Significance of Some Trace Elements in Seminal Plasma of Infertile Men in Gaza Strip

أهمية بعض العناصر النادرة في بلازما السائل المنوي لمرضى قلة الخصوبة في قطاع غزة

Prepared by:
Ahmed Khamis Jarad

Supervised by:
Dr. Abdalla A. Abed
Dr. Nizam M. El-Ashgar

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١٤٢٧ هـ – ٢٠٠٦ م
قال تعالى:

"بسم الله الرحمن الرحيم، يقال تعالى:

"الله الملك، و миров، فشيئاً و كافراً ي_DP


"الرزق


صدق الله العظيم"
Declaration

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Signature Name Date
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Significance of some trace elements in seminal plasma of infertile men in Gaza strip

Abstract

Infertility is considered a worldwide problem which is thought to be caused by multiple factors. The role of trace elements in male fertility has been considered. It has been indicated that certain levels of zinc, copper, iron, and magnesium are necessary for both spermatogenesis and steroidogenesis. The suggested roles of these elements in male infertility lead to the introduction of these elements as supplementary elements for improvement of male fertility.

This study is aimed to compare the levels of zinc, copper, iron and magnesium in the seminal plasma of infertile group and control group, to find out relationships in male infertile group between these elements and both seminal characteristics, serum reproductive male hormones (Luteinizing hormone, Follicle stimulating hormone, and testosterone), and also to understand the biological significance of such elements in male infertility.

Seventy two infertile male subjects, without any treatment who had regular unprotected intercourse for at least 12 months without conception, aged 20-50 years, were selected from the central infertility center Al Shifa hospital, Gaza, Palestine. Seventy two known fertile males selected as control group from general population (their wives had given birth to a child within one year).

Semen samples analyzed according to WHO criteria and seminal plasma trace elements were analyzed by atomic absorption spectrophotometry. Serum samples for endocrine studies were measured by (Enzyme Linked Immunosorbent Assay) ELISA, using kits supplied by Diagnostic System Laboratories (DSL), USA.

Our results showed that the mean values of zinc, copper and magnesium, were significantly lower in infertile men compared to controls (68.9 mg/L, 300µg/L and 67.1 mg/L) versus (122 mg/L, 377 µg/L and 120 mg/L respectively), while iron mean values were significantly higher in infertile men seminal plasma compared to the controls 412 µg/L versus 329 µg/L. Almost all studied seminal parameters
(sperm count, forward motility, weak motile and non motile) were significantly lower in infertile group compared to controls (P<0.001, P<0.001, P<0.05, P<0.001, respectively) as well as hormonal parameters (LH and Testosterone) in the infertile group were significantly lower compared to the control group (p<0.05). There was no significance difference between means of FSH levels (p>0.05).

Correlation analysis in infertile group showed that while seminal plasma zinc and magnesium levels are directly proportional to sperm count (r=0.376, P=0.001 and r=0.293, P=0.013 respectively), and testosterone (r=0.293, P=0.012 and r=0.324, P=0.003 respectively), it is indirectly proportional to seminal volume (r=-0.251, P=0.034 and r=-0.369 P=0.001 respectively). In contradiction copper did not show any significant relations with any of the seminal or hormonal parameters. Seminal plasma levels of iron were found to show correlations with sperm count and FSH levels. Seminal plasma iron levels were directly proportional to sperm count (r=0.290, P=0.014) and indirectly proportional to FSH levels(r=-0.349, P=0.003). Trace elements inter-correlations showed that both magnesium and zinc levels are directly proportional to each other (r=0.744, P=0.0001).

The results obtained in our study indicate that both zinc and magnesium are important for both spermatogenesis and steroidogenesis. The results also indicate that both zinc and magnesium are important extracellularly (in the seminal plasma) while iron is important intracellularly (in sertoli cells). While magnesium and zinc are important for steroidogenesis, increased serum plasma levels of FSH increases the uptake of iron in sertoli cells.

To the best of our knowledge, this is the first study in Gaza Strip investigating the significance of trace elements in seminal plasma and serum endocrine parameters in infertile men.

**Key words:** infertility, zinc, copper, iron, magnesium, Luteinizing hormone, Follicle stimulating hormone, Testosterone, seminal plasma, Gaza strip.
أهمية بعض العناصر النادرة في بلازما السائل المنوي لمرضى قلة الخصوبة في قطاع غزة

***مستخلص***

يعتبر عدم الإجابة مشكلة عالمية لها أسباب عدة ومنها دور العناصر النادرة الذي تم بحثه في خصوبة الرجال، حيث يتم الوصول إلى أن معدل بعض العناصر مثل الخارسنين والنحاس والماغنيسيوم ضروري بمكانة لتحليل الحيوان المنوي وإنتاج الهرمونات الاستروئيدية اللازمة لذلك. ونظراً لدورها مهم في خصوبة الرجال تم إعطاءها كمساعدات للعلاج.

تهدف الدراسة الحالية لمقارنة معدل الخارسنين والنحاس والماغنيسيوم في بلازما السائل المنوي لرجال قليل الخصوبة مع عينة ضابطة وإيجاد علاقات في مجموعة الدراسة بين هذه العناصر من جهة ونسبة نتائج تحليل السائل المنوي وهورمونات الذكرية في الدم (LH, FSH &Testosterone) من جهة أخرى كما وتهدف إلى دراسة وفهم الأهمية الجينية لهذه العناصر في هذه المشكلة.

حالات الدراسة 72 رجل قليل الخصوبة لا يتعاطوا أي علاج، ومبشرتهم الجنسية تم دون أي مواضع وقفة ليوم من بعد الإجابة 12 شهراً، القناع العمري ما بين (20 إلى 50 عاماً) تم اختيارهم من فئة معالجة مشاكل الذكرية في المستشفى المركزي مستشفى الشفاء- غزة.

الناتج الذي تم الحصول عليه أوضح أن الوسط الحسابي لمعدل الخارسنين والنحاس والماغنيسيوم كانت أقل 68.9 mg/L, 300µg/L and 122mg/L, 377µg/L and 120 mg/L مقارنة بـ 67.1mg/L وجدت أبعاد أعلى في مجموعة الدراسة عنها في العينة الضابطة ونسبة الهرمونات الذكرية في الدم (LH, FSH &Testosterone) (329 µg/L) وفي مجموعات الدراسة أقل كثيرون عندها في العينة الضابطة وبفرق إحصائي واضح كما وجدنا أن هناك فرق إحصائي واضح ما بين معدل (LH) في مجموعات الدراسة عنها في العينة الضابطة بالنسبة لمعدل الهرمون (r=0.293, P<0.05) ملم نجد أي فرق إحصائيا واضحة بين كلتا المجموعتين.

تم دراسة العلاقات بين المتغيرات في مجموعة الدراسة أظهرت النتائج أن معدل كل من الخارسنين والماغنيسيوم تتناسب طردية مع عدد الحيوانات المنوية وكمال معامل ارتباط بيرسون (r) (r=0.376, P<0.001) (r=0.293, P<0.01) (r=0.324, P<0.01) يتسببون طردية كذلك مع معدل هرمون الذكرية (Testosterone) في محل الدم وكان معامل ارتباط بيرسون (r=0.293, P<0.05) وبعلاقة إحصائية واضحة كما أن هذين
العنصران يتباينان عكسياً مع حجم السائل المنوي (r=0.376, P<0.001 and r=0.293, P=0.013).

