration (10^6 per ml)	16
Total sperm count (10^6 per ejaculate)	39
Total (progressive + non-progressive) motility (%)	42
Progressive motility (%)	30
Non-progressive motility (%)	1
Vitality (%)	54
Normal forms (%)	4

In general, male infertility may be due to congenital or acquired urogenital abnormalities, genital tract infections, malignancies, scrotal temperature due to varicocele, endocrine disturbances, genetic or epigenetic abnormalities, or

immunological factors. Furthermore, there are many risk factors for male infertility, such as environmental and lifestyle changes (Jungwirth *et al.*, 2015; Sharma, 2017).

2.2. Composition of semen

Normal seminal fluid is greyish-white, opalescent, and slightly viscous. It is a collection of structures released by male reproductive organs such as the testes, epididymis, and MAGs. Therefore, semen can be divided into cellular and non-cellular components, which are acquired by cell centrifugation to obtain the SP (Kumar and Sharma, 2017).

Cellular components account for about 10% of the semen volume and include sperm, epithelial cells of the urogenital tract, immature germ cells, and leukocytes. Non-cellular elements represented in SP comprise about 90% of the semen volume and consist of inorganic ions [zinc (Zn^{+2}), magnesium (Mg^{+2}), calcium (Ca^{+2}), copper (Cu^{+2}), potassium (K^{+}), and sodium (Na^{+})] and organic compounds such as proteins, lipids, sugars (fructose), hormones, and cytokines (Juyena and Stelletta, 2012; Drabovich *et al.*, 2014).

2.2.1. The role of seminal plasma (SP)

The majority of research into the processes of male fertility has focused on sperm, which is critical. However, another essential component of fertility, the non-sperm elements of semen, has long been considered to exist only to maintain sperm (De Jonge and Barratt, 2006).

Functions of SP include: supplying spermatozoa with a nourishing and protective medium as they travel via the female reproductive tract (Selvam *et al.*, 2019); promoting spermatozoa maturation, motility, and control of spermatozoa function and capacitation through a cascade of modifications in the cell membrane, resulting in sperm hyperactivity and the capability to attach to the oocyte zona pellucida (Poiani, 2006; Bedford, 2015; Korsheed, 2018). This connection causes an acrosomal reaction that results in the cleavage of the zona pellucida glycoproteins and sperm-oocyte fusion (Szczykutowicz *et al.*, 2019). Moreover, the seminal immunomodulatory factors regulate maternal immune tolerance for successful fertilization, early embryo development, and implantation (Wang *et al.*, 2020).

2.2.2. Biomarkers of male accessory glands (MAGs)

Seminal vesicles secrete the majority of SP, approximately 70%, and their secretions are fructose, ascorbic acid, proteins, and prostaglandins. The prostate gland secretes 20% of the SP, which includes citric acid, Zn^{+2}, prostate-specific antigen, Ca^{+2}, and acid phosphatase. Cooper's gland contributes 1% of its mucous output consisting of sialic acid and galactose. On the other hand, epididymides produce 9% of SP, with their most significant secretions being free L-carnitine, NAG, and glycerophosphocholine **(Figure 2.1)** (Flint *et al.*, 2015; Papanna *et al.*, 2015).

The prostate and seminal vesicles are MAGs. While the epididymis is an organ on the testes' posterior edge where sperm develops and is stored, it can be referred to as an accessory gland due to its secretory ability (Ricardo, 2018). Each gland produces particular substances that can be used as diagnostic markers to give information about the functional state of these glands (Najafi and Malini, 2011). According to the WHO, citric acid, fructose, and NAG are the most diagnostic biomarkers of the functions of the prostate, seminal vesicles, and epididymis, respectively (WHO, 1999; WHO, 2010).

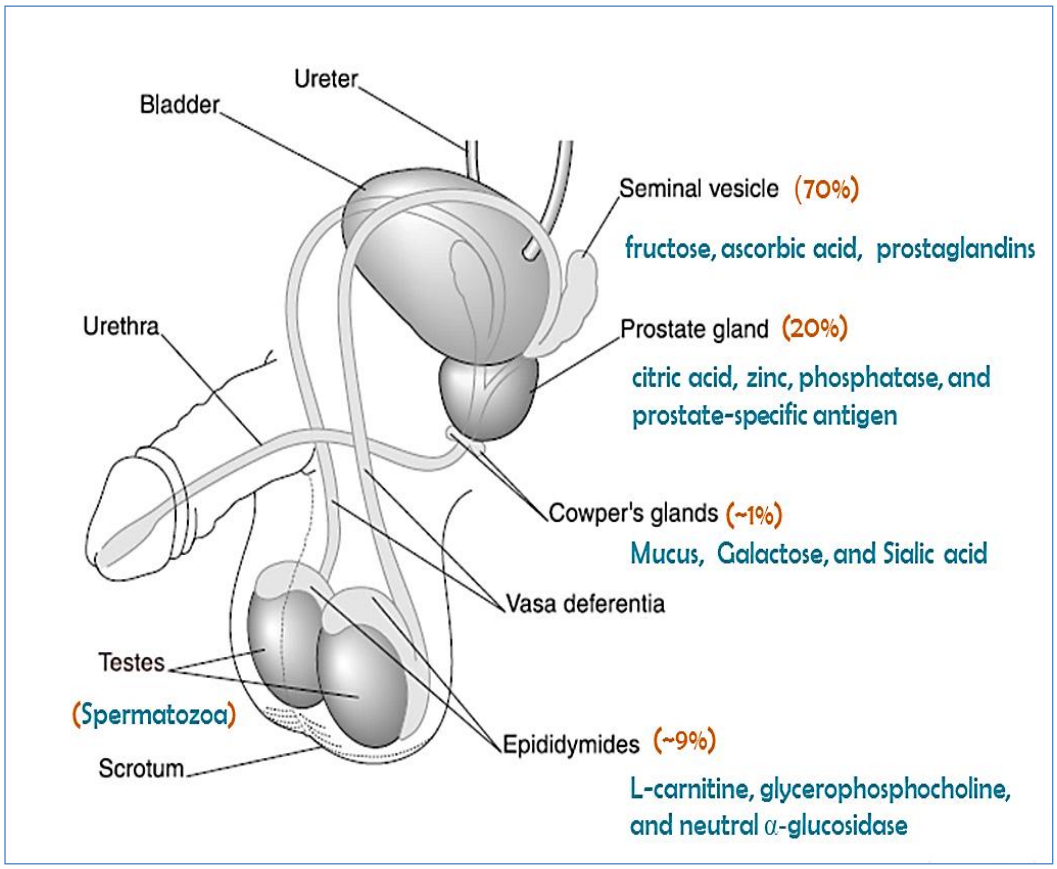


Figure 2.1. Male sex accessory glands and their main products (Barone and Pollack, 2009; edited by the researcher).

2.2.2.1. Seminal citric acid

Citric acid is produced by the prostate at puberty before active spermiogenesis (Kanyo and Sas, 1975). The principal function of citric acid is to maintain the pH and osmotic balance of semen, which preserve membrane function and sperm shape (Said *et al.*, 2009; Huang *et al.*, 2013). Citrate is an anionically charged anion because it has a high affinity for Ca^{+2}, Mg^{+2}, and Zn^{+2} ions (Owen and Katz, 2005). It regulates ionised Ca^{+2} levels in SP to prevent premature sperm capacitation and acrosome activation (Jequier, 2011).

Citric acid has antioxidant and anti-inflammatory features in tissues affected by environmental causes (Abdel-Salam *et al.*, 2014); it also promotes the production of glycosaminoglycans in numerous tissues and is associated with the coagulation and liquefaction of human semen. Consequently, citric acid is important for sperm motility and hyaluronidase activity (Huang *et al.*, 2013). Some studies have shown a positive relationship between seminal citric acid concentration and sperm count, morphology, and motility (Toragall *et al.*, 2019; AL-Khazali *et al.*, 2020; Shemshaki *et al.*, 2021).

The citric acid value is 10 mg or more per ejaculate (Sonbol and Elhanbly, 2021). Citrate levels are a reliable predictor of prostate function, being very high in cases of benign prostatic hyperplasia and very low in cases of prostate cancer or inflammation (Kavanagh, 1994; Alshahrani *et al.*, 2013).

2.2.2.1.1. Citrate metabolism

In humans and other animals, the normal function of the prostate gland highly depends on citrate metabolism (Costello and Franklin, 2002). The primary role of the typical human prostate is to accumulate and secrete very high amounts of citrate, which accounts for the exceptionally high citrate range in the prostatic fluid that is around 400–1500 times higher than in the blood plasma. This ability is possessed by glandular secretory epithelial cells in the prostate-peripheral zone, also known as "citrate-producing" cells (Costello and Franklin, 2009).

Citrate is an essential intermediary in cell metabolism in all other cells, but it is an end product of metabolism in prostate cells. It is due to the reduced capability of prostatic mitochondria to oxidize citrate, resulting in an imbalance in citrate synthesis and oxidation (Costello and Franklin, 2000).

Citrate accumulation occurs in prostatic epithelial cells as a result of an increase in Zn^{+2} accumulation in these cells, which inactivates mitochondrial aconitase that oxidizes citrate to isocitrate as the first reaction in the Kreb's cycle. Thus, the Kreb's cycle in these cells gets truncated, and citrate is secreted in the prostatic fluid. On the other hand, the hormones T and prolactin control the formation of prostate citrate (Costello and Franklin, 1991b; Costello and Franklin, 1998).

The prostate secretory epithelial cells require exogenous substrates to provide the six-carbon source for continued citrate synthesis. Aspartate serves as a 4-carbon source of oxalacetate (OAA) through a unique mitochondrial glutamate-aspartate-citrate pathway, and glucose serves as a 2-carbon source of acetyl CoA (AcCoA) through pyruvate oxidation for net citrate production **(Figure 2.2)** (Costello and Franklin, 1989; Costello and Franklin, 1991a).

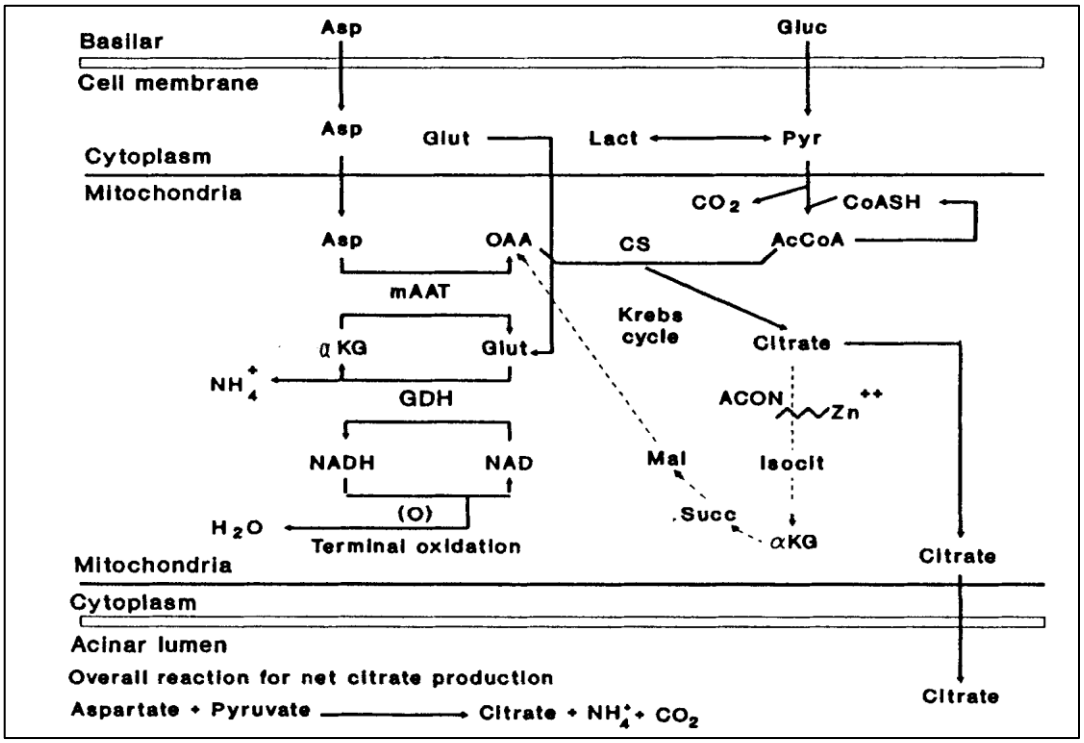


Figure 2.2. The pathway of net citrate production in prostate secretory epithelial cells. Asp, aspartate; **Gluc,** glucose; **Glut,** glutamate; **Lact,** lactate; **Pyr,** pyruvate; **OAA,** oxalacetate; **AcCoA,** acetyl CoA; **Isocit,** isocitrate; **aKG,** a-ketoglutarate; **Succ,** succinate; **Mal,** malate; **mAAT,** mitochondria1 aspartate aminotransferase; **GDH,** glutamate dehydrogenase; **CS,** citrate synthase; **ACON,** aconitase **(Costello and Franklin, 1991b).**

2.2.2.2. Seminal fructose

Since the beginning of mammalian semen studies, including men, glucose has been thought to be the principal sugar abundantly present in semen (Mann and Lutwak-

Mann, 1951). However, D-fructose was identified as the primary sugar in SP by Mann (1946). Seminal vesicles produce a large amount of fructose, while the ampulla of the deferens secretes a small amount (Trang *et al.*, 2018). Additionally, seminal vesicles secrete small amounts of glucose, sorbitol, and ribose, but their combined concentration is less than 1/20th that of fructose. Although androgenic hormones regulate the secretion of fructose from seminal vesicles, numerous variables, such as ejaculatory frequency, blood glucose levels, and healthful state, can influence seminal fructose concentration (Gonzales, 1989; Plant and Zeleznik, 2014).

The total of seminal fructose is 13 μmol (2.4 mg) or more per ejaculate (Flint *et al.*, 2015). Seminal fructose levels below normal indicate seminal vesicle dysfunction. Thus, a measure of fructose content in SP can assist in determining the secretory efficiency of the seminal vesicles, endocrine disorders, and any potential ejaculatory duct blockage (Buckett and Lewis-Jones, 2002; Toragall *et al.*, 2019).

2.2.2.2.1. Fructose metabolism

Fructose is the primary energy source for spermatozoa. It derives in the seminal vesicles from blood glucose with three alternative metabolic pathways for its production: glycogenolysis, direct phosphorylation to generate glucose-6-phosphate, and a non-phosphorylative pathway for the production of sorbitol **(Figure 2.3)** (Ahmed *et al.*, 2010; Kumar and Sharma, 2017).

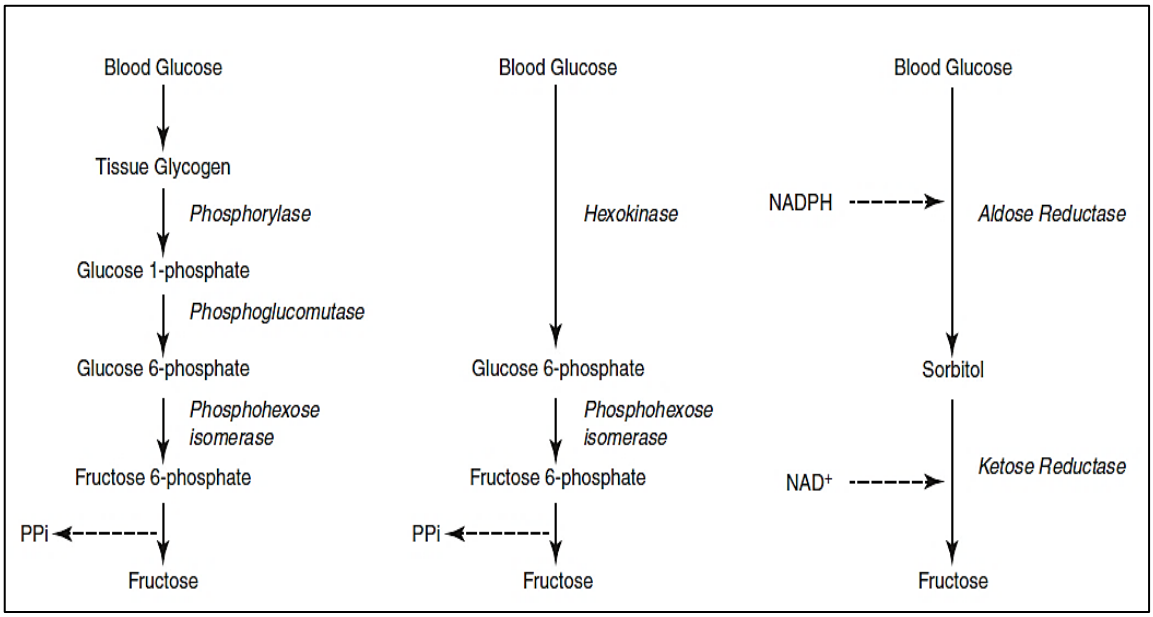


Figure 2.3. Biosynthesis of fructose from blood glucose by seminal vesicles (Kumar and Sharma, 2017).

Fructolysis is a mechanism of both the aerobic and anaerobic metabolism of spermatozoa. Because the vaginal environment is hypoxic, fructose is metabolised anaerobically to lactate. Fructolysis begins with the phosphorylation of fructose by adenosine triphosphate (ATP) to form fructose 6-phosphate, and then further metabolism occurs through fructose 1,6-diphosphate, phosphoglyceric acid, pyruvic acid, and lastly, lactic acid **(Figure 2.4)**. As a result, fructolysis provides the energy for sperm motility (Mann, 1946; Mann and Lutwak-Mann, 1951).

It has been reported that a positive correlation exists between the fructolysis rate and sperm density and motility in human semen (Mann *et al.*, 1980). On the other hand, many previous studies indicate a negative correlation between seminal fructose and sperm concentration, sperm vitality, and sperm progressive motility. Thus, the lower fructose concentration is attributed to sperm consuming more fructose as a source of energy (Ali *et al.*, 2011; Amidu *et al.*, 2012; Trang *et al.*, 2018).