بالمقارنة فإن عنصر النحاس لم يظهر أي علاقة إحصائية واضحة مع أي من نتائج السائل المنوي أو هرمونات الدم أما عنصر الحديد في بلازما السائل المنوي فقد أظهرت الدراسة علاقة طر دبة مع عدد الحيوانات المنوية (FSH) والعلاقة ملحوظة وعلاقة عكسية مع هرمون (r=0.290, P<0.05).

وكان معامل ارتباط بيرسون (r=0.349, P<0.01) وعلاقة بوب والدالة الإحصائية وعلاقة الارتباك المعامل وكمية البول (FSH) يمكن تحديد هذه العناصر ببعضها البعض في مجموعة الدراسة فقد أظهرت الدراسة علاقة طر دبة بين عنصر الخارشير والماغنيسيوم في بلازما السائل المنوي (r=0.744).

أما علاقة هذه العناصر ببعضها البعض في مجموعة الدراسة فقد أظهرت الدراسة علاقة طر دبة بين عنصر الخارشير والماغنيسيوم في بلازما السائل المنوي (r=0.744).

إن النتائج التي حصلنا عليها في دراستنا هذه تحدد بأن عنصري الخارشير والماغنيسيوم هي مهمة لكل من تخلق الحيوان المنوي والهرمونات الاسترويدية كما أظهرت أهمية كل من العنصران في خارج الخليا (Sertoli cells) بينما الحديد فتكون أهميته لخلايا من الداخل (FSH) للمiaux والهرمونات الذكرية وبالتالي مشتقاتها وان زيادة هرمون في بلازما الدم يزيد من قابلية دخول الحديد في الخلايا المنتجة للحيوان المنوي.

يشار إلى أن هذه الدراسة الأولى في قطاع غزة تدرس أهمية العناصر النادرة في بلازما السائل المنوي وهرمونات الذكورة في مصل الدم لمرضى قلة الخصوبة.
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<tr>
<td>AAS</td>
<td>Atomic Absorption Spectrophotometry</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine Diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic Adenosine Monophosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DSL</td>
<td>Diagnostic System Laboratories</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked Immunosorbent Assay</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle Stimulating Hormone</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>L</td>
<td>Liter</td>
</tr>
<tr>
<td>LH</td>
<td>Leuitinizing Hormone</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>ml</td>
<td>milliliter</td>
</tr>
<tr>
<td>mm</td>
<td>Millimeter</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide Adenine Dinucleotide, reduced</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>P-value</td>
<td>Probability</td>
</tr>
<tr>
<td>r</td>
<td>Pearson’s correlation coefficient</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide Dismutase</td>
</tr>
<tr>
<td>TMB</td>
<td>Tetramethylbenzidine</td>
</tr>
<tr>
<td>WHO</td>
<td>World health organization</td>
</tr>
<tr>
<td>β</td>
<td>Slope of linear regression</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µl</td>
<td>Microliter</td>
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To my beloved parents and family

To my wife

To my sons,
Khalid, Mohanad, Yousif, and Mohamed

And

To my daughters
Iman, Reem, and Niveen
Acknowledgment

I would express my thanks to Dr. Abdalla Asaf Abed, my supervisor, who did not spare any effort to overcome all the difficulties aroused during the theoretical parts and for his constructive scientific advice.

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CHAPTER-1
Introduction

1.1. Male Infertility prevalence

World health organization defined infertility as failure of conceiving a child for at least 12 month of unprotected intercourse (109).

Infertility has been shown to have a high prevalence worldwide (affects one in six). WHO (1999) has reported that male factor infertility plays a role in approximately 50 % of infertile couples. Male infertility has multiple causes and the commonest single defined cause is sperm dysfunction (97). However, reports has referred to the worsening of this problem due to the deterioration of the human semen quality by as much as 3% per year, leading to fears that male reproductive problems may be on an increase (6).

Despite the problem in assessing the prevalence of infertility in developing countries, between 8 – 12 % of couples around the world have difficulty conceiving a child at some point in their lives, affecting 50 – 80 million people (108).

In Palestine infertility is a tragic and a costly problem, it costs about 8.8 % of the abroad referral of ministry of health, about 2.9 million US $ per year (63).

1.2. Etiology

The etiologies of male infertility include gene mutations, aneuploidies, infectious diseases, ejaculatory duct occlusion, varicocele, radiation, chemotherapy and erectile dysfunction (70).

Diminished semen quality is one of the primary etiologies of male infertility however it is generally poorly understood. Environmental, occupational and lifestyle characteristics such as age and diet have been implicated in the etiology of diminished semen quality (23).
The role of trace elements on the quality of human semen, and their mechanism of action have been a focus of study for many researchers (90).

1.3. Trace elements

Trace elements have been defined as those elements occurring in the human body but constituting lower than 0.01 % of the body weight (85). Essential trace elements to human health including iron, copper, selenium, manganese, chromium, molybdenum and iodine. Trace elements have been shown to play essential roles in major cellular activities i.e. antioxidants. Zinc is another essential trace element with many enzymatic functions, including antioxidant actions, and involved in chromatin scaffold proteins, DNA synthesis, protein synthesis and cell division (68).

1.4. Trace elements and male infertility

Although there are some studies which demonstrate the significance of trace elements in male fertility, the biological role of these elements is not fully understood (2).

Zinc was found to have high levels in semen from mammals, and zinc has been found to be critical to spermatogenesis. Deficiency of zinc is associated with hypogonadism and insufficient development of secondary sex characteristics in human (79). Also, deficiency of zinc was found to cause failure in spermatogenesis due to atrophy of the seminiferous tubules in the rat (22).

However, high concentrations of zinc have been reported to depress oxygen uptake in the sperm cells (26), head-tail attachment/detachment and nuclear chromatin condensation /decondensation is also influenced by seminal zinc (9). Also sperm motility has been suggested to be affected by zinc levels (54).
The generation of oxidants, also described as reactive oxygen species (ROS), in the male reproductive tract has been a real concern because of their potential toxic effects, at high levels, on sperm quality and function (87,92).

A number of reports indicate the significance of trace elements in male infertility and its effect on the level of antioxidants (91). ROS are needed for the regulation of normal sperm functions, such as sperm capcitation, the acrosome reaction, and sperm-oocyte fusion (3).

High levels of ROS were detected in the semen of 25% to 40% of infertile men (75). Spermatozoa are particularly susceptible to the damage induced by excessive ROS because their plasma membranes contain large quantities of polyunsaturated fatty acids (81), and their cytoplasm contains low concentrations of scavenging enzymes (17), in addition, the intracellular antioxidant enzymes cannot protect the plasma membrane that surrounds the acrosome and the tail, forcing spermatozoa to supplement their limited intrinsic antioxidant defenses by depending on the protection offered by the seminal plasma (87).

One function of zinc and copper is to protect cell against free radical through the enzyme superoxide dismutase (SOD) as shown by the finding that SOD, a cuprozinc enzyme accelerates the dismutation of free radicals (71).

In extracellular and intracellular compartments the binding of iron and copper ions to specific proteins prevents any form of metal-dependent catalysis. In the presence of ROS; metals can be released from binding proteins, inducing reactive radical species (35). Increased levels of metal ions in semen (101) or blood plasma (97) appear to be significantly and positively correlated with male infertility (33).

Spermatogenesis in mammals requires the action of a number of peptide and steroid hormones (sex hormones), each of which plays an important role in normal functioning of the seminiferous epithelium. Sex hormones are not critical only for
regulation of male germ cell development, but also for proliferation and function of the somatic cell types required for proper development of the testis (61).

Among the most common somatic cells that are affected by sex hormones are the interstitial steroidogenic leydig cells, whose primary function appears to be production of testosterone(62). The sertoli cells, whose direct contact with proliferating and differentiating germ cells within the seminiferous tubules makes them essential for providing both physical and nutritional support for spermatogenesis (30).

FSH and LH are secreted by the anterior pituitary and act directly on the testis to stimulate somatic cell function in support of spermatogenesis (78). LH is known to act on leydig cells to produce testosterone while FSH acts on sertoli cells to promote spermatogenesis (30).

1.5. Aim of the study
The overall aim of the current study is to investigate the significance of some trace elements in seminal plasma of infertile men in Gaza strip with the following specific objectives:

1. Compare the seminal plasma levels of trace elements (zinc, copper, magnesium, and iron) in infertile group with a control group.
2. Determine whether there are relationships between these trace elements levels with seminal fluid parameters.
3. Determine whether there are relationships between trace elements levels in seminal plasma with the hormonal profiles (LH, FSH and Testosterone) in serum of the infertiles.
4. Compare the concentration of such divalent cations with each others in order to understand how trace elements in seminal plasma of the infertiles affect each other levels.
CHAPTER-2
Literature review

2.1. Seminal fluid
Seminal fluid is a complex milieu surrounding spermatozoa composed of testicular tissues, epididymis, seminal vesicles and prostate, seminal fluid fulfill a dual role, providing optimal conditions for fertilizing and suppressing host immune response and at the same time protects spermatozoa from infection. From immunological point of view of particular importance are cytokines released by various cell subset in male urogenital tract including spermatozoa and leukocytes; lymphocytes produce interleukins and their receptors, along with a member of compounds such as prostaglandins, peptide hormone, growth factors, enzymes, transport proteins and also steroids (5).

Of biochemical parameters in seminal plasma related to male reproductive system are fructose (indicates the androgen activity of testicles), acid phosphatase (prostate function), lactate dehydrogenase (specific for spermatozoids), and creatine kinase (measures cellular maturity). Acidobasic balance factors and ions (PH, sodium, potassium, chloride, phosphorus, calcium, magnesium and sulphur) which are important as metabolic parameters (47).

Seminal plasma is well endowed with an array of antioxidants that act as free radical scavengers to protect spermatozoa against oxidative stress, this defense mechanism compensates for the loss of sperm cytoplasmic enzymes occurring when the cytoplasm is extruded during spermiation, which in turn diminishes endogenous repair mechanisms and enzymatic defences. Seminal plasma contains a number of enzymatic antioxidants such as superoxide dismutase, catalase and glutathione peroxidase. In addition, it contains a variety of non-enzymatic antioxidants such as vitamin C (ascorbic acid), vitamin E (α-tocopherol), pyruvate, glutathione and carnitine (88).
2.2. The endocrine control of spermatogenesis

The understanding of the hormonal roles in male reproduction is necessary for the developments of better treatment for infertility. The adult male testis has two important roles, fertility and virility (86).

The function of the testis depend on the action of the pituitary gonadotrophins, FSH and LH, which are stimulated by hypothalamic gonadotrophin releasing hormone. Testosterone is secreted by the Leydig cells under LH stimulation and is essential for promoting spermatogenesis. While FSH has a role in the development of immature testis (74).

2.3. Gonadotrophic regulation of spermatogenesis

Spermatogenesis is under the control of peptide and steroid hormones, each of which plays an important role in the normal functioning of seminiferous epithelium. Male sex hormones are critical not only for regulation of male germ cell development, but also for the proliferation and function of the somatic cell types required for proper development of the testis (61). These include

1. Interstitial steroidogenic leydig cells, whose primary function appears to be the production of testosterone (62).
2. The myoid cells that surround the seminiferous tubules and provide physical support and contractile motions to these structures (58).
3. and sertoli cells, whose direct contact with proliferating and differentiating germ cells within the seminiferous tubules makes them essential for providing both physical and nutritional support for spermatogenesis (30).

Each of these cell types is a direct target for one or more of the hormones whose actions are essential for male fertility.
Figure (1): Hormonal regulation of spermatogenesis
2.4. Role of peptide hormones in spermatogenesis

In addition to the fact that FSH and LH has a specific α− subunit , the specificity of the action of these hormones is due to the differential distribution of their receptors, while FSH receptors are limited to sertoli cells, LH receptors are confined to leydig cells (78,20,53).

Genetic and pharmacological studies in rodents indicate that the primary role of FSH in spermatogenesis is stimulation of sertoli cells proliferation during prepubertal development (38). Sertoli cell number largely determines the number of germ cells (89).

Finding out the role of LH in testosterone synthesis is indicated by treatment of LH-receptors knockout mice with exogenous testosterone ,mice able to fully rescue spermatogenesis in the absence of LH- receptors function (53).

Inhibins, which are glycoprotein members of transforming growth factor-β family , participate in the control of spermatogenesis by a negative feedback influence on secretion of FSH (95).

2.5. Role of steroid hormones in spermatogenesis

Testosterone and its metabolites, dihydrotestosterone and estradiol are considered the primary male sex steroid. They function to control both gonads and germ cell development. Although, in males, testosterone carries out the primary morphological development and reproductive function, estradiol plays some role in the maintenance of male fertility. The primary function of estradiol through estradiol receptor alpha in the male reproductive tract is to regulate luminal fluid reabsorption in testis and efferent ducts linking the testis and epididymis (39,52).

Testosterone production is regulated by LH, whose release from pituitary stimulates the steroidogenic leydig cells in the testis to release testosterone (62).
Sertoli cells are the only somatic cell type in direct contact with differentiating germ cells, which are under control of FSH; they provide both physical and nutritional support for spermatogenesis, which occurs in the intercellular spaces between sertoli cells (30).

Adhesion between germ cells and sertoli cells may be androgen dependent, as testosterone withdrawal leads not only to retention and phagocytosis of mature, elongated spermatids, but also to the premature release of round spermatids (69).

2.6. Trace elements and their role in metabolism

2.6.1. Zinc

Zinc exists in all biological systems, tissues and fluids of the body including the seminal plasma of humans. Zinc is now known to be an important biological factor for the function of many cell types. Zinc is essential for growth and development, testicular maturation, neurological function, wound healing and immuno-competence. Over 300 zinc enzymes have been discovered covering all six classes of enzymes and in different species of all phyla (13,14,102).

Zinc has structural, regulatory or catalytic roles in many enzymes (103). Additionally, it maintains the configuration of a number of non-enzymatic proteins such as pre-secretory granules of insulin, some mammalian gene transcription proteins (98) and thymulin. Well known zinc containing enzymes include SOD, alkaline phosphatase and alcohol dehydrogenase.

2.6.1.1. Zinc and infertility

Despite the presence of studies which have demonstrated the significance of zinc in male infertility, the biological role of zinc in male reproduction need to be further studied. Seminal plasma as well as sperms contents of zinc is high and it is noted that sperm content of zinc increases as they cross from testicles to the urethra (48,104).
Human cases of hypogonadism caused by zinc deficiency were first reported by Prasad, the male patient had severe hypogonadism coincidentally with markedly short stature, after zinc supplementation was started the pubic hair appeared in all of them within 7 to 12 weeks, the genetalia increased to normal size and secondary sexual characteristics developed within 12 to 24 weeks in all of them (80).
Reports had indicated that zinc increases the levels of testosterone, zinc deficient rats show hypogonadism which results primarily from leydig cell failure. Zinc enhances human chorionic gonadotrophin induced production of cAMP and consequently testosterone in rat testis (67).

Zinc may increase the conversion of androstenedione to testosterone in the periphery (8).