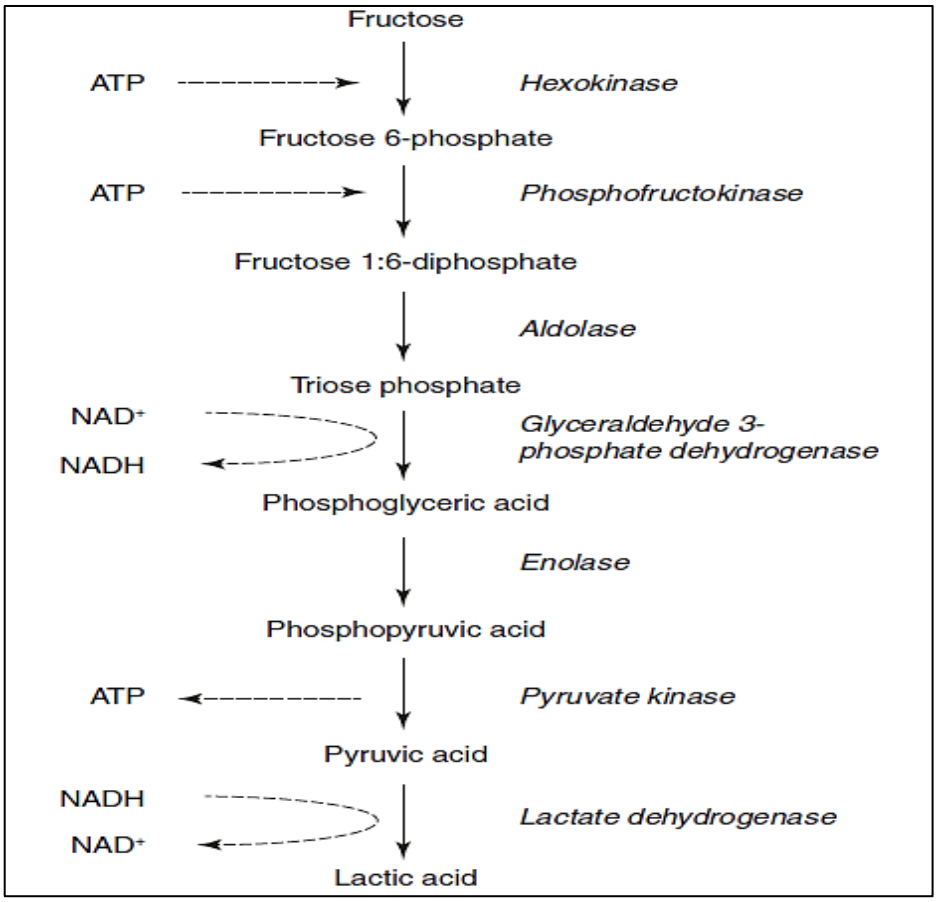


Figure 2.4. The fructolysis pathway in mammals' sperm (Kumar and Sharma, 2017).

Spermatozoa can also efficiently metabolize glucose and mannose substrates thanks to the enzymatic machinery at their disposal (Mann, 1946). However,

mammalian spermatozoa require a high energy supply after ejaculation (Goss, 2018). Several authors have reported that sperm can utilize metabolic substrates of non-hexose compounds, such as lactate, pyruvate, and citrate, to maintain their energy needs through pathways other than glycolysis, such as activation of the Krebs cycle (Medrano *et al.*, 2006; Visconti, 2012).

2.2.2.3. Seminal neutral alpha glucosidase (NAG)

Sheth and Rao (1962) were the first who discover an α-glucosidase in human semen. There are two isoforms of α-glucosidase in the SP: the acidic form of α-glucosidase originates from the prostate, and the neutral type is produced mainly by the epididymis (WHO, 2010). Alpha-glucosidase is a carbohydrate hydrolase that converts α-1,4-linked oligosaccharides (disaccharides and starch) into glucose (Dias *et al.*, 2004).

In the epididymis, the spermatozoa undergo many molecular changes on their surface to achieve progressive motility and the ability to fertilize. NAG activity is considered more specific and sensitive to epididymal disorders (Azenabor *et al.*, 2016). It is highly produced in the corpus and cauda sections of the epididymis when spermatogenesis begins (Castellon *et al.*, 2000).

Although the exact role of NAG in sperm function is unknown, some researchers suggest that it contributes to sperm maturation by modifying surface glycoproteins (Dias *et al.*, 2004). Furthermore, a positive correlation between NAG activity and zona pellucida binding ability has been observed in humans. Thereby, this enzyme is consistent with an efficient epididymal function in terms of sperm maturation and the acquisition of fertilization ability (Ben Ali *et al.*, 1994; Yassa *et al.*, 2001).

It has been reported that the presence of NAG correlates with the glycogen content of various organs or genital secretions, providing energy for sperm maturation and increasing their vitality in cervical mucus with lower glucose levels (Grandmont *et al.*, 1983; Fourie *et al.*, 1993). One study showed that increased sperm motility of more than 40% is related to increased seminal NAG activity and decreased spermatozoal ATP (Fourie *et al.*, 1991). In addition, several studies have shown that the activity of NAG is positively correlated to the main semen parameters, including spermatozoa count, morphology, motility, and progressive motility (Sundaram *et al.*, 2016; Qiu *et al.*, 2018).

The reference limit for NAG is 20 or more (mIU/ejaculate) in healthy men. NAG deficiency may indicate epididymal inflammation or abnormal sperm maturation (Cooper *et al.*, 1990). In most cases, quantitative NAG measures can localize the blockage and differentiate between obstructive and non-obstructive azoospermia (Sandoval *et al.*, 1995).

2.2.3. Hormonal regulation of MAGs secretions

The primary regulators of male reproductive functions are gonadotropin-releasing hormone (GnRH), luteinizing hormone (LH), and follicle-stimulating hormone (FSH). FSH receptors are present on Sertoli cell membranes, while LH receptors are present on Leydig cell membranes. They coordinate to maintain normal spermatogenesis and steroidogenesis (Terranova, 2000).

The main androgen in men is the T hormone, secreted by Leydig cells under the influence of LH. It plays an essential role in the maintenance of spermatogenesis, and the most remarkable feature of T is its ability to stimulate the secretory activity of the MAGs. Furthermore, the pituitary gland secretes prolactin, which acts in tandem with T to regulate Zn^{+2} uptake and protein synthesis as well as the secretion of citric acid, fructose, and NAG (Biswas *et al.*, 1978; Mann and Lutwak-Mann, 1981; Castellon and Huidobro, 1999; Costello and Franklin, 2002).

The MAGs rely specifically on the continuous use of dihydrotestosterone (DHT) as a T-derived androgen by the enzyme 5α-reductase. DHT has a fivefold higher affinity for the androgen receptor (AR) than T. When DHT or T binds to the AR, it translocates to the nucleus, interacts with chromatin, and triggers specific gene transcription **(Figure 2.5)**. This, in turn, leads to the production of specific protein products, known as the "hormone response". Also, primary gene transcript processing, mRNA stability, and the translation may all be affected by hormones (Mann and Lutwak-Mann, 1981; Brooks, 1983).

On the other hand, testicular estrogen is created by T aromatization. Estrogen has a high impact on testicular function and sperm quality through hormonal cross-talk and direct action on testicular cells via estrogen receptors (ER), including alpha (ERα) and beta (ERβ), which exist on testicular cells **(Figure 2.5)** (Molina, 2006; Dutta *et al.*, 2019).

Recent studies have demonstrated that several variables can affect male fertility by inducing OS on an individual hormonal axis or by disrupting the cross-talk between different endocrine systems, thus reducing the production of T. Accordingly, low T leads to failure in spermatogenesis and the normal secretion of MAGs, which play crucial roles in sperm maturation (Darbandi *et al.*, 2018; Leisegang *et al.*, 2021).

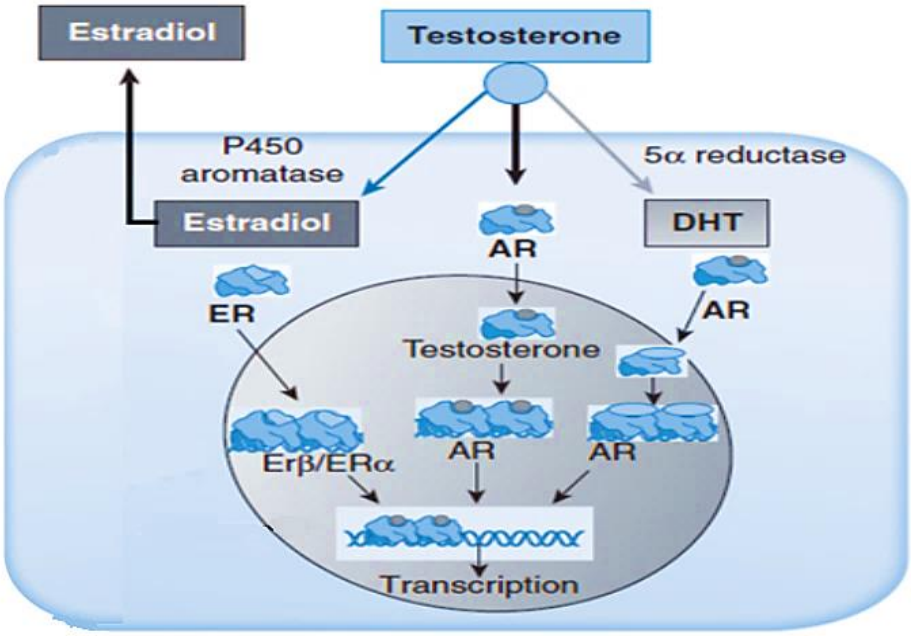


Figure 2.5. Mechanisms of action of testosterone (Molina, 2006).

2.3. Oxidative stress (OS) and male infertility

Numerous clinical issues, such as cancer, connective tissue disease, aging, infection, inflammation, and male infertility, are associated with OS (Saleh and Agarwal, 2002). According to the current literature, OS is responsible for 30% to 80% of male infertility cases. OS occurs when ROS production exceeds the antioxidant defense mechanisms' capacity (Tremellen, 2008).

Scottish scientist MacLeod (1943) reported the first evidence that spermatozoa were exposed to OS when cultured in an oxygen-rich environment, where their motility rapidly decreased. Therefore, excessive generation of ROS disrupts spermatozoa activities through their interference with cellular biomolecules such as lipids, proteins, nucleic acids, and sugars (Wang *et al.*, 2003; Henkel, 2011). On the other hand, low levels of ROS are needed for spermatozoa to acquire fertilization capacity (Saleh and Agarwal, 2002).

2.3.1. Reactive species and free radicals

ROS are hazardous products of oxygen metabolism and oxidative phosphorylation in mitochondria, as well as during the action of some enzymatic systems, such as cytochrome P450 and xanthine oxidase. Therefore, antioxidants help maintain a typical redox balance by neutralizing or removing excessive ROS (Tunc, 2011; Cruz, 2014).

ROS are a vast group of chemicals that include radicals such as superoxide anion ($O_2^{\bullet-}$) and hydroxyl radicals ($^{\bullet}OH$), as well as non-radicals such as hydrogen peroxide (H_2O_2). Moreover, another type of free radical is nitrogen-derived, referred to as reactive nitrogen species (RNS), such as nitric oxide (NO) and peroxynitrite ($ONOO^-$) **(Table 2.2)** (Agarwal and Prabakaran, 2005; Bansal and Bilaspuri, 2011).

Table 2.2. List of ROS and RNS present as non-radicals and free radicals (Agarwal and Prabakaran, 2005).

Types of reactive oxygen species that exist as radicals and free radicals			
Radicals		Non-Radicals	
Hydroxyl	$OH^{\bullet}$	Peroxynitrite	$ONOO^-$
Superoxide	$O_2^{\bullet-}$	Hypochloric acid	HOCl
Nitric Oxide	$NO^{\bullet}$	Hydrogen Peroxide	H_2O_2
Thyl	$RS^{\bullet}$	Singlet Oxygen	$^{-1}O_2$
Peroxyl	$RO_2^{\bullet}$	Ozone	O_3
Lipid peroxyl	$LOO^{\bullet}$	Lipid peroxide	LOOH
Types of reactive nitrogen species that induces oxidative stress			
Nitrous oxide	$NO^{\bullet}$	Nitrosyl cation	NO^+
Peroxynitrite	$OONO^-$	Nitrogen dioxide	$NO_2^{\bullet}$
Peroxynitrous acid	ONOOH	Dinitrogen trioxide	N_2O_3
Nitroxyl anion	NO^-	Nitrous acid	HNO_2
Nitryl chloride	NO_2Cl		

Free radicals are described as any atom or molecule containing one or more unpaired electrons that are very unstable and react rapidly with other molecules in an attempt to grab the required electron. Generally, free radicals assault and seize an electron from the closest stable molecule. When an attacked molecule loses an electron, it turns into a free radical, initiating a chain reaction. Therefore, the process may cascade once it has begun, eventually culminating in the destruction of a live cell (Hammadeh *et al.*, 2009; Ogbuewu *et al.*, 2010).

2.3.1.1. ROS sources in seminal fluid

ROS can arise from various sources, such as defective spermatogenesis, infection, varicocele, smoking, excessive alcohol and drug use, ionizing radiation, psychological stress, excessive exercise, and environmental pollution. Generally, these sources are categorized as endogenous and exogenous **(Figure 2.6)** (Barati *et al.*, 2020).

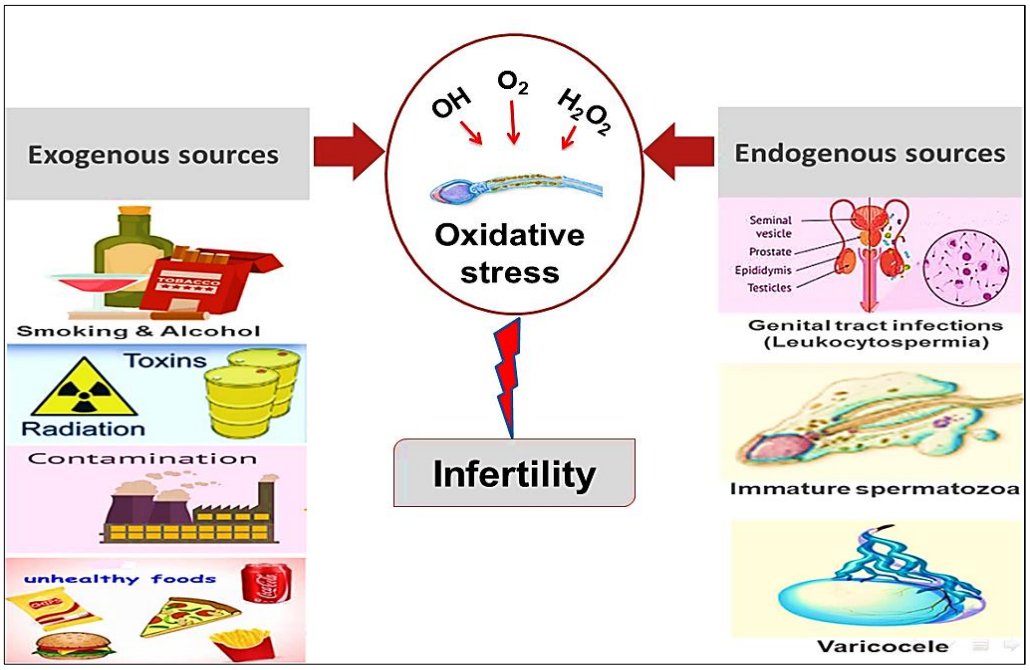


Figure 2.6. Main causes (exogenous and endogenous) of oxidative stress.

2.3.1.1.1. Endogenous sources

There are two major sources of endogenous seminal ROS production: spermatozoa and leukocytes (Tremellen, 2008).

2.3.1.1.1.1. Leukocytes

The leukocytes play a significant role in inflammation and infection conditions and are the principal generator of ROS in the semen. Each milliliter of semen contains up to $1x10^6$ leukocytes with peroxidase activity, and any value more than this is considered leukocytospermia (Henkel, 2011; Barati *et al.*, 2020). The peroxidase-positive leukocytes are polymorphonuclear (PMN) leukocytes, which comprise 50%–60% of all seminal leukocytes, while macrophages constitute another 20%–30%. The prostate and seminal vesicles are the primary sources of peroxidase-positive leukocytes in the semen (Esfandiari *et al.*, 2003), while the macrophages primarily originate from the testicular interstitium and epididymis (Tunc, 2011).

Macrophages and PMN granulocytes can significantly damage sperm through the generation of ROS and the release of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukins (IL-8 and IL-6) (Agarwal *et al.*, 2020). ROS production arises when leukocytes break down pathogens by activating the myeloperoxidase pathway. Activated leukocytes enhance nicotinamide adenine dinucleotide phosphate (NADPH) generation through the hexose monophosphate shunt (HMPS), enabling them to create 100 times more ROS than non-active leukocytes (Agarwal *et al.*, 2014b).

2.3.1.1.1.2. Immature spermatozoa

Spermatozoa can also produce ROS independently of leukocytes, and this ability appears to be primarily dependent on the maturity level of the sperm cell. The primary morphological change that occurs in the spermatozoon during the epididymal transit, which is the disposal of the cytoplasmic droplet, a remnant of the cytoplasm associated with testicular sperm (Tvrda *et al.*, 2018).

In immature aberrant sperm, excess cytoplasm remains in the mid-piece, resulting in the retention of several glucose metabolism-regulating enzymes, such as glucose-6-phosphate dehydrogenase (G6PD). Thus, G6PD increases metabolism, which leads to increased NADPH production that initiates more ROS generation via the HMPS **(Figure 2.7)** (Hammadeh *et al.*, 2009; Agarwal *et al.*, 2020). Indeed, the primary producers of ROS in immature sperm are NADPH oxidase (NOX) in the plasma membrane and NADH-dependent oxidoreductase (diaphorase) in the mitochondria (Barati *et al.*, 2020).

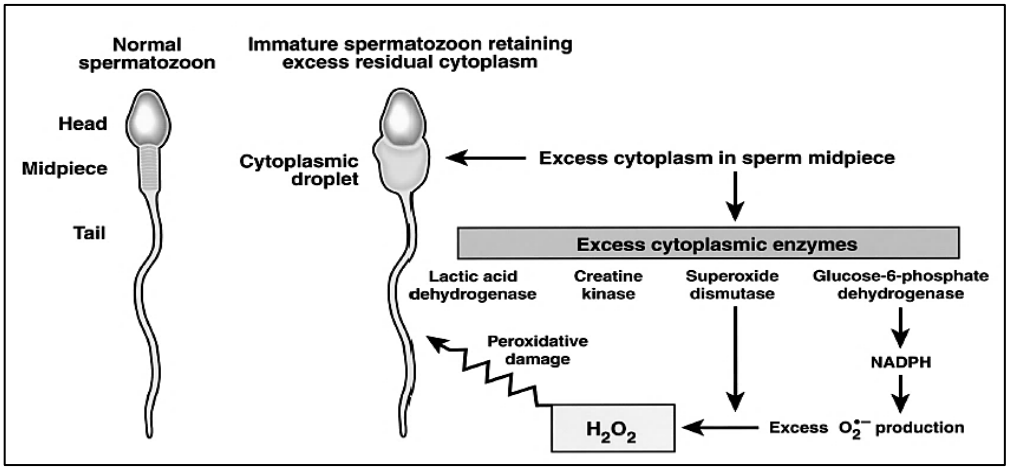


Figure 2.7. Mechanism of increased production of ROS by abnormal spermatozoa (Saleh and Agarwal, 2002).