Zinc also interferes with the metabolism of testosterone by decreasing its hepatic clearance and reducing hepatic 5-alpha reductase activity (51). Zinc may increase not only the serum concentration of testosterone but also may affect the action of androgens, since the DNA-binding domain of the androgen receptors is a cysteine-rich zinc finger protein (31). This increase in the level of testosterone is primarily due to its testicular action as indicated by more than one study (79).

Zinc is also involved in a number of functions of importance to sperm physiology; zinc supplied from the prostate secures a high content of zinc in the sperm nucleus that contributes to the stability of the quaternary structure of the chromatin and preserves genomic integrity (48).

Zinc contributes to the stable attachment of sperm head to tail and its removal induces head-tail detachment (9). Once oocyte penetration has taken place, the spermatozoan nucleus undergoes decondensation and forms the pronucleus, this decondensation process requires reduction of the chromatin zinc content since zinc inhibits the process (43).
Zinc deficiency causes deterioration of the seminiferous tubules, failure of spermatogenesis and decreased testosterone secretion in the rat (104).

Zinc deficiency in men using zinc-restricted diet had shown that the baseline sperm count and total sperm count per ejaculate decreased significantly after zinc restriction and returned to normal, 6 to 12 months after zinc supplementation. This study demonstrated that dietary restriction of zinc could affect testicular function adversely (1).

Serum testosterone concentration and seminal volume per ejaculate were lower in healthy male volunteers fed zinc–restricted diet for 35 days (42).

2.6.2. Copper

It is well established that trace element copper is essential for life. Copper in living organisms, including humans, forms an essential component of many enzymes (cuprous-enzymes) and proteins. The biochemical role of copper is primarily catalytic, with many copper metaloenzymes acting as oxidases to achieve the reduction of molecular oxygen for example cytochrome- C oxidase and SOD. Studies have also shown that copper is required for infant growth, host defense mechanisms, bone strength, red and white cell maturation, iron transport, cholesterol and glucose metabolism (100).

Copper plays additional roles that are less well understood and may be in part non-enzymatic, such as in angiogenesis, nerve myelination and endorphin action (55).

2.6.2.1. Copper and male infertility

Although it has been hypothesized that copper play a role in spermatogenesis, the seminal plasma levels of copper in infertiles were not different from control
groups (107). In the same study there was not any correlation between seminal plasma levels of copper and any of the seminal analysis parameters. However in another study, despite the finding that there was no significant difference between the seminal plasma levels of copper, there was a correlation between the levels of copper and sperm concentration, and progressive motility (46).

Despite the poor study of correlations between the levels of reproductive hormones and copper seminal plasma levels, reports had shown that hormonal treatment of infertile subjects did increase the levels of copper in seminal plasma, which indicates the significance of copper in male reproduction function (106).

Copper is also known to act as an anti-oxidant as part of the copper/zinc SOD enzyme which acts to lower the levels of reactive oxygen species. The presence of copper in the seminal plasma could inhibit lipid peroxidation of the plasma membrane of spermatids (113).

2.6.3. Iron

Iron has the capacity to accept and donate electrons readily interconverting between ferric and ferrous forms; this capacity makes it a useful component of cytochromes, oxygen-binding molecules (hemoglobin and myoglobin) and many enzymes. Iron can also damage tissues by catalyzing the conversion of hydrogen peroxide to free-radical ions that attack cellular membranes, proteins and DNA. Iron ions circulate bound to plasma transferrin and accumulated within cells in the form of ferritin. Iron protoporphyrin (heme) and iron-sulfur clusters serve as enzyme cofactors (65).

2.6.3.1. Iron and male infertility

The seminal plasma concentration of iron in the infertile population were studied, reports had shown that in teratospermic patients compared with control group
there were no difference between teratospermic and control seminal plasma iron concentration and iron may be needed for lipid peroxidation (50).

Iron concentration in seminal plasma of asthenospermia were higher than controls was found (40).

Iron roles in male reproduction can be summarized by the model which describes how iron is transferred into sperms. The proposed model (Fig.2) includes basal transferrin receptors(TfR) on Sertoli cells, movement of iron through the cell, secretion of ferric ions associated with a newly synthesized testicular transferrin(tTf) and incorporation of iron in the newly synthesized transferrin into ferritin in developing germ cells (30).

![Figure 2](image)

**Figure 2**: Iron transport into sertoli cells by transferrin in testis (Griswold, 1998).

Lipid peroxidation usually leads to the formation of reactive oxygen species particles which are oxidative and leads to sperm damage. The most common types of lipid peroxidation are a non-enzymatic membrane lipid peroxidation and enzymatic NADPH and ADP dependent lipid peroxidation (24).

Iron is known to enhance enzymatic processes of lipid peroxidation. In spermatozoa it is known that ferrous ions promote lipid peroxidation.(16).
Beside membrane effects, lipid peroxidation can damage DNA and proteins, either through oxidation of DNA bases (primarily guanine via lipid peroxy or alkoxyl radicals) or through covalent binding to malondialdehyde resulting in strand breaks and cross linking (24).

The oxidation damage to mitochondrial DNA is well known to occur in all aerobic cells which are rich in mitochondria and this may include spermatozoa, the redox status of human spermatozoa is likely to affect phosphorylation and ADP generation with a profound influence on its fertilizing potential (15).

2.6.4. Magnesium
Total body magnesium content is approximately 35 grams, of which 50-60% resides in bone in the normal adult. One third of skeletal magnesium is exchangeable, and it is this fraction that may serve as a reservoir for maintaining a normal extracellular magnesium concentration (21).

Extracellular magnesium accounts for about 1% of total body magnesium, the normal serum magnesium concentration is 1.8-3.3 mg/dl. Magnesium is a required cofactor for over 300 enzyme systems (105).

It is required for both anaerobic and aerobic energy generation and for glycolysis, either indirectly as a part of the magnesium-ATP complex or directly as an enzyme activator (28). Magnesium has also been shown to be required for mitochondria to carry out oxidative phosphorylation (105).

The mitochondrial enzymes utilize the magnesium chelate of ATP and ADP as the actual substrates for phosphate transfer reactions. Magnesium transport into and out of the cells appears to require the presence of carrier mediated transport systems (32). The complex of magnesium from the cell is coupled to sodium transport and requires energy.
Magnesium transport in mammalian cells may be influenced by hormonal and pharmacological factors including β-agonists, growth factors, and insulin (32). It has been suggested that abnormally regulated magnesium uptake system controls intracellular magnesium concentration in cellular compartments. Magnesium concentration in these compartments would thus serve to regulate the activity of magnesium-sensitive enzymes.

Magnesium presence is important for maintaining an adequate supply of purine and pyrimidine nucleotides required for increase DNA synthesis that occurs during proliferation (83).

Replicating cells must be able to synthesize new protein, and this synthesis has been reported to be highly sensitive to magnesium depletion, many hormones, neurotransmitters and other cellular effectors regulate cellular activity via the adenylate cyclase system, and the activation of adenylate cyclase require the presence of magnesium. This is also an evidence for magnesium binding through which magnesium directly increases adenylate cyclase activity (59).

Magnesium is necessary for sodium-ATPase activity, which is responsible for active transport of potassium (19). Magnesium has been called nature's physiological calcium channel blocker (44).

2.6.4.1. Magnesium and male infertility

There were a number of studies which investigate the significance of magnesium in fertility aspects. There were no significant difference occurs in levels of magnesium levels in normozoospermic, oligospermic and azoospermic semen (73).

In another study magnesium levels in seminal plasma were associated with premature ejaculation and that decreased levels of magnesium give rise to vasoconstriction due to increase thromboxane level. This leads to increased
endothelial intracellular calcium and decreased nitric oxide which leads to premature ejaculation (72).

Positive correlation between the concentration of zinc and magnesium and the quantity of spermatozoa along with negative correlation with the percentage of motile spermatozoa were found(7).

Seminal levels of magnesium were lower in infertile groups than controls, which indicate the significance of magnesium in male fertility (76,18).
CHAPTER-3
Materials and Methods

3.1. Study design
This study presents a case control study in which the case group was infertile men
with oligospermia or asthenospermia and the control group was fertile men and
their wives were pregnant or had delivery of a child within the previous 12 months.

3.1.1. Subjects
We studied two groups of subjects:
   a) Case group: this group consists of 72 infertile subjects aged 20-50 years
      with oligospermia or/and asthenospermia.
   b) Control group: this group consists of 72 fertile men aged 20-50 years their
      wives were pregnant or had delivery of a child within the previous 12
      months.

3.1.2. Exclusion criteria
All subjects needed to fill an exclusion criteria: taken according to questionnaire
(Appendix 1). Infertile subjects excluded from the study group if they have the
following criteria, Azoospermia, Prostatitis, any infection in genitourinary tract,
sexually transmitted diseases, surgery in pelvic area, exposed for long time to
heat, smoking or they are under any medication (i.e. Antibiotics, Estrogen,
Testosterone, Radioactive iodine and Steroids).

3.1.3. Setting
The study was conducted at the central infertility clinic (Andrology) at Shifa
Hospital in Gaza.

3.1.4. Ethical Considerations
An authorization to carry out the study was obtained from Helsinki (Declaration of
Helsinki the most widely accepted guideline on medical research involving human
subjects) using an agreement letter prepared from the Islamic University of Gaza
(Appendix 4).
3.1.5. Data collection

Every subject filled a questionnaire and then signed a consent form (Appendix 3). Seminal fluid collection and analysis were carried out in strict compliance with the WHO guidelines. Serum hormonal profiles of LH, FSH and testosterone as well as seminal plasma measurement of the trace elements zinc, copper, iron and magnesium were carried out.

3.2. Variables Determination

3.2.1. Seminal fluid analysis

Semen quality was assessed in accordance with the WHO(1999) guidelines. Seminal fluid samples were obtained with masturbation after a maximum interval of sexual abstinence between 2 and 7 days, the complete ejaculate collected in a sterile wide opening container and delivered to the laboratory preferably within 30 minutes, but at least within one hour after ejaculation.
Sample container uniquely labeled (name and number unique for the sample) concomitantly with the requisition and sample record form, the sample placed on an orbital mixer in the incubator (+37°C) for 25-30 minutes after ejaculation. Sample examination begins at 30 minutes after ejaculation.

Sample volume determined by a graded pipette with 0.1 accuracy. Liquefaction checked to be completed. Viscosity checked by estimation of how fast the sample runs out of the pipette.

Examination of a wet preparation performed directly after complete liquefaction, 10µl of well-mixed semen on a clean microscopic slide and covered with a cover glass on top (22X22mm, #11/2) this gives the preparation a depth of about 20 µm, examination began as soon as the flow in the preparation ceased.

3.2.1.1. Sperm count
A well-mixed semen sample was diluted (1:20) with 5% sodium bicarbonate (50 grams of sodium bicarbonate powder, diluted in 10 milliliters of 36-40% formaldehyde solution (saturated), and all constituents dissolved in one liter of distilled water).

The dilution was mixed well on vortex and charged into Improved Neubauer Haemocytometer, both sides completely filled with the dilution rested for 10–15 minutes in a humid box to allow the spermatozoa to sediment to the grid of the counting chamber.

Spermatozoa counted in both sides of the chamber in the four large white blood cells squares, using light microscope at magnification of 40X, the mean is taken and multiplied by the dilution factor and the area for the chamber counted to express the result as millions sperm per milliliter.
3.2.1.2. Sperm motility
Motility was assessed by light microscopy (10 μl of semen was delivered onto a glass slide and covered with a 22X22mm cover slip) and graded as follow: class a and b, fast and weak forward motility, class c, non-progressive motility, class d, immobile spermatozoa. At least 200 spermatozoa were classified in duplicate i.e. at least 400 spermatozoa in total, at least five fields assessed in each count.

3.2.2. Hormonal assay
Blood sample drawn from each participant, 3 ml of venous blood allowed to clot then centrifuged and serum samples stored at -20°C until the determination of LH, FSH and Testosterone performed by ELISA using DSL Webster, Texas, USA kits.

3.2.2.1. Determination of serum LH
Principle of the test
Serum LH was determined after an enzymatically amplified (one-step) Sandwich –type immunoassay, samples were incubated with anti-LH antibody in microtitration wells which have been coated with another anti-LH antibody. After incubation and washing, the wells were incubated with the substrate tetramethylbenzidine (TMB). An acidic stopping solution was then added and the point of enzymatic turnover of the substrate which was determined by dual wavelength absorbance measurements at 450 nm and 620 nm. The measured absorbance is directly proportional to the concentration of LH present.
Test procedure

A. Materials supplied

DSL Active-kit catalogue number DSL-10-4600

<table>
<thead>
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<th>Reagent</th>
<th>Concentration mIU/ml</th>
</tr>
</thead>
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</tr>
<tr>
<td>LH standard B</td>
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<td>LH standard C</td>
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<tr>
<td>LH Control level I</td>
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</tr>
<tr>
<td>LH control level II</td>
<td>High</td>
</tr>
</tbody>
</table>

B- Preparation of reagents

1. Wash solution

Exactly 100 µl of the wash concentrate was pipetted into a clean container and diluted by adding 900 µl of deionized water (10X) , stable for one month at room temperature (~ 25°C).

2. Antibody-enzyme conjugate solution was diluted at a ratio of one part into fifty parts of the assay buffer according to the number of wells used, freshly diluted just prior to use in the assay.

3. Microtitration wells

Coated wells were selected for the assay.
C. Assay procedure (Non-shaking assay procedure)

All specimens and reagents were allowed to reach room temperature (~25 °C) and mixed thoroughly by gentle inversion before use. Standards, controls, and unknown were assayed in duplicate.

1- Microdilution strips to be used were marked.

2- Antibody-enzyme conjugate solution was prepared by diluting the conjugate concentrate with the conjugate diluent.

3- The standards, controls and the unknowns of 50µl were pipetted to the appropriate wells.

4- Antibody-enzyme conjugate solution of 100 µl was added to each well using a semi-automatic dispenser.

5- Wells for were incubated 90 minutes at room temperature (~25 °C).

6- Contents of each well was aspirated and washed five times with the wash solution using an automatic microplate washer. Blotted dry by inverting plate on absorbent material.

7- The TMB chromogen solution of 100 µl was added to each well using semi-automatic dispenser.

8- Wells were incubated for 20–25 minutes at room temperature (~25 °C).

9- The stopping solution of 100 µl was added to each well using a semi-automatic dispenser.

10- Absorbance of the solution in the wells was read within 30 minutes using a microplate reader set to 450 nm with a background wavelength correction set at 620 nm. TC 98 ELISA strip reader were used (Germany).

D. Results calculation

1- The mean absorbance for each standard, controls and unknowns were calculated.

2- The log of the mean concentrations for each of the standards were plotted along the X-axis versus log of the mean absorbencies of LH along the Y-axis,
using a linear curve–fit for data reduction or the data can be plotted linear versus linear and a smoothed line curve-fit can be used.

3- The best fitting curve was drawn through the mean of the duplicate points.