2.3.1.1.2. Exogenous sources

Exogenous ROS may be generated by a range of lifestyle and environmental factors, including unhealthy foods, excessive smoking, and alcohol use, as well as radiation and contaminants. These variables may increase ROS production to hazardous levels, causing oxidative damage to sperm and thus resulting in male infertility (Tvrda *et al.*, 2018).

2.3.1.2. Physiological role of ROS on sperm

The function of reproductive cells depends on aerobic metabolism, which uses oxygen, so the most abundant ROS in spermatozoa are $^{\bullet}OH$, $O_2^{\bullet-}$, H_2O_2, and peroxyl ($ROO^{\bullet}$), which are necessary for cell homeostasis but whose metabolites can alter sperm function. Therefore, spermatozoa create trace amounts of ROS essential for various physiological processes, including capacitation, hyperactivation, acrosome reaction, and sperm-oocyte fusion **(Figure 2.8)** (Cruz, 2014; Du Plessis *et al.*, 2015).

ROS are necessary for appropriate chromatin packing and stability during the development of spermatozoa, which can act as oxidizing agents to promote the synthesis of disulfide bonds between the cysteine residues of protamine—small nuclear proteins that replace histones during spermatogenesis (Erenpreiss *et al.*, 2006). Since spermatozoa lack repair mechanisms, chromatin condensation is a crucial protective mechanism, as ROS protect male gametes from subsequent oxidative attacks (Tvrda *et al.*, 2018).

ROS, Ca^{+2}, and tyrosine kinase levels increase during sperm capacitation. These substrates increase cyclic adenosine monophosphate (cAMP) levels, which in turn activate protein kinase A (PKA), the enzyme responsible for the phosphorylation of tyrosine (O'Flaherty *et al.*, 2006; Cruz, 2014). Hence, tyrosine phosphorylation leads to sperm hyperactivity and the acrosome reaction, allowing sperm–oocyte fusion (Wagner *et al.*, 2018).

2.3.1.3. Pathological role of seminal ROS

When very high levels of ROS surpass the body's natural antioxidant defenses, sperm suffer several damages, including lipid peroxidation (LPO), sperm DNA fragmentation (SDF), and apoptosis **(Figure 2.8)** (Makker *et al.*, 2009).

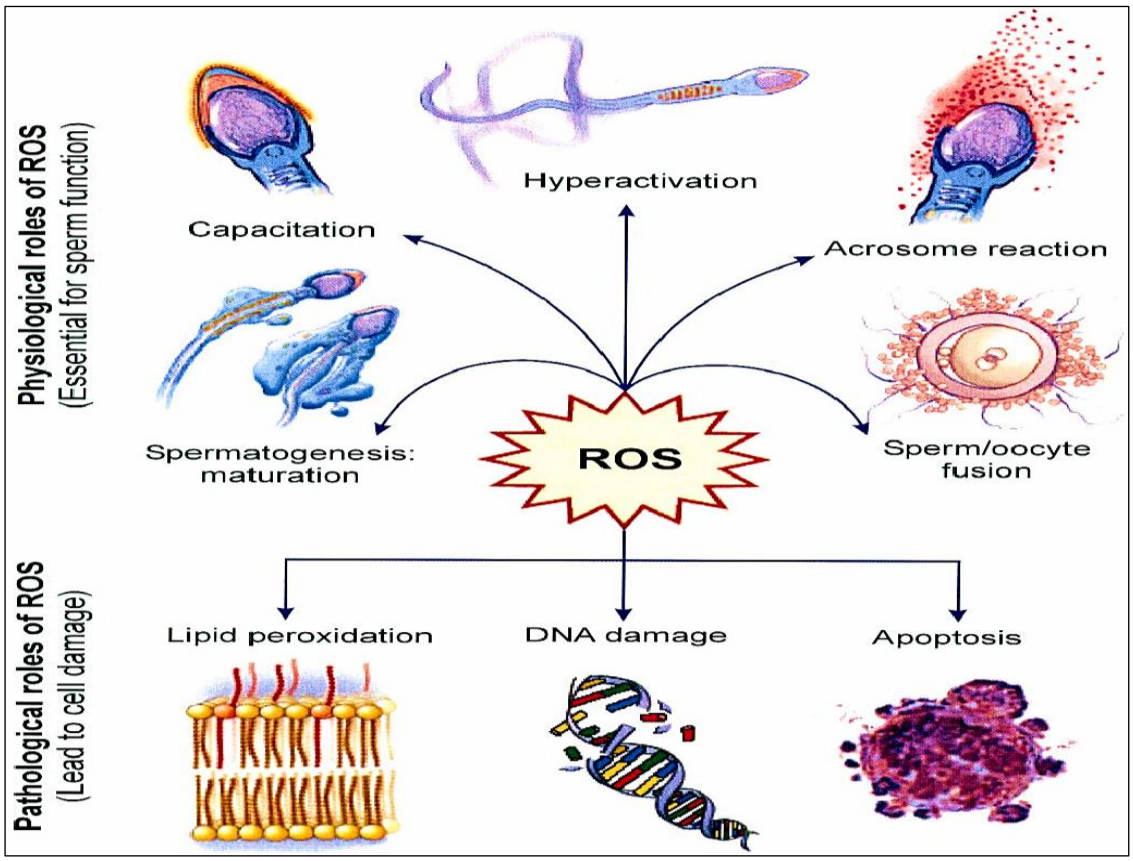


Figure 2.8. The physiological and pathological roles of ROS in spermatozoa (Kothari *et al.*, 2010).

LPO: The sperm plasma membrane contains polyunsaturated fatty acids (PUFAs) with unlinked double bonds within the methylene groups. The presence of this double bond weakens the carbon-hydrogen bond, making hydrogen more vulnerable to oxidative attack. As a result of increased levels of ROS in the sperm, the fluidity of the sperm membrane weakens and becomes specifically impenetrable, which leads to disruption of its receptors and enzymes, hence impairing sperm functions (Tvrda *et al.*, 2018).

The LPO process comprises three stages: initiation, propagation, and termination **(Figure 2.9)**. In the first stage, free radicals react with fatty acid chains, removing hydrogen from adjoining methylene groups and forming water molecules and lipid-free radicals that may react with oxygen molecules to create $ROO^{\bullet}$ radicals (Evans *et al.*, 2021). These radicals may interact with other adjacent fatty acids to generate more free radicals, thus propagating the process. This process terminates when two radicals react to generate a stable, non-radical product (Agarwal *et al.*, 2012). However, malondialdehyde (MDA) is produced as a byproduct of LPO, which is commonly used in laboratory tests to measure peroxidative damage in sperm (Makker *et al.*, 2009).

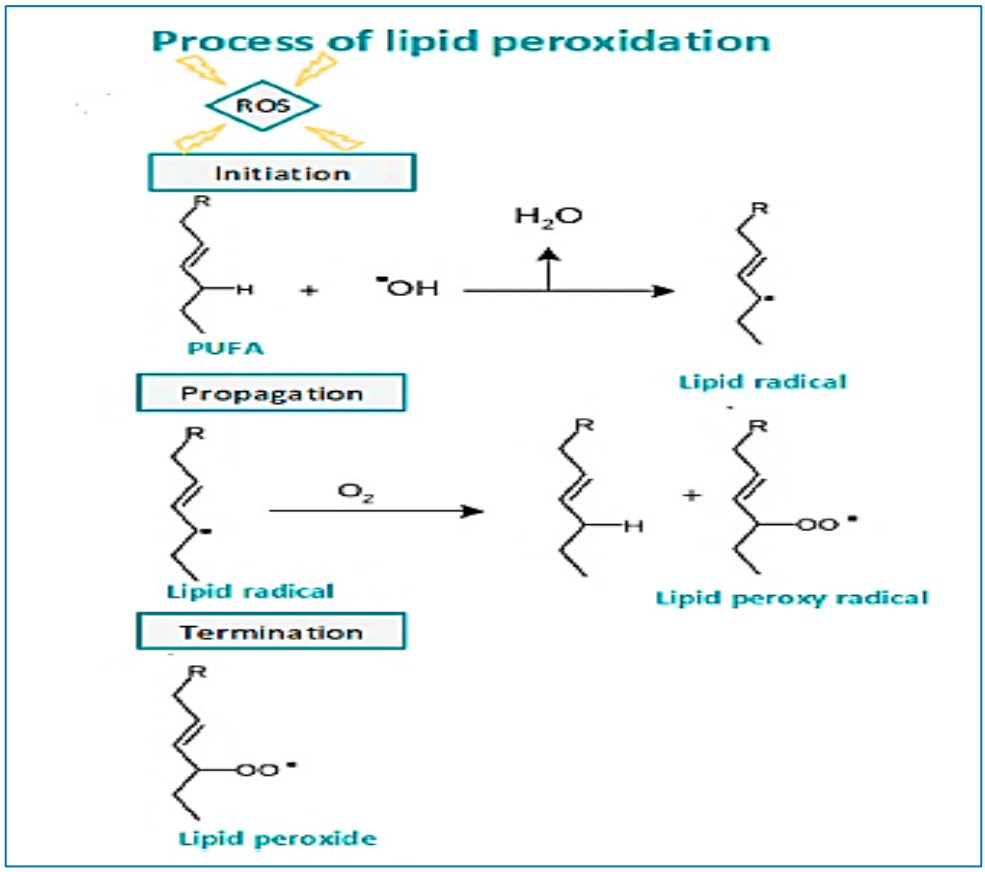


Figure 2.9. Stages of the lipid peroxidation process (Evans *et al.*, 2021).

The SDF is caused by high exposure to ROS and low levels of antioxidants in the body, leading to inadequate nuclear maturation, DNA breaks, poor DNA integrity, or chromosomal aneuploidies (Townsend, 2016). Similarly, ROS may result in mutations in mitochondrial DNA (mtDNA), which lead to defects in mitochondrial energy metabolism and thus affect sperm motility in vivo (Tvrda *et al.*, 2018). DNA damage is connected with apoptosis, a low fertilization rate, a high probability of a miscarriage, and morbidity in offspring (Khomami, 2018).

Many cell death signalling and regulatory pathways are activated during apoptosis due to DNA fragmentation. ROS can induce double-strand DNA breaks and subsequently lead to spermatozoa apoptosis. Additionally, ROS destroy sperm mitochondrial membranes, releasing the signalling molecule cytochrome C, which activates apoptotic caspases and annexin-V (Takeshima *et al.*, 2021). Therefore, cytochrome C release during the apoptotic pathway increases ROS levels further, causing DNA damage and fragmentation and possibly enhancing the apoptotic cycle (Wagner *et al.*, 2018).

Accordingly, Wang *et al.* (2003) found a positive relationship between sperm damage, increased OS, and germ cell apoptosis in patients with MFI. Furthermore, another study revealed a significant negative correlation between elevated ROS levels and sperm concentration, total motility, and normal morphology (Moein *et al.*, 2007).

2.3.1.4. OS as a cause and result of male genital tract inflammation

Male accessory gland infections (MAGI) are detected in 5–12% of male infertility cases (Tunc, 2011). MAGI is caused by two factors: microbial and inflammatory. Microbial forms include bacteria, fungi, and viruses, while inflammatory responses are characterized by leukocytospermia, high levels of pro-inflammatory cytokines (IL-1, IL-6, IL-8, TNFα, etc.), increased ROS production, and/or excessive antisperm (La Vignera *et al.*, 2011).

In the late '60s, the first indication emerged that infection of the MAGs might impair their secretory function (Weidner *et al.*, 1999). In addition, several studies have demonstrated that infections of the male genital tract (MGT) result in a decrease in the concentration of some markers of MAGs activity in semen, especially citric acid, fructose, and glucosidase, indicating that these changes occur throughout the inflammation (Cooper *et al.*, 1990; Marconi *et al.*, 2009; Djordjevic *et al.*, 2018).

Numerous male infertility causative variables pave the way for male reproductive function impairment through the shared mechanisms of OS and inflammation (Dutta *et al.*, 2022). Both OS and inflammation are mutually reinforcing pathophysiological processes in which one causes the other to happen. Both of these processes may occur concurrently in the pathophysiology of male infertility (Dutta *et al.*, 2021). Varicocele, lifestyle factors, obesity, and metabolic syndrome all induce an inflammatory response, attract leukocytes, and generate ROS (Agarwal *et al.*, 2018).

During the inflammatory phase, inflammatory stimuli activate transcription factors, further promoting inflammatory processes in MGT and thus generating more ROS. On the other hand, excessive ROS production may cause the oxidation of membrane phospholipids and proteins, activating transcription factors that enhance the synthesis of pro-inflammatory mediators. As a result, inflammation and OS create a feedback loop **(Figure 2.10)** (Dutta *et al.*, 2021; Dutta *et al.*, 2022).

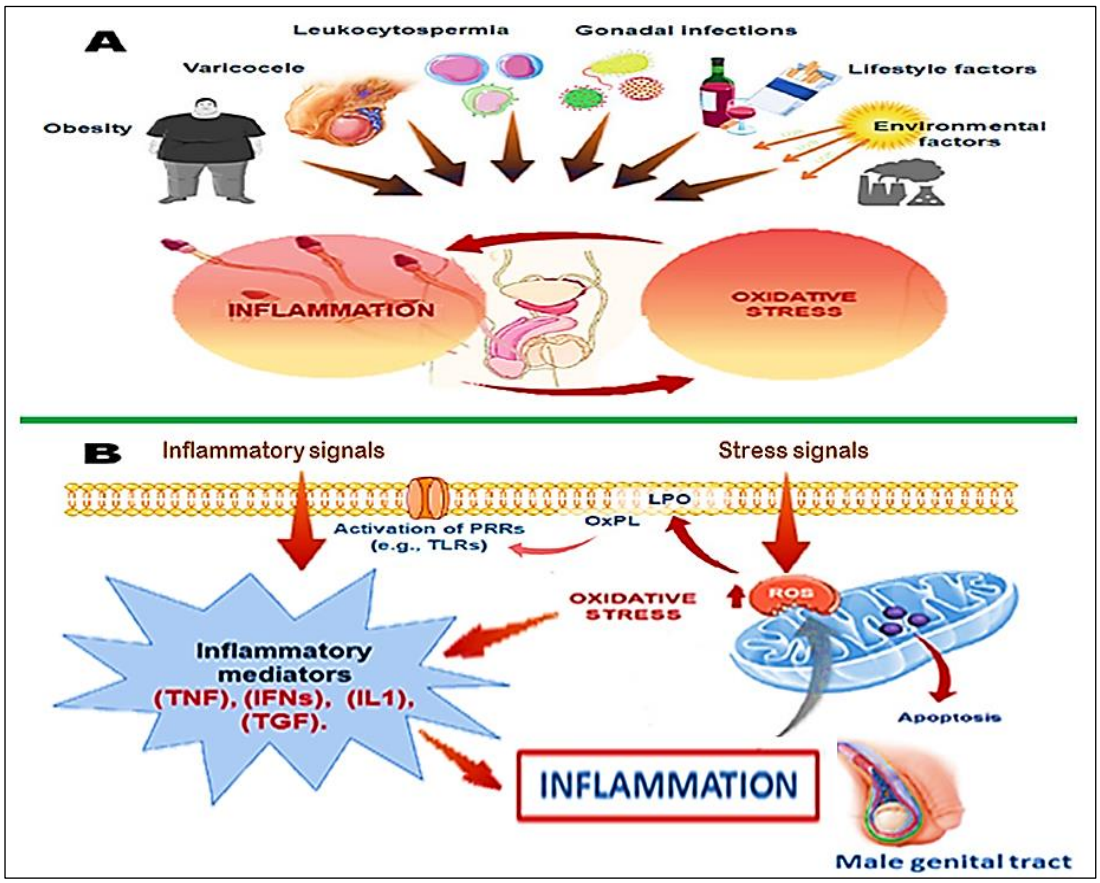


Figure 2.10. Inflammation and oxidative stress as primary mediators of prevalent causes of male infertility. (A) Exogenous and endogenous factors impacting the male fertility via induction of OS and inflammation. **(B)** Inflammatory and oxidative signals activate pattern recognition receptors (PRRs) in the MGT, resulting in the expression of inflammatory mediators that cause exaggerated inflammation and can also act as OS stimuli, thereby creating a vicious loop impairing male fertility **(Dutta *et al.*, 2021; edited by the researcher).**

2.3.2. Antioxidant defenses

The semen contains a variety of antioxidant defense mechanisms that prevent the detrimental effects of ROS and protect the sperm from OS (Cruz, 2014). Antioxidants are molecules that prevent the oxidation chain reaction by donating their electrons to free radicals. The electron-donating antioxidants do not convert to hazardous free radicals due to their capacity to tolerate the change in their electrons without becoming reactive. There are two types of seminal antioxidants: enzymatic and non-enzymatic (Ogbuewu *et al.*, 2010).

Enzymatic antioxidants are composed of SOD, CAT, GPx, and GR. These enzymes protect human sperm against LPO by decreasing lipid hydroperoxide formation. Antioxidants such as SOD, CAT, and GPx convert $O_2^{\bullet-}$ and H_2O_2 into O_2 and H_2O. GPx acts on regenerating reduced glutathione (GSH) from its oxidized form

(GSSG) using NADPH produced in the HMPS **(Figure 2.11)** (Hammadeh *et al.*, 2009; Lourenco and Fernandes, 2015).

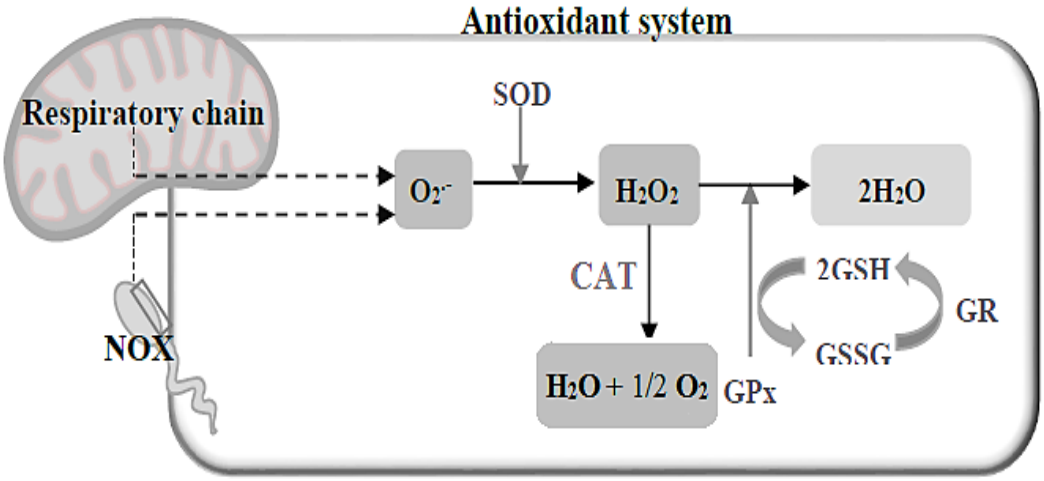


Figure 2.11. The action of antioxidant enzymes in neutralizing oxidizing factors (Lourenco and Fernandes, 2015).

Many non-enzymatic antioxidants enhance total seminal antioxidant activity, depending on the body's dietary intake of synthetic antioxidants and dietary supplements (Bansal and Bilaspuri, 2011). They include Zn^{+2}, vitamins E and C, albumin, carnitine, and other nutrients. These antioxidants neutralize the activity of free radicals,, either by oxidizing themselves or by directly inhibiting free radicals generation (Tunc, 2011). Several male gonads are the primary source of seminal antioxidants, but they cannot be the exclusive secretors **(Table 2.3).**

Table 2.3. The seminal antioxidant types and the main male reproductive glands that secrete them (Ribeiro *et al.*, 2021).

Reproductive Glands	Antioxidants
Epididymis	Carnitines
Seminal Vesicle	Vitamin C
Prostate	Zinc Selenium Glutathione peroxidase Glutathione reductase Superoxide dismutase Catalase Albumin

3. Materials and methods

3.1. Materials

The materials of this study are shown in the following table:

Table 3.1. List of laboratory chemical reagents, commercial kits, equipment, and tools.

1. The chemical reagents	**Manufacture**
Methylene Blue, 1% (w/v)	Sigma-Aldrich (USA).
Eosin Y, 0.5% (w/v)	Sigma-Aldrich (USA).
Ortho-Toluidine	Sigma-Aldrich (USA).
2. Commercial kits	**Manufacture**
Citric Acid Test	FertiPro, Beernem (Belgium).
Fructose Test	FertiPro, Beernem (Belgium).
EpiScreen Plus	FertiPro, Beernem (Belgium).
CANros	Candore BioScience (India).
3. Equipment and tools	**Manufacture**
Light microscope	Carl Zeiss LR113484 (Germany).
Hemocytometer	Neubauer (Germany).
Makler counting chamber	Sefi-Medical Instruments (Israel).
Centrifuge	Eppendrof AG, Hamburg (Germany).
Vortex	Stuart SA8 (UK).
Incubator	Memmert 854 W (Germany).
Water bath	Thermo Electron Corporation (USA).
UV/ Vis Spectrophotometer	JUNWAY 6505 (Germany).
Plate reader	Das srl (Italy).

3.2. Methods

3.2.1. Semen samples collection

Samples of 104 were collected from fertility laboratories in Tripoli, Libya (Yashfeen Centre and Al Shark Lab). The men who participated in the study were over 25 years old; 25 were fertile, and 79 were infertile. The samples were collected by masturbation after 2–5 days of ejaculatory abstinence into a sterile container. Then, analyses relevant to this study were performed. The following flow chart shows the study design.

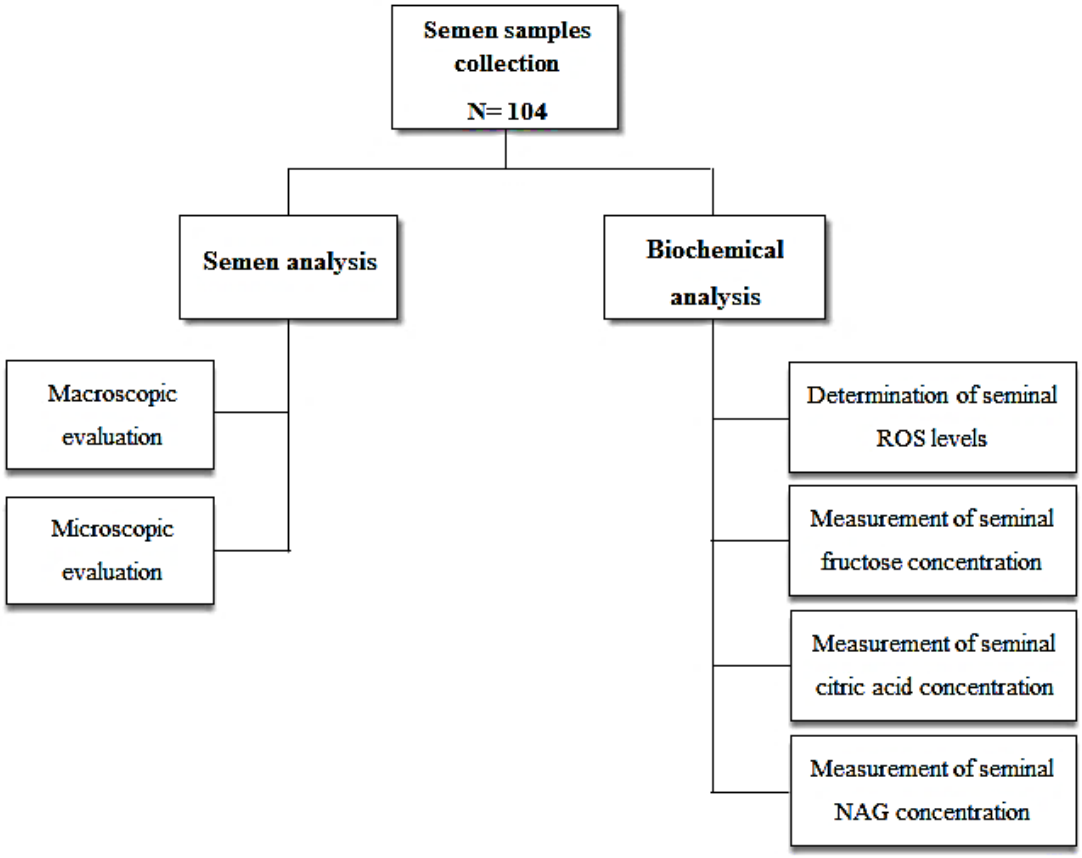


Figure 3.1. Flow chart showing the study methods.

3.2.2. Semen analysis

Semen analysis was performed on all samples according to the WHO procedure (2021).

3.2.2.1. Macroscopic examination

The macroscopic evaluation started immediately after liquefaction to avoid dehydration or temperature changes that might affect the semen quality.

3.2.2.1.1. Semen liquefaction

After collection, samples were incubated at 37 °C for 15–20 minutes to liquefy, which might take up to 60 min or more in some cases. When the liquefaction process lasted more than 60 min, it was recorded as abnormal.

3.2.2.1.2. Semen viscosity

After liquefying the sample, the viscosity was evaluated by aspirating it with a wide-bore (about 1.5 mm in diameter) plastic pipette, allowing the semen to fall naturally, and measuring the length of any thread. When the viscosity of the semen is normal, it falls in the form of discrete, tiny droplets. If the sample is abnormal, the drop forms a thread more than 2 cm long.

3.2.2.1.3. Appearance and semen volume

Concerning appearance, a normal sample shows a homogeneous and grey-opalescent appearance. The colour of the sample may also have some changes: whitish means there are a lot of sperm or leukocytes; reddish-brown means there are red blood cells; and yellowish means an infection or taking certain vitamins or drugs.

The volume was measured by transferring the sample from the container to a 15-mL Falcon tube and reading the gradients directly.

3.2.2.1.4. Semen pH

The pH of the semen was determined using pH test strips at a uniform time, 30 min after collection or within 1 hour after ejaculation. Normal semen pH is alkaline, ranging between 7.2 and 8.2. A pH value of less than 7.2 may indicate a deficiency of alkaline seminal vesicular fluid or be due to urine contamination.

3.2.2.2. Microscopic examination

The microscopic examination includes the analysis of sperm motility, concentration, morphology, and vitality.

Sperm concentration and motility were calculated using a Makler chamber and following the procedures provided by the manufacturer (Sefi-Medical Instruments). The Makler chamber is a quick and easy tool for obtaining reliable sperm count and motility.

3.2.2.2.1. Sperm concentration

The sample of semen was well mixed, and a small drop of it was put in the middle of the disc area using a pipette. The droplet spread across the entire surface of the disc with a thickness of 10 microns once the cover glass was firmly in place. Next, the Makler chamber was examined at x200 magnification.

Initially, sperm heads were counted in a strip of 10 squares of the grid, and the number counted represented their concentration in millions per mL. Then, this count was repeated on one or two additional strips to determine the mean. In the samples of oligospermia, sperm were counted in the entire grid area. Then five zeros were added to the calculated number to determine the concentration in millions per ml. Lastly, the total number of sperm in the ejaculate was counted by multiplying the amount of semen by the concentration of sperm.

Simultaneously with the determination of sperm concentration, the presence of seminal debris in the sample was evaluated at four levels: none (level 0), a few (level 1), moderate (level 2), and excessive (level 3).

3.2.2.2.2. Sperm motility

Motility was estimated immediately after the droplet was placed in the chamber and examined under a microscope at x200 magnification. First, the non-motile sperm were counted in sixteen squares in the centre of the grid. Then, moving sperm were counted and classified into motile progressive or motile non-progressive Finally, the percentage of each type of motility was calculated (progressive motility, non-progressive motility, and immobility).

3.2.2.2.3. Sperm morphology

A smear of semen was prepared by placing 10 μL of semen on the end of a clean slide, and a drop of semen was pulled along the slide surface using the cover slide. The slides were then allowed to air dry before being fixated for 30 min in an ethanol fixative. Next, methylene blue staining was done to determine the ratio of normal to abnormal forms under a microscope in five fields. Anomalies in the sperm were also classified into head defects, midpiece defects, and tail defects.

3.2.2.2.4. Sperm vitality

This parameter was assessed based on the measurement of the integrity of the sperm membrane by eosin dye exclusion. An aliquot of 5 μL of ejaculate was removed and combined with 5 μL of eosin solution on a microscope slide. Then the slide was examined at ×400 magnification to assess the percentage of live and dead spermatozoa, with stained sperm considered dead and unstained sperm considered alive.

3.2.2.2.5. Leukocytes

Leukocytes were estimated using the ortho-toluidine dye, which determines the property of the peroxidase enzyme in granulocytes.

First, the working solution was made by mixing 9 μL of the ortho-toluidine solution with 1 mL of saturated NH_4Cl solution, 1 mL of 148 mmol/L Na_2EDTA, and 10 μL of 30% (v/v) H_2O_2. After that, 10 μL of semen sample was mixed with 90 μL of working solution [1+9 (1: 10) dilution] and vortexed for about 10 seconds. Then, 10 μL of the mixture was put into the new Neubauer chamber (Hemocytometer). Finally, the chamber grid was examined at ×400 magnification, and at least 200 peroxidase-positive cells were counted in each replicate. The peroxidase-positive cells stained brown, but the peroxidase-negative cells remained unstained.

The number of peroxidase-positive cells was counted to get the number of cells per mL (1000 cells/mL) as follows:

$WBC\ (10^6/mL) = (N/n) \times 10$

N = the number of peroxidase-positive cells counted.

n = the total number of grids counted.

10 = dilution factor.

3.2.3. Biochemical analysis

The biochemical analysis includes the analysis of ROS, citric acid, fructose, and NAG. The SP was separated from the spermatozoa by centrifugation at 1000 rpm for 10 min and then transferred to new tubes and stored at -20° C to measure markers of MAGs. The study analyses were conducted on all samples except for NAG activity,

which was only measured in 70 samples (20 from fertile men and 50 from infertile men).

3.2.3.1. Determination of seminal ROS levels

The ROS were measured on freshly collected samples using a commercially available kit (CANros), and the manufacturer's recommendations were followed (Candore BioScience, India). The Agarose N-Gel tube was incubated at 90°C–100°C for 2 min or until the gel melted, then at 37°C for 5 min before adding and mixing 200 µL of the semen sample. After 55 min of incubation, the colour was immediately examined and compared to the colour code listed in the kit's catalogue **(Appendix I).**

This test depends on the reduction of Nitro Blue Tetrazolium (NBT) dye to detect the total ROS produced by leucocytes and spermatozoa in human semen. When NBT reacts with the free radicals contained in the semen sample, it creates colors ranging from light pink to dark purple. As a result, ROS levels were divided into four categories: negative (level 0), low (level 1), moderate (level 2), and high (level 3).

3.2.3.2. Measurement of seminal citric acid concentration

Seminal citric acid was measured using a commercially available kit (Citric Acid Test), and the manufacturer's recommendations were followed (FertiPro, Beernem, Belgium).

First, 100 µL of isopropanol was mixed with 100 µL of thawed SP. After 20 min of centrifugation, 50 µL of supernatant was pipetted into a cuvette tube, followed by the addition of 400 µL of ferric chloride. Fe3+ ions interact in the presence of citrate to form a yellow complex. Finally, the optical density (OD) of the reaction and the standard was measured at 405 nm, and the seminal citric acid concentration was calculated using the following equation:

$$\text{Citric Acid mg/ml} = \frac{\text{OD Sample}}{\text{OD Standard}} \text{ x 4mg/ml}$$

3.2.3.3. Measurement of seminal fructose concentration

Seminal fructose was measured using a commercially available kit (Fructose Test™), and the manufacturer's recommendations were followed (FertiPro, Beernem, Belgium).

Initially, 100 µL of the SP and 100 µL of fructose standards were pipetted into separate Eppendorf tubes, where fructose standards were used to prepare a standard curve with a range of fructose concentrations from 0 to 5 mg/mL, calculated according to the given instructions **(Table 3.2)**. Then, 500 µL of a trichloroacetic acid (TCA) was added to the samples and standards. After 10 min of centrifuging, 20 µL of the supernatant was pipetted into an empty Eppendorf tube and, sequentially, 200 µL of hydrochloric acid and 20 µL of indole were added to all tubes. The tubes were sealed and incubated for 30 min at 37°C in a water bath, then 200 µL of NaOH was added to stop the reaction.

Table 3.2. Fructose standards solutions

Concentration	Fructose standard	Water	
5 mg/ml	100µl	0µl	Standard 3
2,5 mg/ml	50µl	50µl	Standard 2
1 mg/ml	20µl	80µl	Standard 1

Finally, 200 µL of each tube was pipetted into an empty well and read at 450 nm. Seminal fructose concentrations were determined by comparing the measured OD values against the standard curve using the standard values **(Appendix II).**

3.2.3.4. Measurement of seminal NAG concentration

Seminal NAG was measured using a commercially available kit (EpiScreen Plus™), and the manufacturer's recommendations were followed (FertiPro, Beernem, Belgium).

First, each sample was pipetted into two Eppendorf tubes with a volume of 20 µL of each. Then, a reaction solution and an inhibitor solution were prepared in separate Eppendorf tubes for each sample. The reaction solution contained 3 µL of a 50x substrate solution [Para (4)-Nitrophenyl-alpha-D-glucopyranoside (PNPG) in Dimethyl Sulfoxide (DMSO)] in 147 µL of a reaction buffer (pH 6.8), supplemented with 1% sodium dodecyl sulfate (SDS). On the other hand, the inhibitor solution contained 3 µL of a 50x substrate solution in 147 µL of an inhibitor solution (a reaction buffer containing glucose), which acted as a negative control.

After adding 130 µL of the reaction solution to the sample reaction tube and 130 µL of the inhibitor solution to the sample inhibitor tube, the tubes were vortexed and placed in a water bath for 2 hours at 37 ºC. Next, the dilutions for the standard curve were then made by dissolving 100 µL of standard stock solution (reagent 5) in 2400 of standard dilution buffer (reagent 6) to produce the highest standard of 200 µM that serves as the starting solution for preparing the other standards, as indicated in the table below:

Table 3.3. NAG standards solutions

200 µM standard (µL)	Reagent 6 (µL)	Final concentration (µM)
375	125	150
250	250	100
125	375	50
25	475	10
0	500	0

After incubation, the reaction was stopped by adding 1 mL of NaOH and vortexing the sample tubes. Finally, 200 µL of all standards and samples were pipetted into a microtiter plate, and the absorbance was read at 405 nm. Seminal NAG activity was determined by comparing the measured OD values against the standard curve using the standard values **(Appendix III).**

3.2.4. Statistical analysis

Data were analyzed using the Statistical Package for Social Sciences software (IBM SPSS for Windows version 23.0). The Shapiro-Wilk test was used to assess the normality of the data distribution. Since not all variables had a normal distribution, their values were presented as medians and interquartile ranges (IQR). The Spearman's rank-order correlation (Rho) test was used for correlation analysis, whereas the Mann-Whitney test was employed to compare infertile and fertile groups. A P-value < 0.05 was deemed statistically significant.

4. Results

The results are presented in **3 distinct steps**: the first is a comparison of sperm characteristics between fertile and infertile groups. Second, the comparison of MAGs biomarkers between the study groups and the display of possible correlations between these biomarkers and semen parameters. Finally, the comparison of ROS levels between study groups and their association with sperm parameters and MAGs biomarkers.

4.1. Semen parameters

The results demonstrated a significant difference between the two study groups, with baseline semen parameters mostly aligned with those in the WHO 2021 guideline **(Appendix IV).** In terms of age, infertile men were considerably older than fertile men (median = 39; IQR = 35–45) and (median = 34; IQR = 30–36), respectively (P <0.001). Furthermore, the median values of most semen parameters, such as sperm concentration, total sperm count, sperm vitality, sperm motility, progressive sperm motility, and normal sperm morphology, were statistically significantly lower in the infertile group compared to the fertile group. However, there was no significant difference in the median pH and WBCs between both study groups, with P = 0.280 and P = 0.563, respectively. On the other hand, a significant increase in semen volume was observed in the infertile group (median = 3.8; IQR = 2.8–5) compared to the fertile group (median = 2.5; IQR = 2–3) (p <0.001). In addition, the liquefaction time and level of seminal debris were higher among infertile men than in fertile men (P = 0.040 and P <0.001, respectively) **(Appendix IV).**

4.2. Biomarkers of MAGs

As shown in **Table 4.1**, the citric acid level (mg/ml) was lower in the infertile group (median = 3.2; IQR = 2–4.4) compared to the fertile group (median = 5.9; IQR = 4.9–7; P <0.001) (**Figure 4.1)**. However, median citric acid (mg/ejaculate) did not change significantly between the two study groups (P = 0.086) (**Appendix V).** In addition, the fertile group had a significantly higher NAG activity (mIU/mL) than the infertile group (median 11.9; IQR 8–14.4) and (median 5.7; IQR = 4–8.7), respectively (P <0.001) (**Figure 4.1)**. Despite this, the median total NAG (mIU/ejaculate) showed no significant difference between the two study groups (P = 0.122) (**Appendix V).**

Moreover, there were no significant differences in median fructose (mg/ml) and total fructose (mg/ejaculate) between both study groups, with P = 0.208 and P = 0.359, respectively **(Table 4.1) (Appendix VI).** Box plots were used in statistically significant results to illustrate the median, sample distribution, and the presence of outliers.