4- LH concentrations of the controls and unknown were determined from the standard curve by matching their mean absorbance readings with the corresponding LH concentrations.

E. Quality Control
DSL controls were used and fall within established confidence limits. The confidence limits for DSL controls were printed on the control vial labels.
Low and high level controls included in each assay.
The TMB solution was colorless. Development of a blue color may indicate reagent contamination or instability.

F. Expected Values
Adult Males: 1.08 – 8.34 mIU/ml.

3.2.2.2. Determination of FSH
Principle of the Test
The DSL – 10-4700 Active FSH ELISA is an enzymatically amplified (two-step) sandwich–type immunoassay. In the assay, standards, controls, and unknown serum samples were incubated in microtitration wells which have been coated with anti-FSH antibody.
After incubation and washing, the wells were treated with another anti-FSH detective antibody labeled with the enzyme horseradish peroxide. After a second incubation and washing step, the wells were incubated with the substrate TMB. An acidic stopping solution was then added and the degree of enzymatic turnover of the substrate was determined by dual wavelength absorbance measurement at 450 and 620 nm. The absorbance measured was directly proportional to the concentration of FSH present. A set of FSH standards was used to plot a standard
curve of absorbance versus FSH concentration from which the FSH concentrations in the unknowns can be calculated.

Test procedure

A- Materials supplied

Materials supplied in DSL Active FSH ELISA kit (Cat No DSL 10-4700)

<table>
<thead>
<tr>
<th>Material</th>
<th>Concentration (Mlu/ ml)</th>
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<td>FSH control level I</td>
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<tr>
<td>FSH control level II</td>
<td>High control</td>
</tr>
</tbody>
</table>

B- Preparation of reagents

1- Wash solution: 100 ml of wash concentrate was poured in a clean container and diluted by adding 900 ml of deionized water. The wash solution is stable for one month at room temperature provided the bottle is kept tightly sealed.

2- Antibody-enzyme conjugate solution: The antibody-conjugate concentrate was diluted at a ratio of one part into 50 parts of the number of wells used. The antibody-enzyme conjugate concentrate was freshly diluted just prior to use in the assay.

3- Microtitration Wells: the number of coated wells required for the assay were selected.

C. Assay procedure (Non-shaking assay procedure)

All specimens and reagents were allowed to reach room temperature and mixed thoroughly by gentle inversion before use. Standards and unknown assayed in duplicate.
1- Microtitration strips to be used were marked.
2- Antibody – enzyme conjugate solution was prepared.
3- The standards, controls and unknowns 100 µl were pipetted to the appropriate wells.
4- Wells were incubated for 60 minutes at room temperature (~25°C).
5- Each well was aspirated and washed five times with the wash solution using an automatic microplate washer. Blotted dry by inverting the plate on absorbent material.
6- The Antibody – Enzyme conjugate solution of 100 µl was added to each well using a semi-automatic dispenser.
7- Wells were incubated 30 minutes at room temperature (~25°C).
8- All wells were aspirated and washed 5 times with wash solution. Blotted by inverting plate on an absorbent paper. 100 µl of the TMB chromogen solution was added to each well.
9- Wells were incubated 20 minutes at room temperature (~25°C). Exposure to direct sunlight was avoided.
10- The stopping solution of 100 µl was added to each well using a semi-automatic dispenser.
11- Absorbance of the solution in the wells was read within 30 minutes, using a microplate reader set to 450 nm. With a background wavelength at 620 nm. TC 98 ELISA reader were used (Germany).

D. Results Calculation
1- The mean absorbance for each standard, control or unknown was calculated.
2- The log of the concentration along the x-axis versus log of the mean absorbance readings for each of the standards along the y-axis were plotted, using a linear curve – fit.
3- The best fitting curve through the mean of the duplicate points was drawn.
4- FSH concentrations of the unknowns and controls from the standard curve were determined by matching their mean absorbance readings with the corresponding FSH concentrations.
E. Quality control
- DSL control, used.
- Low and high level controls included in the assay.
- The TMB solution was colorless.

F. Expected values
Adult male: < 1.50 – 5.84 mIU/ml.

3.2.2.3. Determination of Testosterone

Principle of the Test
The procedure follows the basic principle of enzyme immunoassay where there is competition between an unlabeled antigen and an enzyme–labeled antigen bound to the antibody binding sites. The amount of enzyme–labeled antigen bound to the antibody is inversely proportional to the concentration of the unlabeled analyte present. Unbound materials are removed by decanting and washing the wells. The absorbance measured is inversely proportional to the concentration of testosterone present in the serum. A set of testosterone standards is used to plot a standard curve of absorbance versus testosterone concentrations in the unknowns can be calculated.

Test procedure

A- Materials supplied
DSL – Active Testosterone Kit cat .No DSL 10-4000

<table>
<thead>
<tr>
<th>Material</th>
<th>Concentration (ng / ml)</th>
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<tr>
<td>Testosterone standard B</td>
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<tr>
<td>Testosterone standard C</td>
<td>0.50</td>
</tr>
<tr>
<td>Testosterone standard D</td>
<td>2.50</td>
</tr>
<tr>
<td>Testosterone standard E</td>
<td>5.00</td>
</tr>
<tr>
<td>Testosterone standard F</td>
<td>10.00</td>
</tr>
<tr>
<td>Testosterone standard G</td>
<td>25.00</td>
</tr>
<tr>
<td>Testosterone control level I</td>
<td>Low</td>
</tr>
<tr>
<td>Testosterone control level II</td>
<td>High</td>
</tr>
</tbody>
</table>
B- Preparation of Reagents

1- Wash solution
The wash concentrate into a clear container of 60 ml was poured and diluted by adding up to 1500 ml of deionized water (25 x), stable for one month at room temperature (~ 25°C).

2- Enzyme conjugate solution
Diluted at a ratio of one part Enzyme conjugate concentrate into 50 parts of the conjugate diluent, according to the number of wells used.

3- Microtitration wells
Coated wells required for the assay were selected.

Assay procedure
All specimens and reagents were allowed to reach room temp. (~ 25°C) and mixed thoroughly by gentle inversion before use. Standards and unknowns assayed in duplicate.

1- Microtitration strips to be used were marked.
2- The standards, controls and unknowns of 50µl were pipetted into the appropriate wells.
3- Enzyme conjugate solution was prepared.
4- The enzyme conjugate solution of 100 µl was added to each well using a semi-automatic dispenser.
5- The Testosterone antiserum of 100 µl was added to each well using a semi-automatic pipette.
6- Wells shaking at a fast speed (500-700 rpm) on an orbital microplate shaker were incubated, at room temperature (~ 25°C) for one hour.
7- Each well was aspirated and washed 5 times with wash solution using an automatic microplate washer. Blotted dry by inverting plate on absorbent material.
8- The TMB chromogen solution of 100 µl was added to each well using a semi-automatic dispenser.
9- Wells shaking at (500 -700 rpm) on an orbital microplate shaker were incubated at room temperature. (~25°C) for 30 minutes. Exposure to direct sunlight was avoided.

10- The stopping solution (0.2 Molar sulfuric acid) of 100 µl was added to each well using semi – automatic dispenser.

11- Absorbance of the solution in the wells was read within 30 minutes using a microplate reader set to 450 nm. With background wavelength correction set at 620 nm. TC 98 ELISA strip reader were used (Germany).

**Results Calculation**

1- The mean absorbance of each standard, control or unknown was calculated.

2- Log – linear graph paper was used, log concentration was plotted on the x – axis versus the linear optical density reading for each standards on the y – axis using 4-parameter curve fit for the data reduction.