Table 4.1. Comparison of MAGs biomarkers concentrations between infertile and fertile groups.

	Fertile group	**Infertile group**	**P – value**
	Median (25%-75% range)	**Median** (25%-75% range)	
Citric acid (mg/mL)	5.9 (4.9 – 7)	3.2 (2 – 4.4)	<0.001
Total citric acid (mg/ejaculate)	17.9 (10.7 – 22.7)	11.3 (6 – 22.3)	0.086 [ns]
Fructose (mg/mL)	4.9 (3.5 – 6)	3.5 (2 – 5.9)	0.208 [ns]
Total fructose (mg/ejaculate)	12.3 (7.5 – 17)	13 (7 – 26.9)	0.359 [ns]
NAG (mIU/mL)	11.9 (8 – 14.4)	5.7 (4 – 8.7)	<0.001
Total NAG (mIU/ejaculate)	32.3 (21 – 39.5)	23 (12.2 – 35.7)	0.122 [ns]

NAG, neutral alpha glucosidase. [ns]—not significant.

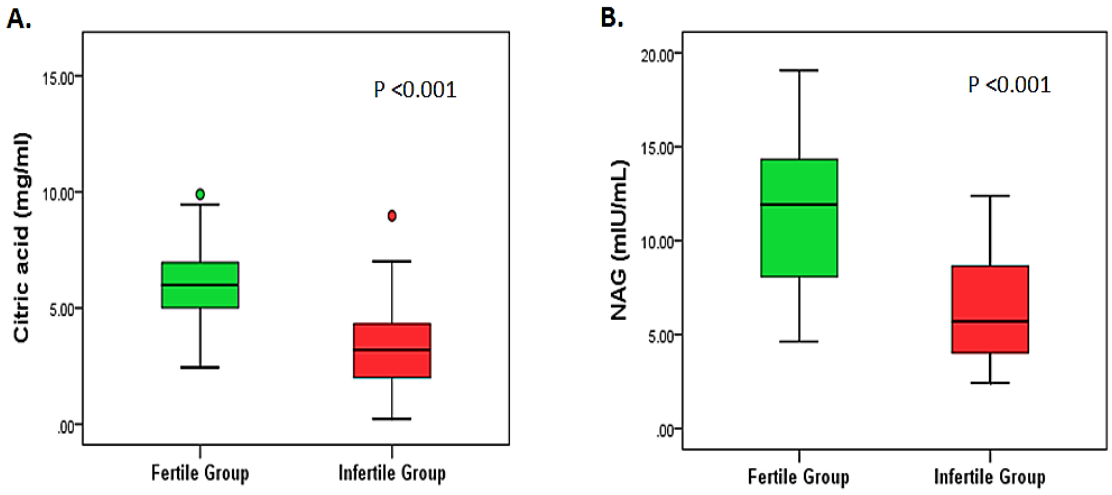


Figure 4.1. Comparisons of citric acid and NAG levels per mL between the infertile and fertile groups. A: Citric acid levels (mg/mL), **and B:** NAG activity (mIU/mL). ◦— data points with values greater than Q3 + 1.5 × interquartile range.

4.2.1. Association between the MAGs biomarkers with semen parameters.

The correlation values between MAGs biomarkers and semen parameters are summarized in **Table 4.2**

Table 4.2. Spearman correlations between MAGs biomarkers and semen parameters

		Total citric acid	Citric acid (mg/ml)	Total fructose	Fructose (mg/ml)	Total NAG	NAG (mIU/ml)
Semen volume	Rho	0.477***	-	0.503***	-	0.395**	-
	P-value	<0.001		<0.001		0.003	
Sperm concentration	Rho	-	0.258**	-	-	-	0.445**
	P-value		0.008				0.002
Total sperm count	Rho	-	-	-	-	-	0.365*
	P-value						0.013
Total motility (%)	Rho	-	0.215*	-	-	-	-
	P-value		0.028				
Progressive motility (%)	Rho	-	0.336***	-	0.200*	-	0.420**
	P-value		<0.001		0.042		0.004
Immotile (%)	Rho	-	-0.209-*	-	-	-	-
	P-value		0.033				
Normal morphology (%)	Rho	-	0.332**	-	-	-	0.485**
	P-value		0.001				0.001
Head defects	Rho	-	-0.292-**	-	-	-	-0.485-**
	P-value		0.003				0.001
Midpiece defects	Rho	-	-0.239-*	-	-	-	-0.336-*
	P-value		0.015				0.022
Seminal debris	Rho	-	-0.281-**	-	-	-	-
	P-value		0.004				

NAG, neutral alpha glucosidase. *** P < 0.001, ** P < 0.01, * P < 0.05 are considered statistically significant.

As shown in **Table 4.2**, semen volume was positively correlated with total citric acid, fructose, and NAG. The Spearman's values and P-values for citric acid, fructose, and NAG were (r = 0.477, P <0.001), (r = 0.503, P <0.001), and (r = 0.395, P = 0.003), respectively **(Figure 4.2)**. Other positive correlations were found between progressive motility and citric acid (mg/ml) (r = 0.336, P <0.001), fructose (mg/ml) (r = 0.200, P = 0.042), and NAG (mIU/ml) (r = 0.420, P = 0.004) **(Figure 4.3)**.

In addition, sperm concentration was positively correlated with citric acid (r = 0.258, P = 0.008) and NAG (r = 0.445, P = 0.002) **(Figure 4.4**). Normal sperm morphology was also positively correlated with citric acid (r = 0.332, P = 0.001) and NAG (r = 0.485, P = 0.001) **(Figure 4.4**). Moreover, total sperm count was positively correlated with NAG (r = 0.365, P = 0.013), and total motility was positively correlated with citric acid (r = 0.215, P = 0.028) **(Appendixes VII and VIII).** On the other hand, citric acid was negatively correlated with immotile sperm (r = -0.209, P = 0.033) and seminal debris (r = -0.281, P = 0.004) **(Figure 4.5).**

Figure 4.6 shows that citric acid and NAG levels had negative correlations with head defects (citric acid: r = -0.292, P = 0.003; NAG: r =-0.485, P = 0.001) and midpiece defects (citric acid: r = -0.239, P = 0.015; NAG: r =-0.336, P = 0.022). It should be noted that no correlation was found between fructose levels and sperm parameters other than those already mentioned.

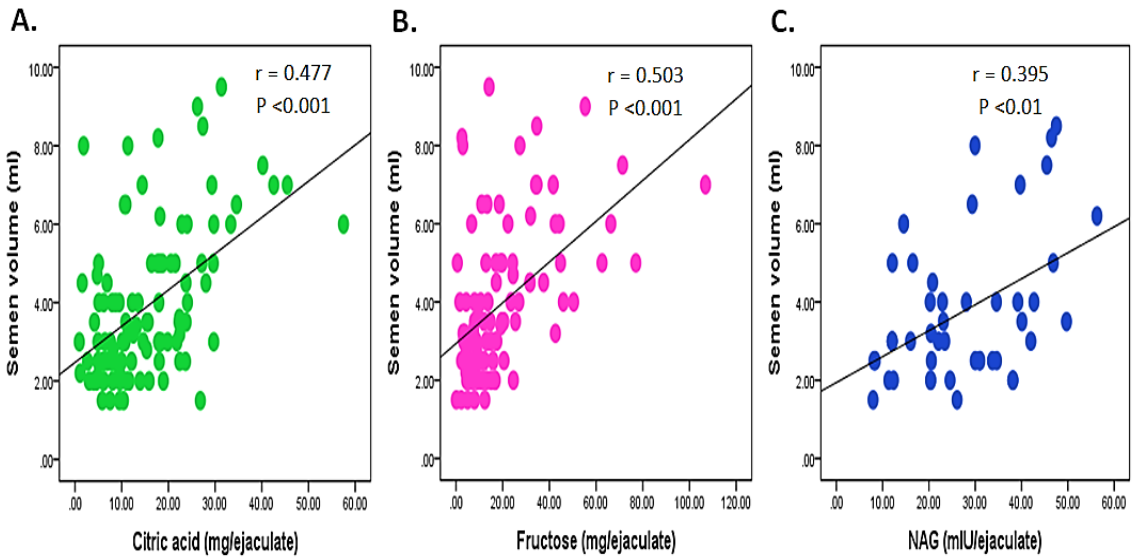


Figure 4.2. Correlation of total citric acid, fructose, and NAG with semen volume. The correlation of semen volum with each: **A.** Citric acid (mg/ejaculate), **B:** Fructose (mg/ejaculate), and **C.** NAG (mIU/ejaculate).

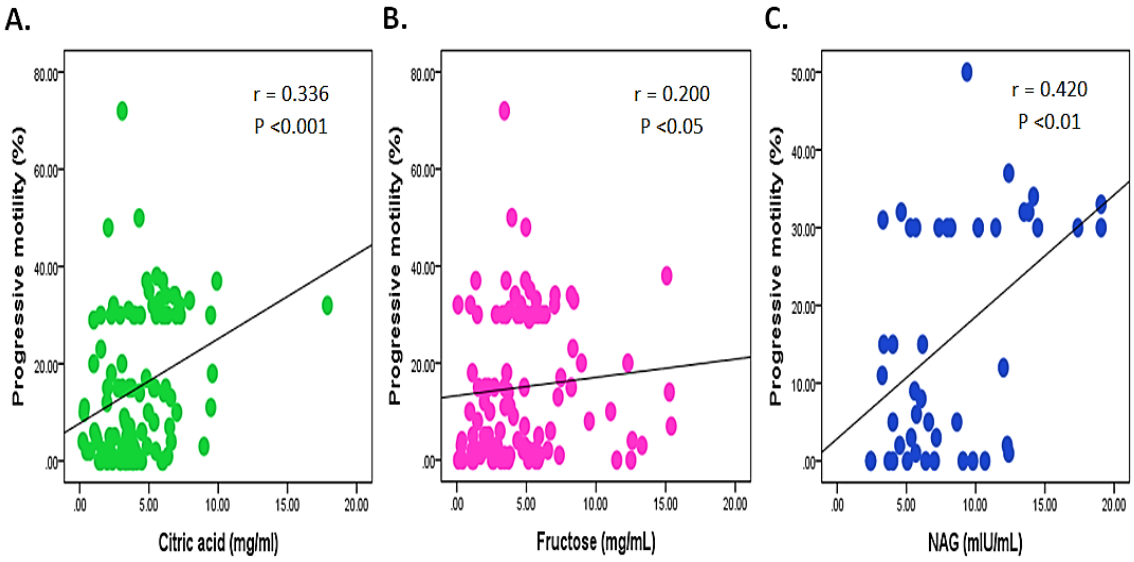


Figure 4.3. Correlation of progressive motility with citric acid, fructose, and NAG. The correlation of progressive motility with each: **A.** Citric acid (mg/mL), **B.** Fructose (mg/mL), and **C.** NAG (mIU/mL).

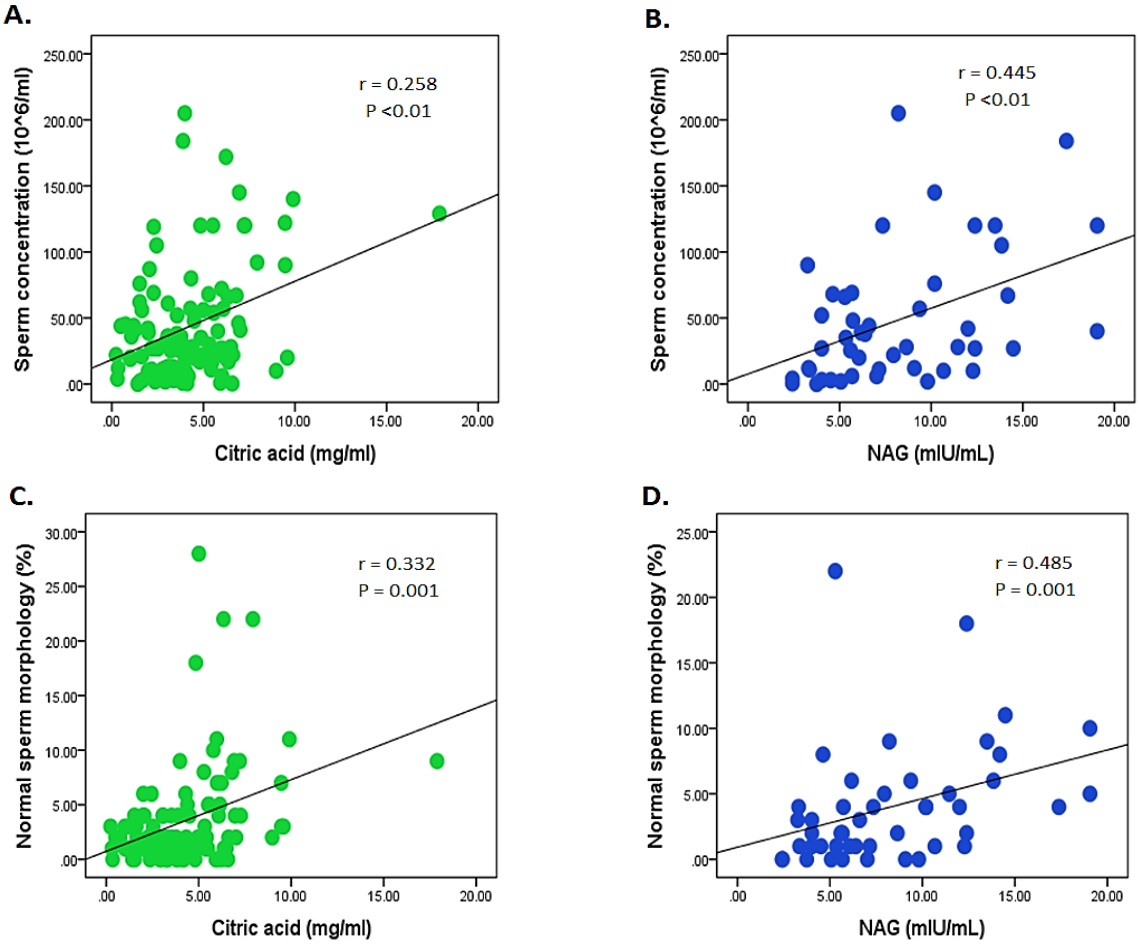


Figure 4.4. Correlation of citric acid and NAG levels with sperm concentration and normal morphology. A: Citric acid (mg/ml) and sperm concentration. **B:** NAG (mIU/mL) and sperm concentration. **C:** Citric acid (mg/ml) and normal morphology. **D:** NAG (mIU/mL) and normal morphology**.**

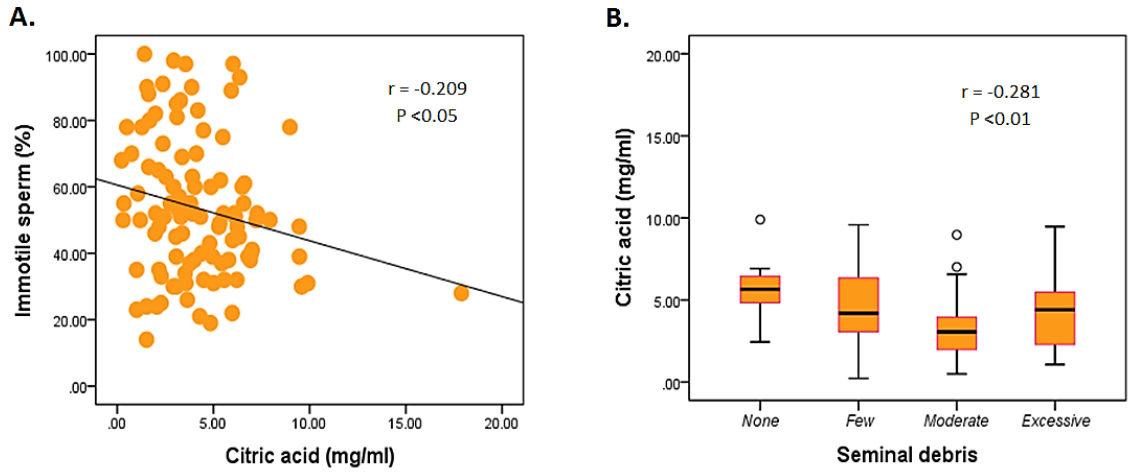


Figure 4.5. The correlation between citric acid, immotile sperm and seminal debris. A: Citric acid (mg/ml) and immotile sperm. **B:** Citric acid (mg/ml) and seminal debris. ◦—data points with values greater than Q3 + 1.5 × interquartile range.

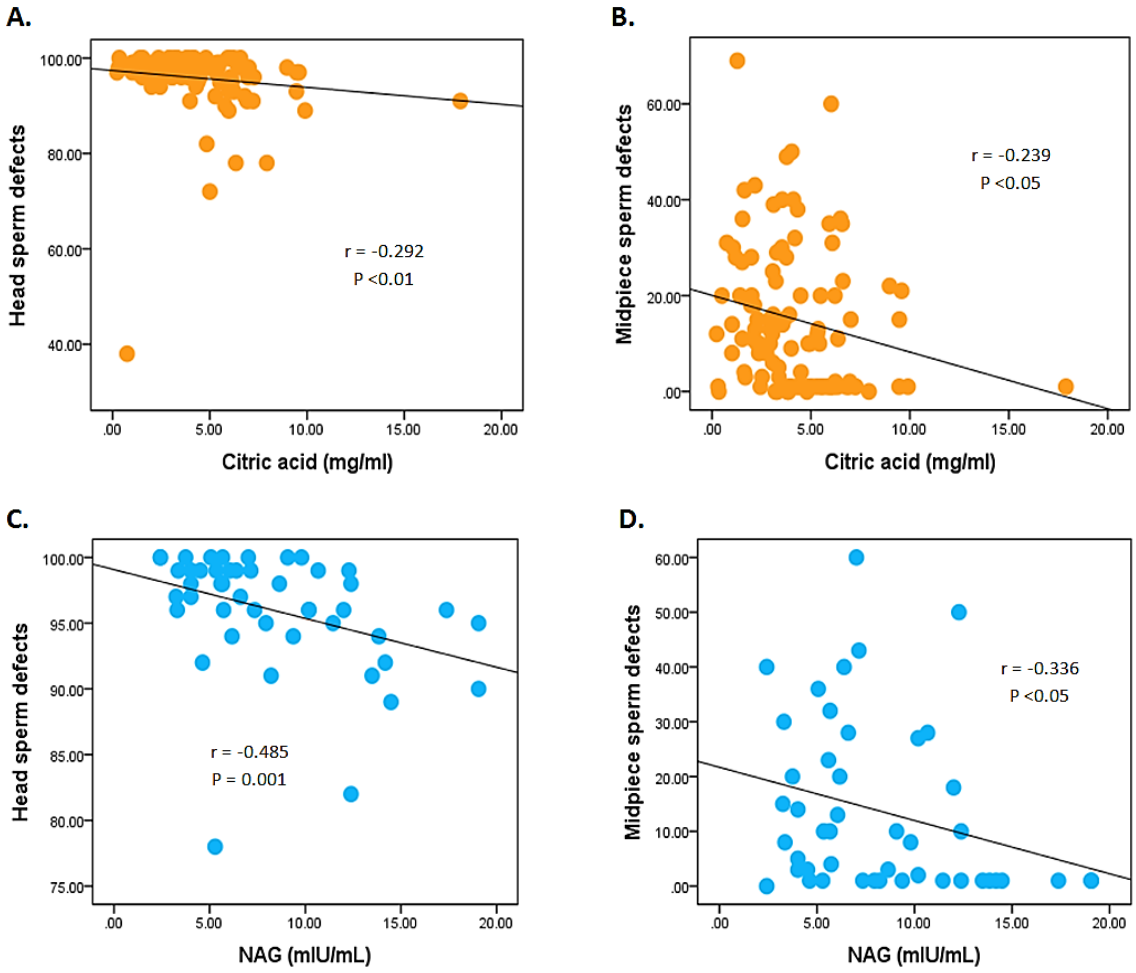


Figure 4.6. Correlation of citric acid and NAG levels with head and midpiece sperm defects. A: Citric acid (mg/ml) and head sperm defects. **B:** Citric acid (mg/ml) and midpiece sperm defects. **C:** NAG (mIU/mL) and head sperm defects. **D:** NAG (mIU/mL) and midpiece sperm defects.

4.3. Seminal ROS levels

Colorimetric scale levels of NBT reduction validated the existence of ROS in the semen samples of infertile men (median = 2; IQR = 1-2) compared to fertile men (median = 0; IQR = 0-0), with a significance level of P <0.001 (**Figure 4.7).**

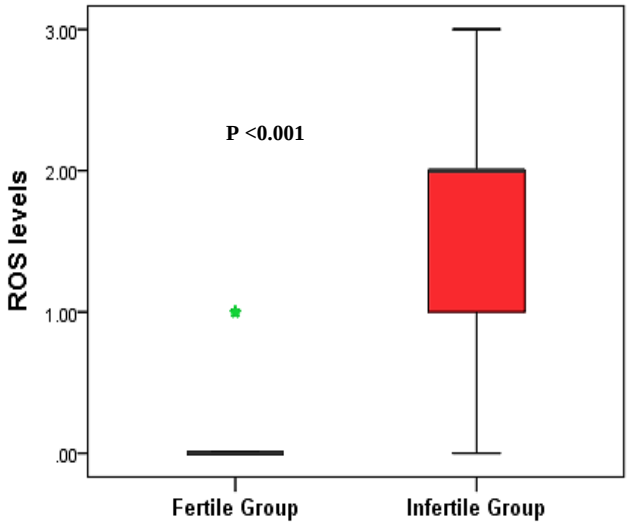


Figure 4.7. Seminal ROS levels in fertile and infertile groups. *—outliers.

4.3.1. Association between ROS levels with semen parameters.

Spearman's test displayed a significant correlation between ROS and major semen parameters **(Appendix IX).** The seminal ROS levels were negatively correlated with each sperm concentration (r = -0.348, P <0.001), total sperm count (r = -0.315, P = 0.001), vitality (r = -0.197, P = 0.046), total motility (r = -0.261, P = 0.008), progressive motility (r = -0.434, P <0.001), and normal morphology (r = -0.366, P <0.001) **(Appendix IX). Figure 4.8** illustrates the difference in sperm concentration, total sperm count, vitality, total motility, progressive motility, and normal morphology among seminal ROS levels.

In contract, the distribution of abnormal semen parameters, including liquefaction time, seminal debris, immotile sperm, head defects, tail defects, and midpiece defects, with seminal ROS levels, is shown in **Figure 4.9.** The ROS levels had significant positive correlations with the liquefaction time (r = 0.233, P = 0.017), seminal debris (r = 0.480, P <0.001), immotile sperm (r = 0.254, P = 0.009), head defects (r = 0.333, P = 0.001), tail defects (r = 0.268, P = 0.006), and midpiece defects (r = 0.416, P <0.001). However, semen volume, seminal pH, and WBCs were not statistically significant with ROS (P >0.05) **(Appendix IX)**.

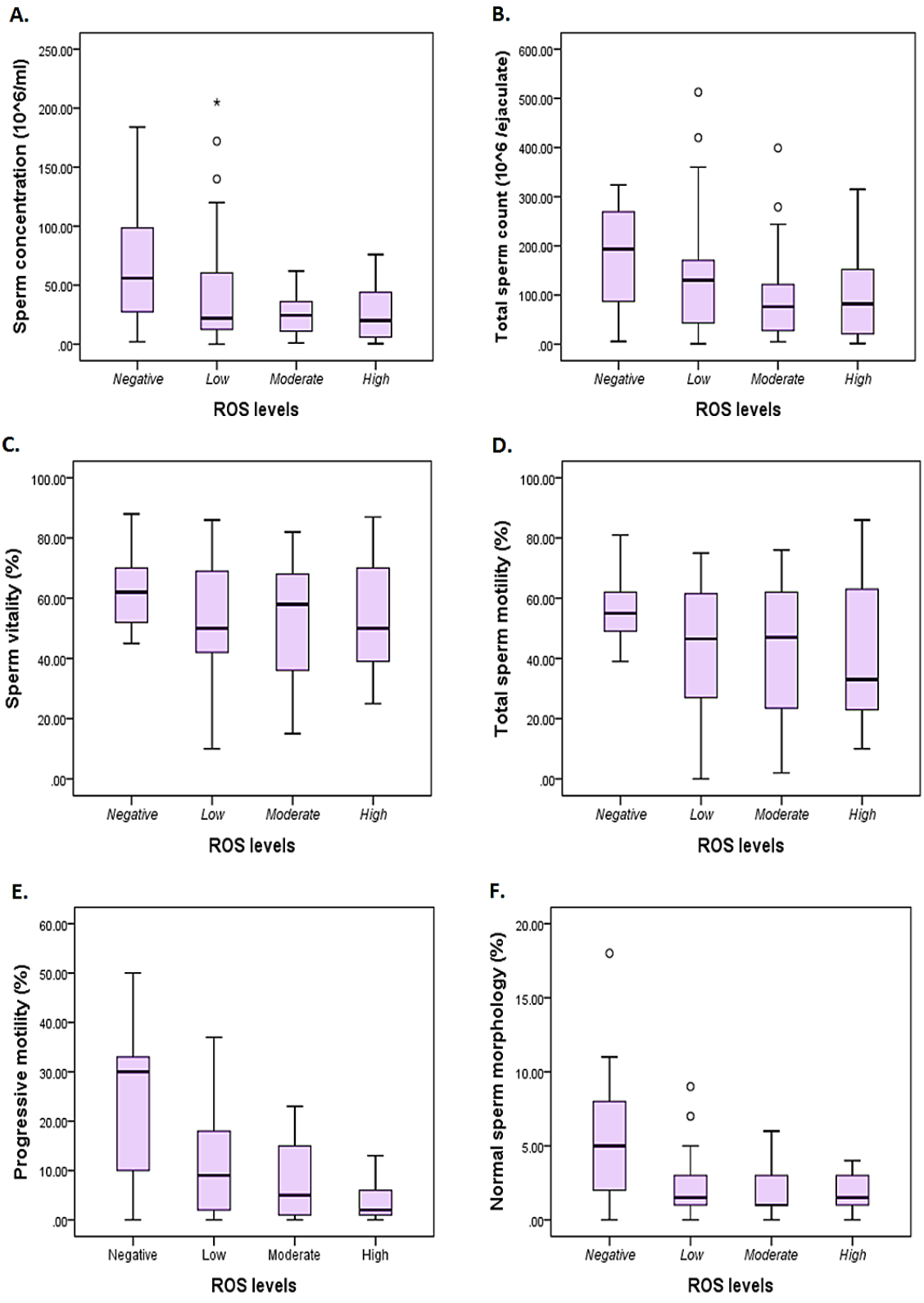


Figure 4.8. Comparison of sperm concentration, total sperm count, vitality, total motility, progressive motility, and normal morphology with seminal ROS levels. A. sperm concentration; **B.** total sperm count; **C.** sperm vitality; **D.** sperm motility; **E.** sperm progressive motility; and **F.** normal sperm morphology. ⁎—outliers. ◦—data points with values greater than Q3 + 1.5 × interquartile range.

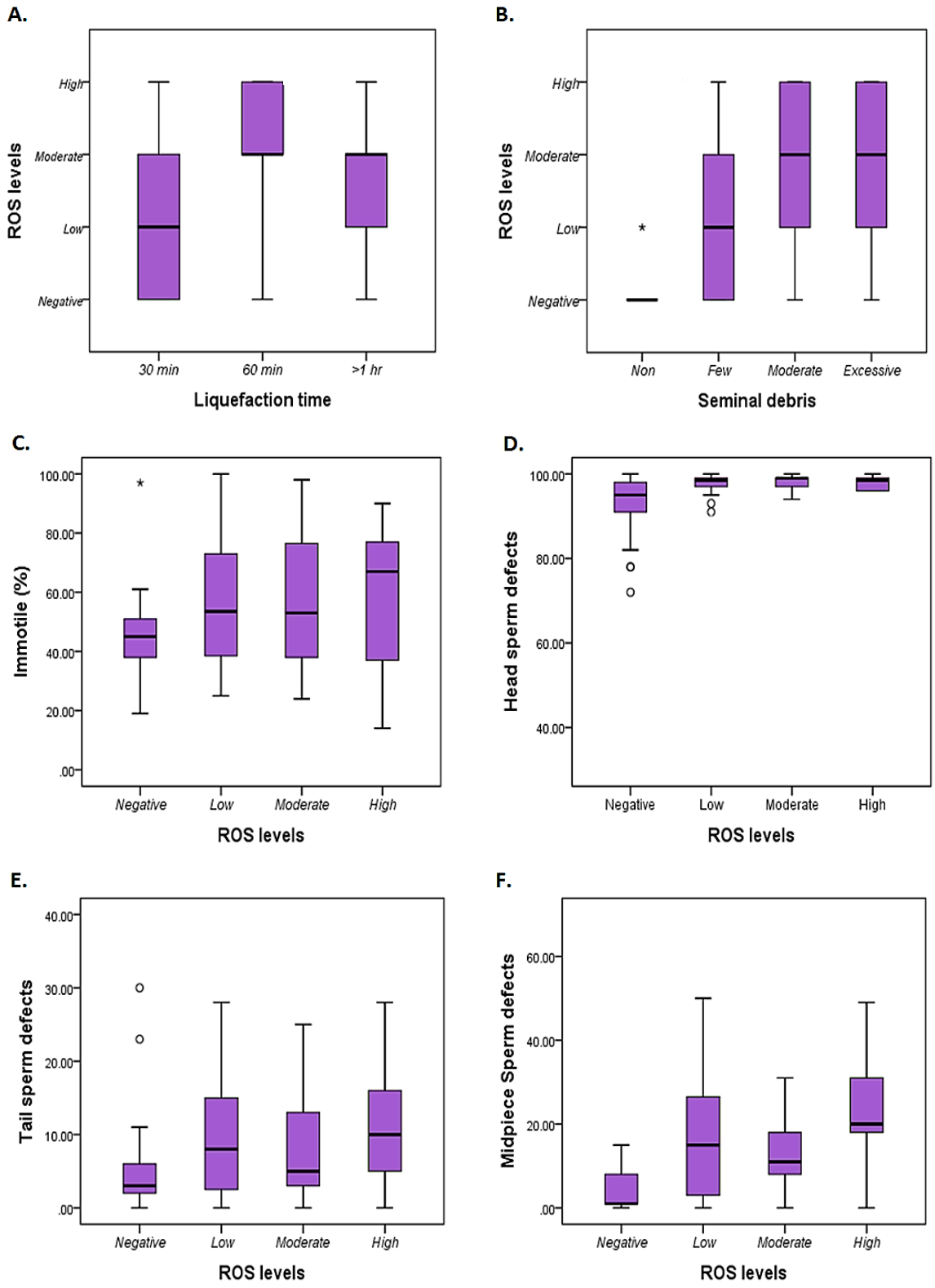


Figure 4.9. Comparison of liquefaction time, seminal debris, immotile sperm, head defects, tail defects, and midpiece defects with seminal ROS levels. A. liquefaction time; **B.** seminal debris; **C.** immotile sperm; **D.** head defects; **E.** tail defects; and **F.** midpiece defects. ⁎—outliers. ◦—data points with values smaller than Q1 − 1.5 × interquartile range or greater than Q3 + 1.5 × interquartile range.

4.3.2. Relationship between the ROS levels, biomarkers of MAGs, and age.

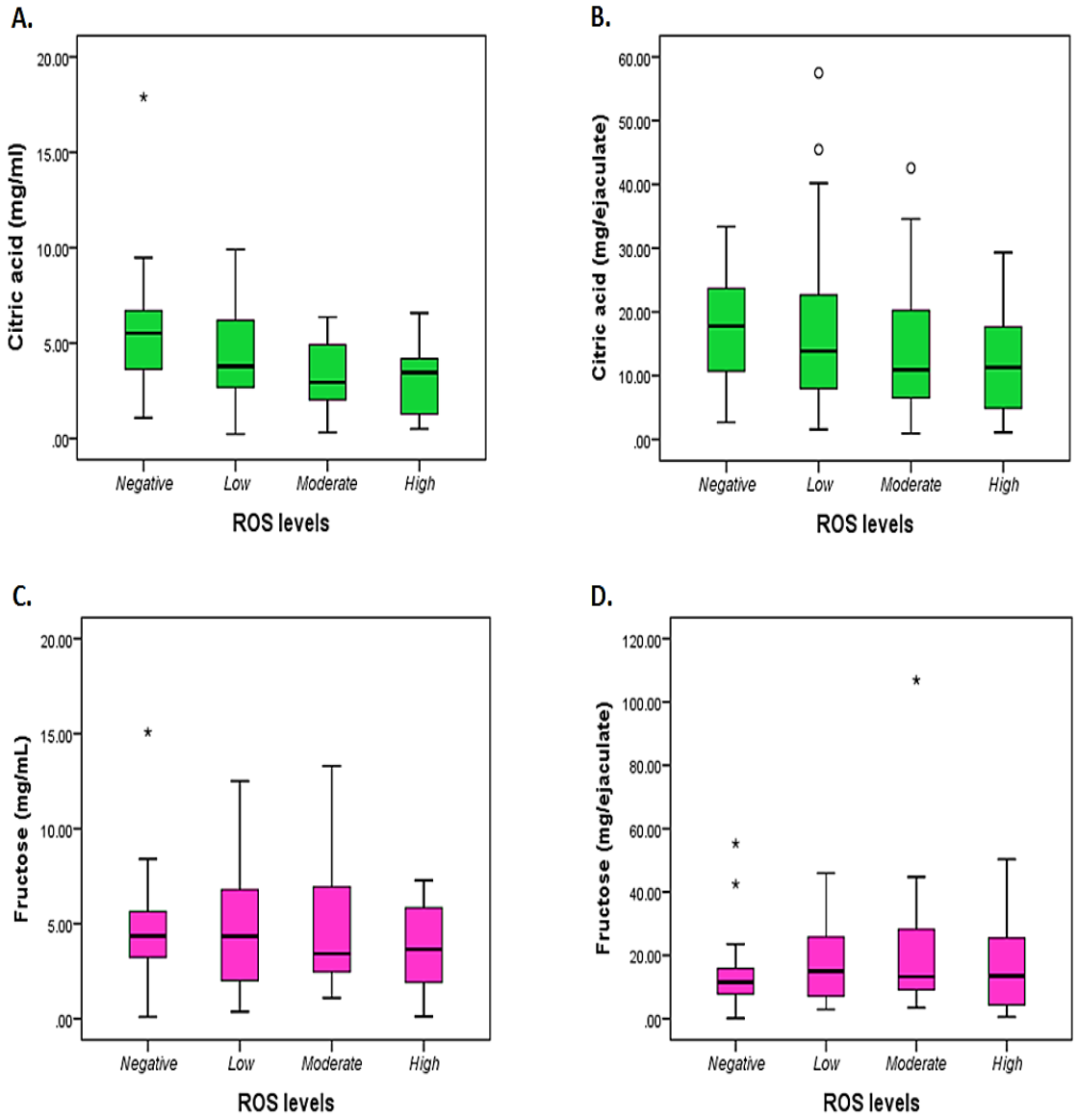
Table 4.3 showed that there was a negative association between seminal ROS levels and both levels of citric acid (r = -0.397, P <0.001; r = -0.239, P = 0.015, per ml and ejaculate, respectively) and NAG (r = -0.418, P = 0.004; r = -0.346, P = 0.019, per ml and ejaculate, respectively). In contrast, there was no association between seminal ROS levels and fructose concentrations (mg/mL, ejaculate) (P >0.05). The comparison of citric acid, fructose, and NAG concentrations among seminal ROS levels is shown, in **Figure 4.10.**

Table 4.3. The correlation between seminal ROS, biomarkers of MAGs, and age.

		Age	Citric acid (mg/ml)	Total citric acid	Fructose (mg/ml)	Total fructose	NAG (mIU/ml)	Total NAG
ROS levels	Rho	**0.202***	**-0.397*****	**-0.239***	-0.054	0.071	**-0.418****	**-0.346***
	p-value	**0.040**	**<0.001**	**0.015**	0.583	0.475	**0.004**	**0.019**
Citric acid (mg/ml)	Rho	**-0.194***	-	0.741***	-0.015	-0.015	0.183	-0.020
	p-value	**0.049**	-	<0.001	0.879	0.878	0.222	0.893
Total citric acid	Rho	-0.106	0.741***	-	0.122	**0.308****	-0.171	**0.330***
	p-value	0.282	<0.001	-	0.216	**0.001**	0.256	**0.025**
Fructose (mg/ml)	Rho	-0.135	-0.015	0.122	-	0.857***	0.202	0.265
	p-value	0.172	0.879	0.216	-	<0.001	0.179	0.075
Total fructose	Rho	-0.059	-0.015	**0.308****	0.857***	-	-0.064	**0.396****
	p-value	0.554	0.878	**0.001**	<0.001	-	0.674	**0.007**
NAG (mIU/ml)	Rho	-0.229	0.183	-0.171	0.202	-0.064	-	0.626***
	p-value	0.126	0.222	0.256	0.179	0.674	-	<0.001
Total NAG	Rho	0.012	-0.020	**0.330***	0.265	**0.396****	0.626***	-
	p-value	0.937	0.893	**0.025**	0.075	**0.007**	<0.001	-

NAG, neutral alpha glucosidase. **ROS,** reactive oxygen species. *** P < 0.001, ** P < 0.01, * P < 0.05 are considered statistically significant.