3- The best fitting curve was drawn through the mean of the duplicate points.

4- The testosterone concentrations of the controls and unknowns were determined from the standard curve by matching their mean absorbance readings with the corresponding testosterone concentrations.

**Quality control**

- DSL controls used and it falls within the established confidence limits on the bottle.
- Low and high controls were used in the assay.
- The TMB solution was freshly prepared.

<table>
<thead>
<tr>
<th>Expected values</th>
<th>Adult males(ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20-30 yrs</td>
<td>2.9-9.9</td>
</tr>
<tr>
<td>30-40</td>
<td>2.0-10.0</td>
</tr>
<tr>
<td>40-50</td>
<td>0.069-5.2</td>
</tr>
<tr>
<td>50-60</td>
<td>0.16-6.6</td>
</tr>
</tbody>
</table>
3.2.3. Trace elements analysis by atomic absorption (AAS)

After performing semen analysis, the spermatozoa were separated from seminal plasma by centrifugation at 7000 gravity for 5 minutes the supernatant separated and stored at -20°C until the time of analysis (12).

To measure the seminal plasma levels for trace elements, seminal plasma samples were thawed and directly diluted for the determination of the trace elements. For zinc and magnesium, seminal plasma were diluted 200 times in 0.1 N nitric acid, while copper and iron levels were diluted 5 times in 0.1 N nitric acid. Levels were determined by an atomic absorption spectrophotometry (Perkins Elmer, A Analyst 100-Germany).

3.2.3.1. Measurement system

Atomic absorption spectrophotometry

Atomic absorption spectrophotometry is commonly used in many analytical laboratories for determination of trace elements in water samples and in acid digests of sediment or biological tissues. Zinc, copper, iron and magnesium were measured in seminal plasma samples.

Assay principle

While a sample is being aspirated into a flame, a light-beam is directed through the flame into a monochromator and onto a detector that measures the amount of light absorbed by the atomized element in the flame. A source lamp (hollow cathode lamp) composed of the element of interest is used because each element has its own characteristic wavelength. This makes the method relatively free from spectral or radiation interferences. The amount of energy at the characteristic wavelength absorbed in the flame is proportional to the concentration of the element in the sample over a limited concentration range. Most atomic absorption instruments are also equipped for operation in an emission mode.
Reagents
Metal-free water was essentially used for the preparation of all reagents. Hydrochloric acid, HCl and nitric acid, HNO₃, (analytical grade) were used for standard preparation and for digestion methods.

3.2.3.2. Preparation of standards
Standard solutions of known metal concentrations were prepared in water with a matrix similar to the tested samples. Standards were bracket the expected sample concentration and fall within the method’s working range. Very dilute standards having concentrations of at least 0.05 mg L⁻¹ were prepared daily from fresh standard stock solutions. If sample digestion is used, standards should be carried through the same digestion procedures. The standard stock solutions described below have a concentration of 100 mg L⁻¹.

Zinc: one tenth gram of zinc metal was dissolved in 20 ml 1:1 HCl and diluted up to 1,000 ml with water.

Copper: one tenth gram of copper metal was dissolved in 2 ml concentrated HNO₃, then 10 ml of concentrated HNO₃ was added and the solution mixture was diluted up to 1,000 ml with water.

Iron: one tenth of iron wire was dissolved in a mixture of 10 ml 1:1 HCl and 3 ml concentrated HNO₃, then 10 ml of concentrated HNO₃ was added and the solution mixture was diluted up to 1,000 ml with water.

Magnesium: an exact weight of 0.1658 g magnesium oxide was dissolved in a minimum of 1:1 HNO₃, then 10 ml of concentrated HNO₃ was added and the solution mixture was diluted up to 1,000 ml with water.

3.2.3.3. Procedure
It is not possible to provide an operating procedure that would be correct for all atomic absorption spectrophotometers because of differences between models of instrument. The manufacturer’s operating manual should be followed. A general procedure contains three components as described below.
A. Adjustment of the Instrument:
1. A hollow cathode lamp for the desired element was installed in the instrument and the wavelength was set to the appropriate setting for the element.
2. Slit width was set according to the manufacturer’s suggested value for the element being measured.
3. Instrument then turned on and the lamp current was adjusted to the level suggested by the manufacturer.
4. Instrument then warmed up, 10-20 minutes, and current readjusted as necessary.
5. Wavelength was adjusted until optimum energy gain is obtained.
6. Lamp was aligned in accordance with the directions in the operating manual.
7. Suitable burner head was installed and its position was adjusted.
8. Air was then turned on and its flow was adjusted to the rate recommended to give maximum sensitivity for the metal being measured.
9. Acetylene then turned on and its flow was adjusted to recommended rate, then ignited and the flame was allowed a few minutes to stabilize.
10. Blank of deionized water that has been given the same treatment and acid concentration as the standards and samples was aspirated and the reading adjusted to zero.
11. A Standard solution was aspirated and the aspiration rate was adjusted to obtain maximum sensitivity.
12. Burner was adjusted horizontally and vertically to obtain maximum response.

B. Preparation of the calibration curve
1. At least five concentrations of each metal ion standard solutions were selected to perform a calibration curve. There should be one concentration greater and one less than that expected in the sample(s).
2. Blank was aspirated and adjusted to the zero value.
3. Each standard was aspirated in turn into the flame and the absorbance was recorded.
4. Calibration curve was performed by plotting the absorbance of the standards against their concentrations. This step is not necessary for instruments with direct concentration readout.

C. Analysis of samples
1. Nebulizer was rinsed by aspirating with water containing 1.5 ml HNO$_3$ per liter. The blank was atomized and set to the zero value.
2. Samples were atomized and their absorbance were determined.
3. Lamps were changed and the procedure repeated for each element.

D. Calculations
Determination the concentration of each metal ion, were based on the calibration curves. Results for trace elements were calculated in $\mu$g L$^{-1}$ while, in mg L$^{-1}$ for the more common metals. Concentrations may be read directly from instruments with a direct readout capability. If a sample has been diluted, appropriate dilution factor were applied. The recommended wavelengths and slit widths for the metal ions determined are given in the following table:

<table>
<thead>
<tr>
<th>Metal</th>
<th>Selected wavelength</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zinc</td>
<td>213.8nm</td>
</tr>
<tr>
<td>Magnesium</td>
<td>285.2nm</td>
</tr>
<tr>
<td>Copper</td>
<td>324.8 nm</td>
</tr>
<tr>
<td>Iron</td>
<td>248.3nm</td>
</tr>
</tbody>
</table>

All slit widths were 0.7 $\mu$m
3.3. Statistical analysis

All experimental data were expressed in mean and standard deviation and data obtained in the study and control group were compared by two – tailed t-test for unpaired data.

Linear regression analysis to correlate seminal variables, trace elements and endocrine parameters in the study group as a whole analyzed by means of SPSS version 8 for windows software package for multiple comparisons and Pearson’s coefficient (r) calculated to determine associations between these parameters. P (probability) lower than 0.05 was considered significant.
CHAPTER-4

4. Results

This chapter presents the results of the study. The study population consists of seventy-two infertile men (case group) and seventy-two fertile men (control group) were included in this study. The case group was selected according to family history and the case group was not able to conceive at least for twelve months of unprotected sex and their wives had no medical problems.

Seminal plasma trace elements (zinc, copper, iron and magnesium), seminal parameters and endocrine parameters were measured. The mean age and standard deviation (SD) of the case group was (33.1 ±7.8) years and the mean age and standard deviations of the control group were (33.9±7.15) years.