Figure 4.11 shows the negative correlation of citric acid concentration (mg/ml) with age (r = -0.194, P = 0.049) **(Table 4.3)**. While **Figure 4.12** presents box plots comparing ROS levels and age, seminal ROS levels exhibited a positive correlation with age (r = 0.202, P = 0.040) **(Table 4.3).** On the other hand, there was a significant positive association between MAGs biomarkers and each other per ejaculate but not per mL. Total citric acid was compared to total fructose (r = 0.308, P = 0.001) and NAG (r = 0.330, P = 0.025), and total fructose was compared to NAG (r = 0.396, P = 0.007), as displayed in **Table 4.3**.



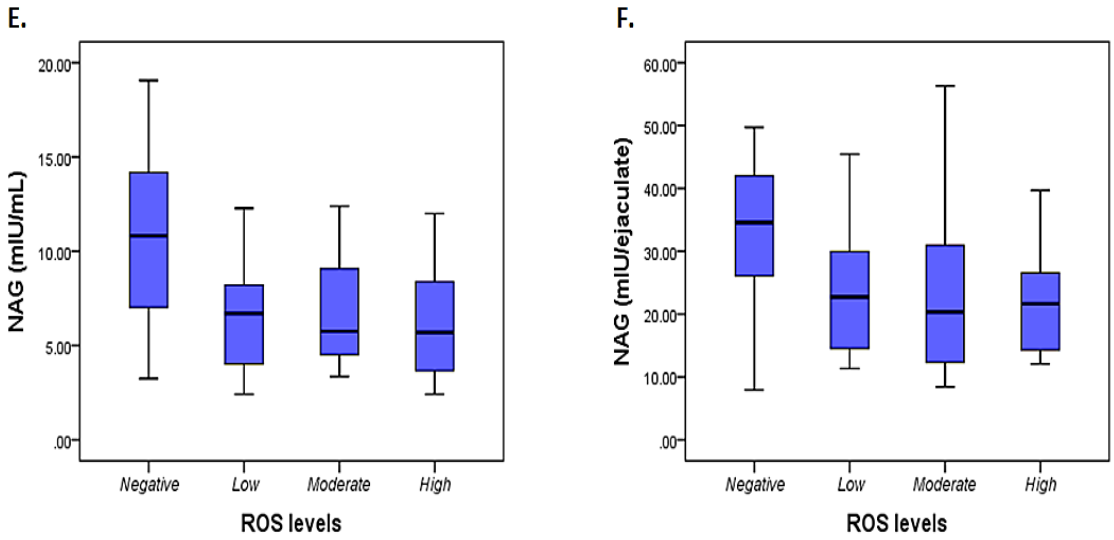


Figure 4.10. Comparison of citric acid, fructose, and NAG concentrations with seminal ROS levels. A. citric acid (mg/ml), **B.** citric acid (mg/ejaculate); **C.** fructose (mg/ml), **D.** fructose (mg/ejaculate); **E.** NAG (mIU/ml), **and F.** NAG (mIU/ejaculate). ⁎—outliers. ◦—data points with values greater than Q3 + 1.5 × interquartile range.

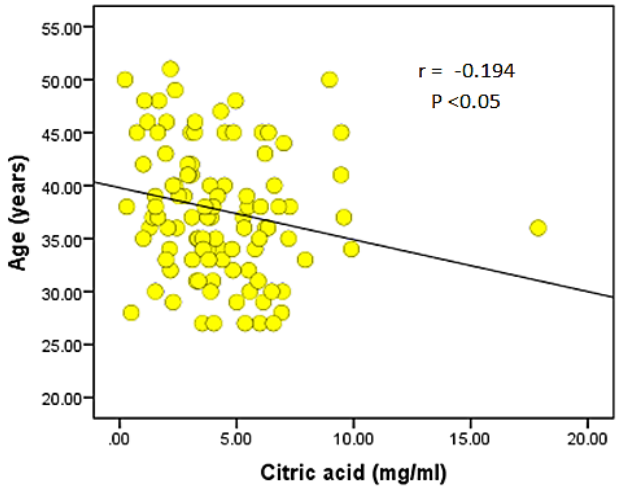


Figure 4.11. Correlation of citric acid concentration with age.

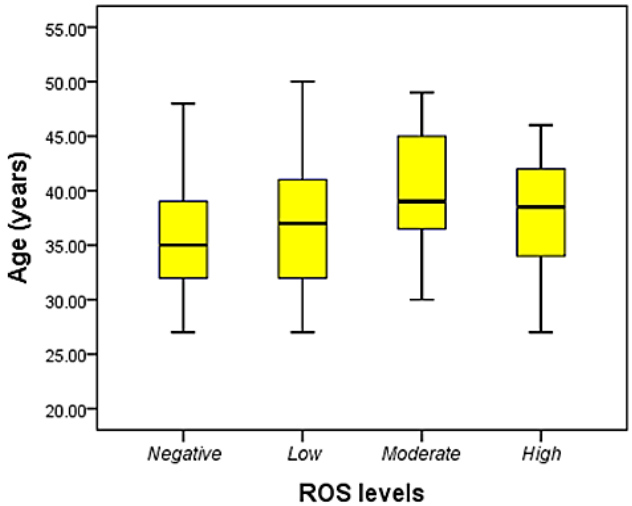


Figure 4.12. Comparison of seminal ROS levels and age.

5. Discussion

The oxidative damage occurs when ROS levels overwhelm local antioxidants (Tremellen, 2008). Therefore, ROS overproduction impairs male reproductive function, either directly by causing sperm damage or indirectly by disrupting endocrine hormones. Nevertheless, the SP contains antioxidant components that help protect sperm from oxidative damage (Darbandi *et al.*, 2018; Ribeiro *et al.*, 2021). To understand how OS affects the secretory capacity of MAGs and semen quality, we analyzed the semen of the study population, followed by the biochemical analysis of essential MAGs biomarkers and ROS in semen.

5.1. Basic semen analysis

Semen analysis is the first and most important test to assess male fertility by checking the quantitative and qualitative characteristics of semen (Caraballo *et al.*, 2019). Cooper *et al.* (2010) indicated that semen analysis should follow WHO guidelines, which provide limited reference values to compare with the results obtained from the patient. In this study, infertile men had lower sperm parameters than the limit values, whereas healthy men's sperm parameters were within the normal ranges **(Appendix IV)**.

In this study, the semen volume of the infertile group (median = 3.8) was significantly higher than that of the fertile group (median = 2.5) ($P < 0.001$) **(Appendix IV)**. Although the upper reference limit value of semen volume is not included in the WHO manual (2021), however, in the WHO manual (1999) and previous literature, the normal range of semen volume produced by masturbation after 2–7 days of abstinence is between 2.0 ml and 6.0 ml. Therefore, larger volumes (>6.0 ml) are described as hyperspermia (Cooke *et al.*, 1995). However, our results revealed that liquefaction time and seminal debris levels were higher among infertile men than in fertile men ($P = 0.040$ and $P < 0.001$, respectively) **(Appendix IV)**. This may be due to a dysfunction of the MAGs that are responsible for the secretion of coagulation and liquefaction proteins (Barbagallo *et al.*, 2021).

The present results showed that the median sperm concentration was 21 (10^6/ml) in the infertile group **(Appendix IV)**; this value is significantly lower than that found in a previous study of Tunisian men (Atig *et al.*, 2012), where the mean sperm

concentration was 36.33 (10^6/ml) in the infertile men. On the other hand, Bousnane *et al.* (2017) indicated that the mean sperm motility and sperm vitality were 35.15% and 35.80%, respectively, in the infertile Algerian men; these values are lower than our data, which showed that the median sperm motility and sperm vitality were 45% and 50%, respectively, in the infertile group **(Appendix IV)**.

Another study in Egypt (Abd Elrahman *et al.*, 2021) revealed that the mean normal sperm morphology was 5% in infertile men; these results are also comparable to ours, which found the median normal sperm morphology to be 1% only in infertile men **(Appendix IV)**. This may be due to the high percentage of head defects in the majority of the subjects participating in this study, with a median of 92% and 99% in fertile and infertile men, respectively.

5.2. Role biomarkers of MAGs on male fertility

Seminal fluid has a significant role in spermatozoa survival and overall fertilization success. Therefore, a change in its biochemical composition can be directly related to infertility (De Jonge and Barratt, 2006). The results of this study showed that both levels of citric acid (mg/ml) and NAG (mIU/mL) were decreased in infertile men versus fertile men ($P < 0.001$) **(Figure 4.1)**, while the fructose levels (mg/ml, ejaculate) remained convergent in both study groups ($P > 0.05$) **(Appendix VI) (Table 4.1)**.

On the other hand, our data did not show a significant difference in the total levels of citric acid (mg/ejaculate) and NAG (mIU/ejaculate) among both study groups ($P > 0.05$), although the median of these total biomarkers was higher in the fertile group than in the infertile group **(Appendix V) (Table 4.1)**. The median citric acid levels (mg/ejaculate) were 17.9 in the control group, compared to 11.3 in the infertile men **(Table 4.1),** which are very close to the values of another study that showed that levels of seminal citric acid (mg/ejaculate) decreased in all cases of infertility, with the mean being 10.12 as opposed to 18 in the control group (Najafi and Malini, 2011).

In the current study, the median NAG activity was 32.3 (mIU/ejaculate) in the fertile men **(Table 4.1).** However, Qiu *et al.* (2018) indicated that the mean of NAG in the fertile men was significantly higher than our data, which was 61.2 (mIU/ejaculate). In contrast, the results of our work contrast with the work done by Said *et al.* (2009), who found that levels of citric acid and NAG did not significantly differ among

Tunisian healthy and infertile men, while there was a significant difference between infertile groups in fructose levels.

The total of MAGs biomarkers is used to assess secretory function and potential damage to MAGs as clinical reflections of overall changes in semen volume, taking into account other factors influencing secretion volumes, such as a long period of sexual abstinence and the naturally high secretion of MAGs (WHO, 2010; Goss, 2018).

This study showed positive associations between semen volume and the total of citric acid ($r = 0.477$, $P < 0.001$), fructose ($r = 0.503$, $P < 0.001$), and NAG ($r = 0.395$, $P < 0.01$) (**Figure 4.2) (Table 4.2),** in agreement with (Elzanaty *et al.*, 2002; Goss, 2018; Fraczek *et al.*, 2020). Moreover, our results found a direct positive correlation between total citric acid, total fructose ($r = 0.308$, $P = 0.001$), and total NAG ($r = 0.330$, $P < 0.05$), as well as between total fructose and total NAG ($r = 0.396$, $P < 0.01$) **(Table 4.3)**. Such consistent results confirm that the functionality of MAGs significantly subsidizes the semen volume.

Citric acid is a reliable measure of prostatic gland function and plays a role in balancing the osmotic equilibrium of semen, which influences membrane function and sperm morphology (Huang *et al.*, 2013). In addition, it serves as a substrate for energy metabolism in sperm by two mechanisms: either by indirectly raising seminal pH, which several studies have shown to enhance sperm motility within the range of 7.2–8.2 or by directly converting excess extracellular citrates to malate or pyruvate by a series of enzymes, which are then utilized in Kreb's cycle in sperm mitochondria, providing energy for flagellar movement (Medrano *et al.*, 2006; Visconti, 2012; Zhou *et al.*, 2015).

In this study, seminal citric acid had a positive correlation with progressive motility ($r = 0.336$, $P < 0.001$**) (Figure 4.3),** total motility ($r = 0.215$, $P < 0.05$) **(Appendix VII)**, sperm count ($r = 0.258$, $P < 0.01$), and normal morphology ($r = 0.332$, $P = 0.001$) **(Figure 4.4) (Table 4.2)**, which is in line with many previous studies (Toragall *et al.*, 2019; AL-Khazali *et al.*, 2020; Shemshaki *et al.*, 2021), while it is conversely with other studies (Kanyo and Sas, 1975; Said *et al.*, 2009).

However, researchers have shown that abnormal T and MAGI may reduce sperm motility, normal morphology, and citric acid levels while increasing seminal debris and viscosity (Cooper *et al.*, 1990; La Vignera *et al.*, 2011; Sonbol and Elhanbly, 2021).

Our results also showed that there was an inverse correlation between citric acid levels and immotile sperm (r =-0.209, P <0.05), seminal debris (r =-0.281, P <0.01) **(Figure 4.5)**, head defects, (r =-0.292, P <0.01), and midpiece defects (r =-0.239, P <0.05) **(Figure 4.6) (Table 4.2)**. This reveals that sperm dysfunction could be the result of a direct effect of testicular damage on prostate secretion.

Seminal fructose measurement is one of the most recommended seminal vesicular markers as well as a diagnostic biochemical marker for obstructive azoospermia (Buckett and Lewis-Jones, 2002; Lu *et al.*, 2007). Several authors have found a negative correlation between seminal fructose and sperm concentration, motility, and progressive motility due to increased fructolysis, which is essential for normal sperm motility (Ali *et al.*, 2011; Amidu *et al.*, 2012; Trang *et al.*, 2018; Toragall *et al.*, 2019).

In this study, there were no positive effects of fructose levels on sperm activity, excluding progressive motility (r = 0.200, P <0.05) **(Figure 4.3) (Table 4.2)**, and this slightly concurs with Sundaram *et al.* (2016), who found a significant positive correlation with sperm concentration, total motility, and progressive motility. Since no decrease in median fructose levels was recorded in the present results, it is potential that the increase in sperm motility is due to alternative energy sources of fructose (Mann, 1946). As mentioned previously, citric acid is a possible energy source because it significantly increases sperm motility.

NAG activity is the most accurate and sensitive indicator of epididymal disorders and can help distinguish between obstructive and non-obstructive azoospermia cases (Sandoval *et al.*, 1995). Some research has indicated that NAG contributes to sperm maturation by modifying surface glycoproteins and provides energy for sperm motility by converting α-1,4-linked oligosaccharides into glucose (Fourie *et al.*, 1991; Dias *et al.*, 2004)

The current study showed a positive correlation between NAG activity and all the major sperm parameters, including progressive motility (r = 0.420, P <0.01) **(Figure 4.3)**, total sperm count (r = 0.365, P <0.05) **(Appendix VIII)**, sperm concentration (r = 0.445, P <0.01), and normal morphology (r= 0.485, P = 0.001) **(Figure 4.4)**. On the other hand, there was a negative correlation between NAG activity and head defects (r =-0.485, P = 0.001) and midpiece defects (r =-0.336, P <0.05) **(Figure 4.6) (Table 4.2)**.

These results are in agreement with several studies (Sundaram *et al.*, 2016; Qiu *et al.*, 2018; Moronkeji and Emokpae, 2020), but are not in line with some studies (Pena *et al.*, 2004; Said *et al.*, 2009). The agreed results in this study confirm that NAG is consistent with an efficient epididymal function in terms of sperm maturation, motility, and the acquisition of fertilization ability through modifying surface glycoproteins and supplying spermatozoal ATP (Yassa *et al.*, 2001; Dias *et al.*, 2004).

5.3. Impact ROS on semen parameter

Recent reports have indicated that 30–80% of infertile men have elevated levels of seminal ROS (Tremellen, 2008). In this study also, the ROS median values were significantly higher in infertile men than in fertile men ($P < 0.001$) **(Figure 4.7)**, which is consistent with several studies (Moein *et al.*, 2007; Venkatesh *et al.*, 2009; Agarwal *et al.*, 2014a). Indeed, seminal ROS play a physiological and pathological role, depending on the concentration in which they are present (Castleton *et al.*, 2022).

Many studies have shown that high levels of ROS cause LPO in the sperm membrane, which makes the membrane less flexible and inhibits sperm motility (Tvrda *et al.*, 2018; Wagner *et al.*, 2018). ROS may also cause mutations in the nuclear and mtDNA of sperm, where by mtDNA damage rapidly depletes ATP, decreases axonemal protein phosphorylation, and inhibits sperm motility (Tafuri *et al.*, 2015). Moreover, elevated ROS levels disrupt mitochondrial membranes, which results in the release of cytochrome C, which activates caspases and, ultimately, apoptosis (Wang *et al.*, 2003; Takeshima *et al.*, 2021).

Our data showed that seminal ROS levels were negatively associated with all characteristics of normal sperm, including their concentration ($r = -0.348$, $P < 0.001$), the total number ($r = -0.315$, $P = 0.001$), vitality ($r = -0.197$, $P < 0.05$), total motility ($r = -0.261$, $P < 0.01$), progressive motility ($r = -0.434$, $P < 0.001$), and normal morphology ($r = -0.366$, $P < 0.001$) **(Appendix IX) (Figure 4.8)**. Meanwhile, seminal ROS levels had significant positive associations with abnormal semen parameters, including liquefaction time ($r = 0.233$, $P < 0.05$), seminal debris ($r = 0.480$, $P < 0.001$), immotile sperm ($r = 0.254$, $P < 0.01$), head defects ($r = 0.333$, $P = 0.001$), tail defects ($r = 0.268$, $P < 0.01$), and midpiece defects ($r = 0.416$, $P < 0.001$) **(Appendix IX) (Figure 4.9)**.

These results are consistent with previous findings (Moein *et al.*, 2007; Agarwal *et al.*, 2014a), while they are contrary to some studies (Pasqualotto *et al.*, 2000;

Venkatesh *et al.*, 2009). Our study suggests that high seminal ROS levels can lower sperm quality through multiple pathways, such as LPO, SDF, and apoptosis. However, hyperviscosity and seminal debris may indicate dysfunction of the seminal vesicles or prostate as a result of oxidative damage (Ricardo, 2018; Barbagallo *et al.*, 2021).

Leukocytes and immature sperm are the primary sources of ROS in semen (Henkel, 2011). Our study did not find a significant correlation between leukocytes and seminal ROS levels ($P > 0.05$) **(Appendix IX)**. This study suggests that the origin of seminal ROS may be caused by spermatogenesis defects. However, most low-grade chronic MGT infections are clinically asymptomatic and have a lot of activated macrophages in the semen, which produce a large amount of ROS (La Vignera *et al.*, 2011). Accordingly, Tunc (2011) reported that the seminal neopterin measurement of macrophage activity could be a better indicator of sperm health than peroxidase activity, which the WHO recommends as an initial screening method (WHO, 2021).

5.4. ROS and MAGs biomarkers

MAGs secrete many seminal antioxidants that prevent the oxidation chain reaction by donating their electrons to free radicals (Ribeiro *et al.*, 2021). Abdel-Salam *et al.* (2014) demonstrated that citric acid acts as an antioxidant and anti-inflammatory in damaged tissues. However, citrate has a high affinity for seminal anionic antioxidants such as Cu^{+2}, Mg^{+2}, and Zn^{+2} (Owen and Katz, 2005). In favor of these reports, our results showed a negative association between seminal ROS levels and citric acid ($r = -0.397$, $P < 0.001$; $r = -0.239$, $P < 0.05$, per ml and ejaculate, respectively) **(Table 4.3)** (**Figure 4.10)**.

Furthermore, a previous study found that treatment with oral supplementation of antioxidant combinations, including Carnitine, lycopene, Zn^{+2}, folic acid, vitamin B12, selenium, fructose, and citric acid, led to improvement of semen parameters in idiopathic conditions, and the ROS levels in these patients decreased from 25% to 9% after taking these supplements for six months (Chattopadhyay *et al.*, 2016). In contrast, this study found no correlation between seminal ROS levels and concentrations of fructose ($P > 0.05$ per mL and ejaculate) **(Table 4.3)** (**Figure 4.10).**

It has been reported that decreased NAG activity is associated with reduced sperm membrane integrity and increased SDF due to the long-term effect of OS on epididymal function (Vivas-Acevedo *et al.*, 2014). Accordingly, the present results showed the

seminal ROS had a negative correlation with NAG activity (r =-0.418, P <0.01; r =-0.346, P <0.05, per ml and ejaculate, respectively) **(Table 4.3)** (**Figure 4.10)**, which is in line with data of a previous study (Mahmoud *et al.*, 1998).

Interestingly, many studies showed a decrease in significant biomarkers (citric acid and NAG) in infertile men with MAGI, alcohol and nicotine consumption, obesity, and varicocele (Marconi *et al.*, 2009; Lourenco and Fernandes, 2015; Lozano-Hernandez *et al.*, 2017; Fraczek *et al.*, 2020). However, none of these studies specifically analyzed the levels of ROS as the primary reference for these conditions. Accordingly, our findings suggest that OS mediates concurrent changes in prostatic and epididymal function, either by interfering with endocrine hormones or having an impact on both glands simultaneously due to infection or inflammation (Mann and Lutwak-Mann, 1981; Darbandi *et al.*, 2018).

Age is another important factor affecting a man's fertility (Harris *et al.*, 2011); some research has demonstrated an age threshold for the appearance of defects in semen parameters and a loss of the delicate balance between ROS production and antioxidant defenses after 34 years of age (Kidd *et al.*, 2001; Nago *et al.*, 2021). On the other hand, it has been reported that ageing affects hypothalamic-pituitary-gonadal axis function, resulting in proliferative disorders of the prostate gland (Verze *et al.*, 2016).

In this study, infertile men were considerably older than fertile men (median age, 39 vs 34 years, respectively, P <0.001) **(Appendix IV)**. In addition, our results showed that age was negatively correlated with citric acid mg/ml (r =-0.194, P <0.05) **(Figure 4.11)**, and positively correlated with ROS levels (r = 0.202, P <0.05) **(Table 4.3) (Figure 4.12)**. All of this suggests that an OS and changes in the endocrine system may be the two causes responsible for age-related semen parameter deterioration.

6. Conclusion and Recommendations

6.1. Conclusion

This study demonstrated that the biochemical status of semen might be responsible for the proper functioning of the sperm through the significant positive correlation of citric acid, fructose, and NAG with major sperm characteristics such as concentration, motility, progressive motility, and normal morphology, all of which indicate the ability of a sperm to fertilize an oocyte.

There is substantial evidence that OS plays a significant role in the pathogenesis of male infertility by affecting sperm activity and MAGs functioning. Our results showed that seminal ROS levels were significantly higher in infertile men, deteriorating all semen parameters. Moreover, this work provided an interpretation of the potential influence of OS on the inadequate secretion of the epididymis and prostate through direct negative correlations between ROS, citric acid, and NAG levels, which may be due to infection, inflammation of these glands or/and androgen deficiency.

6.2. Recommendations

- Assessment of the secretory products of MAGs should be included in the routine semen analysis of the Libyan population, which may help the evolution of new standards that are beneficial for predicting and improving male fertility.
- Clinical ROS measurement should be performed to identify patients with seminal OS who may benefit from antioxidant supplementation.
- Antioxidant supplementation could be taken to overcome oxidative damage (during spermatogenesis, sperm storage or transit in the reproductive tract, or infection), which may help reduce apoptosis and thus improve sperm quality and decrease DNA damage.

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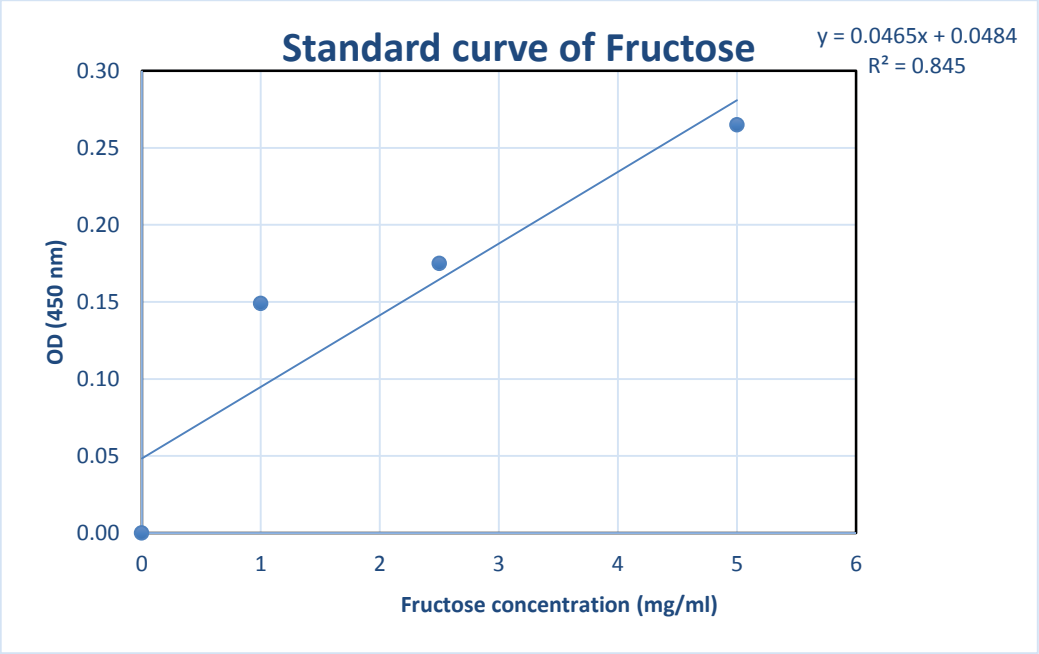
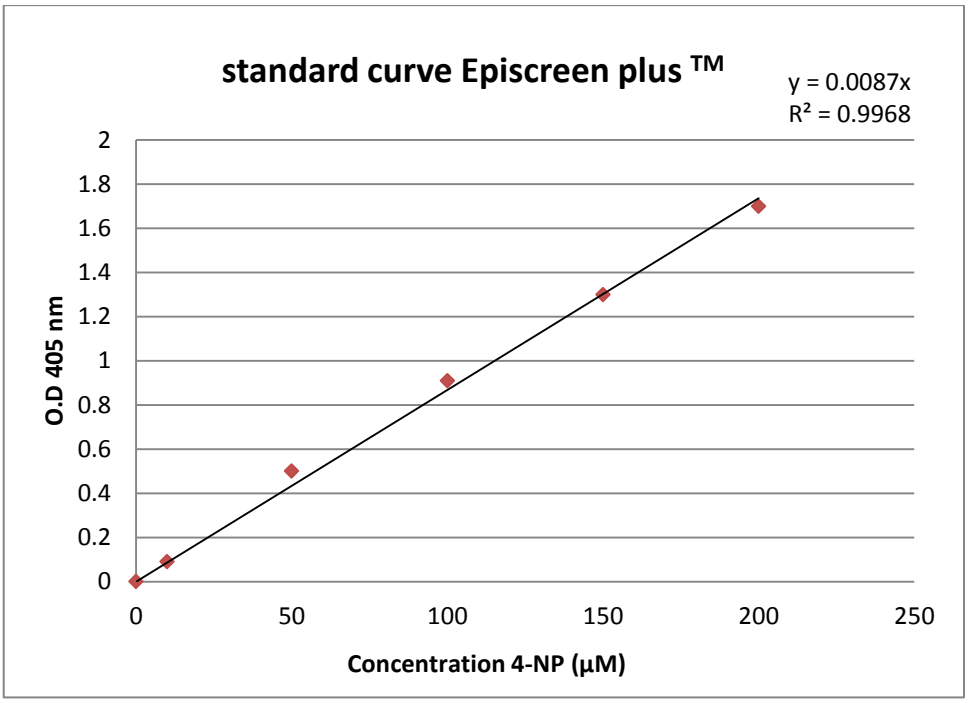
Appendices

Appendix I. Interpretation of the colour codes for NBT-reactivity levels.

Interpretation	Colour	Colour Code
Normal	White / Light pink	
Low level of ROS	Light purple	
Moderate level of ROS	Purple	
High level of ROS	Dark purple	



Appendix II. Standard curve of seminal fructose



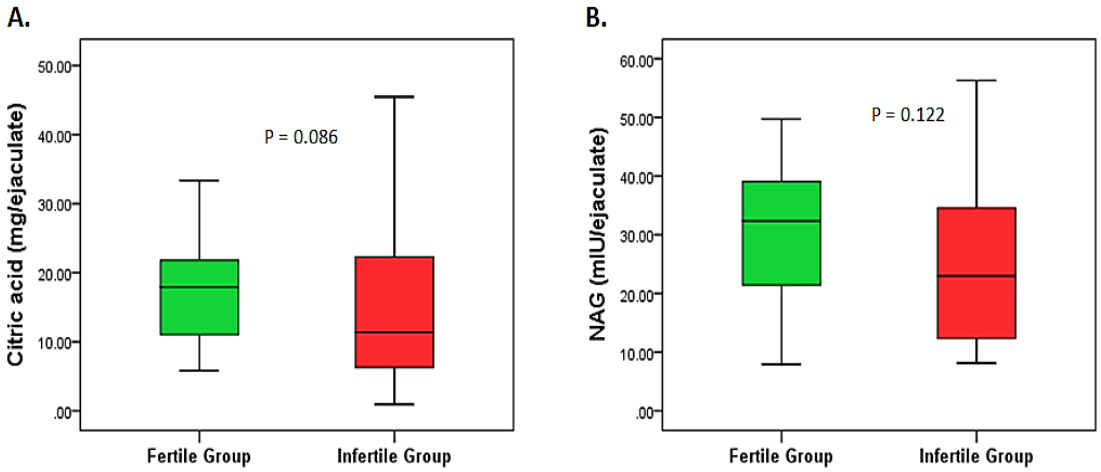
Appendix III. Standard curve of seminal NAG

Appendix IV. Comparison of semen parameters between the infertile and fertile groups.

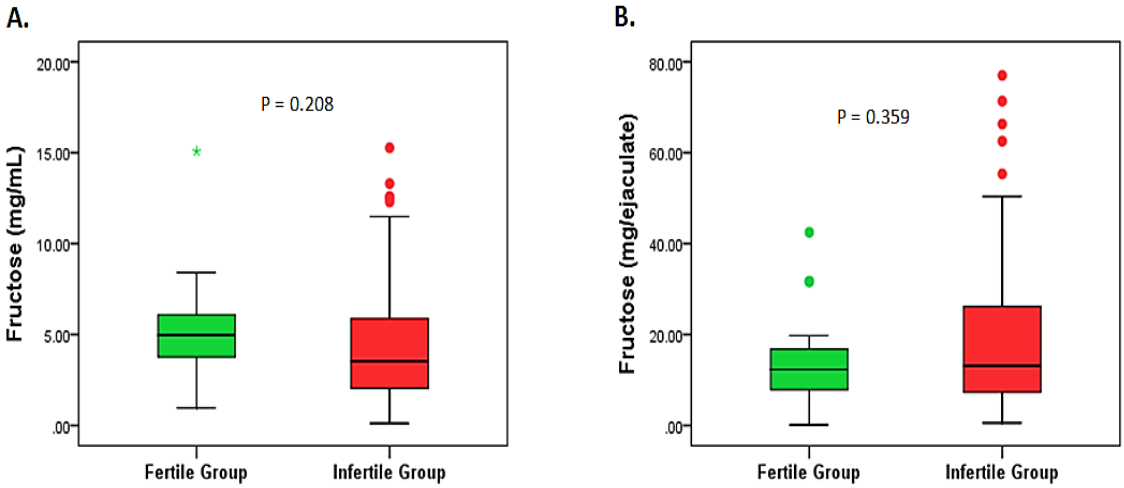
	Fertile Group (n = 25)	**Infertile Group** (n = 79)	**P-value**
	Median (25%-75% range)	**Median** (25%-75% range)	
Age	34 (30 – 36)	39 (35 – 45)	<0.001
Volume (ml)	2.5 (2 – 3)	3.8 (2.8 – 5)	<0.001
Liquefaction	1 (1 – 3)	2 (1 – 3)	0.040
pH	8 (7.5 – 8)	8 (8 – 8)	0.280
Sperm concentration (10^6 per ml)	92 (55 – 125.5)	21 (10 – 38)	<0.001
Total sperm count (10^6 per ejaculate)	244 (98.5 – 303)	90 (34 – 152)	<0.001
Vitality (%)	62 (51 – 72.5)	50 (39 – 70)	0.029
Total motility (%)	56 (49.5 – 68.5)	45 (23 – 61)	<0.001
Progressive motility (%)	32 (30 – 34.5)	5 (1 – 15)	<0.001
Non-progressive motility (%)	25 (18 – 30)	34 (23 – 45)	0.006

Immotile (%)	44 (31.5 – 50.5)	55 (39 – 77)	0.001
Normal morphology (%)	8 (5 – 10.5)	1 (1 – 2)	<0.001
Head defects	92 (89.5 – 95)	99 (98 – 99)	<0.001
Tail defects	2 (2 – 3)	8 (3 – 18)	<0.001
Midpiece defects	1 (1 – 1)	15 (10 – 29)	<0.001
WBCs (10^6 per ml)	1 (0.5 – 1)	1 (0.5 – 1)	0.563
Seminal debris	1 (0 – 1)	2 (1 – 2)	<0.001

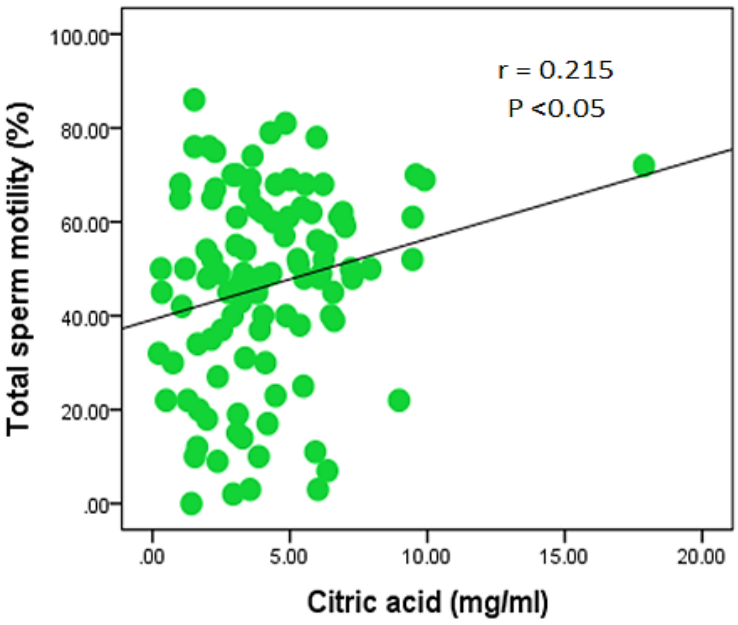
WBCs, white blood cells. **pH,** potential of hydrogen.



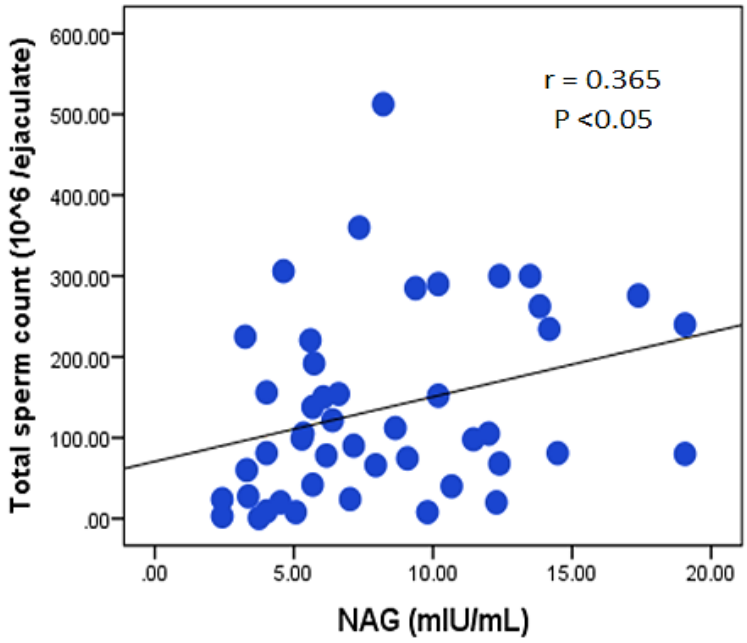
Appendix V. Comparisons of citric acid and NAG levels per ejaculation between the infertile and fertile groups. A: Citric acid levels (mg/ ejaculate), and **B:** NAG activity (mIU/ejaculate).



Appendix VI. Comparison of fructose levels between the infertile and fertile groups. A: Fructose levels (mg/ ml), **and B:** Fructose levels (mg/ejaculate). *—outliers. ◦—data points with values greater than Q3 + 1.5 × interquartile range.



Appendix VII. Correlation of citric acid levels with total sperm motility.



Appendix VIII. Correlation of NAG levels with total sperm count.

Appendix IX. Summarizes the relationship between levels of ROS and semen parameters.

	r- value	P-value
Volume (ml)	0.189 ^{ns}	0.056
Liquefaction	0.233*	0.017
pH	0.040 ^{ns}	0.686
Sperm concentration	-0.348-***	<0.001
Total sperm count	-0.315-**	0.001
Vitality (%)	-0.197-*	0.046
Total motility (%)	-0.261-**	0.008
Progressive motility (%)	-0.434-***	<0.001
Immotile (%)	0.254**	0.009
Normal morphology (%)	-0.366-***	<0.001
Head defects	0.333**	0.001
Tail defects	0.268**	0.006
Midpiece defects	0.416***	<0.001
WBCs (10^{6} per ml)	-0.036- ^{ns}	0.716
Seminal debris	0.480**	<0.001

WBCs, white blood cells. **pH,** potential of hydrogen. *** P < 0.001, ** P < 0.01, * P < 0.05 are considered statistically significant. ^{ns}—not significant.