4.1. Semen analysis

Table (1) shows that sperm parameters excluding the volume of the case group were below the WHO reference range (Appendix 2). Mean sperm count compared with the control group mean count was very much lower than the control group (P<0.001). Forward progression percentage was lower in the case group than the control group (P<0.001). Weak-motile percentage were lower in the case group than the control group (P<0.05), non-motile percentage were lower in the case group than the control group (P<0.001).
Table (1): Comparison between seminal parameters of case group (infertile men) with control group (fertile men)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Case group (N=72) Mean±SD</th>
<th>Control group (N=72) Mean±SD</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume (ml)</td>
<td>3.08±1.12</td>
<td>3.32±0.9</td>
<td>&gt;0.05 NS</td>
</tr>
<tr>
<td>Count (million)</td>
<td>23.9±22</td>
<td>63.1±16.7</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>Forward motility</td>
<td>22.8±14</td>
<td>51.3±5.36</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>Weak motile</td>
<td>21.3±10.6</td>
<td>18.5±5.08</td>
<td>&lt;0.05*</td>
</tr>
<tr>
<td>Non-motile</td>
<td>55.9±18.6</td>
<td>30.3±6.27</td>
<td>&lt;0.001***</td>
</tr>
</tbody>
</table>

All values were expressed as mean ± SD
*Mean difference is significant at P < 0.05
** Highly significant differences at P < 0.01
*** More highly significant at P < 0.001
NS (non significant) P > 0.05

4.2. Hormonal assay

The mean levels of FSH, LH and testosterone were normal in both groups, however LH level were significantly lower in the case group than in the control group (P < 0.05), testosterone mean levels were significantly lower in the case group than the control group (P < 0.05), while the mean levels of FSH in both groups were not significantly different (P > 0.05) as shown in Table (2).
Table (2): Comparison between serum endocrine parameters of case group (infertile men) with control group (fertile men)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Case group Mean±SD</th>
<th>Control group Mean ±SD</th>
<th>P -value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LH</td>
<td>4.52±1.45</td>
<td>5.05±1.23</td>
<td>&lt;0.05*</td>
</tr>
<tr>
<td>FSH</td>
<td>5.33±0.6</td>
<td>5.37±0.65</td>
<td>&gt;0.05 NS</td>
</tr>
<tr>
<td>Testosterone</td>
<td>5.09±1.03</td>
<td>5.4±0.9</td>
<td>&lt;0.05*</td>
</tr>
</tbody>
</table>

All values were expressed as mean ± SD
* mean difference is significant at P<0.05.
** Highly significant difference at P<0.01
*** More highly significant at P<0.001
N.S P>0.05 non significant

4.3. Trace elements in seminal plasma

When compared to control group, case group trace elements zinc, copper and magnesium levels were significantly lower than the control group. Zinc mean concentration in seminal plasma were highly lower than the control group (P<0.001). Copper seminal plasma concentration in case group was lower than the concentration of the control group (P<0.05). Magnesium seminal plasma mean concentration in case group was much lower than the mean concentration of the control group (P<0.001). The mean concentration of iron was significantly higher in case group than the mean concentration of the control group (P<0.05) as shown in Table (3).
**Table (3):** Comparison between seminal plasma trace elements in the case group with the control group

<table>
<thead>
<tr>
<th></th>
<th>Case group N=72 Mean±SD</th>
<th>Control group N=72 Mean±SD</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zinc (mg/L)</td>
<td>68.9±37.7</td>
<td>122±26.1</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>Copper (µg/L)</td>
<td>300±218</td>
<td>377±182</td>
<td>&lt;0.05*</td>
</tr>
<tr>
<td>Iron(µg/L)</td>
<td>412±290</td>
<td>329±223</td>
<td>&lt;0.05*</td>
</tr>
<tr>
<td>Magnesium (mg/L)</td>
<td>67.1±36.4</td>
<td>120±28.2</td>
<td>&lt;0.001***</td>
</tr>
</tbody>
</table>

All values were expressed as mean ± SD

*Mean difference is significant at P<0.05

** Highly significant difference at P<0.01

*** More highly significant at P<0.001
4.4. Correlation analysis

Correlation analysis of the case group done between trace elements and semen parameters and the endocrine parameters.

4.4.1. Zinc correlations

4.4.1.1. Zinc and seminal parameters

As shown in Table (4) zinc was inversely correlated with semen volume (P<0.05), positively correlated with sperm concentration (P<0.001) while no correlation found with the other seminal plasma parameters (P>0.05)

Table (4): linear regression analysis between zinc seminal plasma level and seminal parameters in case group n = 72

<table>
<thead>
<tr>
<th></th>
<th>β (slope)</th>
<th>r</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume</td>
<td>-8.482</td>
<td>-0.251</td>
<td>0.034*</td>
</tr>
<tr>
<td>Count (million)</td>
<td>0.646</td>
<td>0.376</td>
<td>0.001***</td>
</tr>
<tr>
<td>Forward motility</td>
<td>-0.488</td>
<td>-0.182</td>
<td>0.127 NS</td>
</tr>
<tr>
<td>Weak motile</td>
<td>0.702</td>
<td>0.197</td>
<td>0.097 NS</td>
</tr>
<tr>
<td>Non-motile</td>
<td>0.025</td>
<td>5.075</td>
<td>0.835 NS</td>
</tr>
</tbody>
</table>

*Correlation is significant at p<0.05 level

β slope of linear regression

r Pearson correlation coefficient

NS non-significant
Figure (3): Relationship between seminal plasma zinc and semen volume in case group  n = 72.

Figure (4): Relationship between seminal plasma zinc and sperm count in case group  n = 72.
4.4.1.2. Zinc and serum endocrine parameters

Seminal plasma zinc is positively correlated with testosterone levels of the case group (p < 0.05) as shown in Table (5).

**Table (5):** Linear regression analysis between seminal plasma zinc and serum endocrine parameters in case group n = 72

<table>
<thead>
<tr>
<th></th>
<th>β (slope)</th>
<th>r</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LH</td>
<td>5.472</td>
<td>.211</td>
<td>0.075 NS</td>
</tr>
<tr>
<td>FSH</td>
<td>4.532</td>
<td>.074</td>
<td>0.538 NS</td>
</tr>
<tr>
<td>Testosterone</td>
<td>10.694</td>
<td>.293</td>
<td>0.012*</td>
</tr>
</tbody>
</table>

*Correlation is significant at p<0.05 level

β  slope of linear regression
r  Pearson correlation coefficient
NS  non-significant

**Figure (5):** Relationship between seminal plasma zinc and serum testosterone levels in case group n = 72.
4.4.1.3. Zinc and other seminal plasma trace elements

Table (6) shows linear regression analysis between trace elements in seminal plasma of the case group, no correlation were found between trace elements copper and iron with zinc levels, there were a strong positive correlation between magnesium and zinc levels (P<0.001).

Table (6): linear regression analysis between seminal plasma zinc and other trace elements in case group:

<table>
<thead>
<tr>
<th></th>
<th>β (slope)</th>
<th>r</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copper(µg/L)</td>
<td>2.47</td>
<td>0.143</td>
<td>0.230</td>
</tr>
<tr>
<td>Magnesium(mg/L)</td>
<td>0.772</td>
<td>0.744</td>
<td>0.001***</td>
</tr>
<tr>
<td>Iron(µg/L)</td>
<td>9.2</td>
<td>0.072</td>
<td>0.551</td>
</tr>
</tbody>
</table>

*Correlation is significant at p<0.05 level

β slope of linear regression

r Pearson correlation coefficient

NS non-significant
Figure (6): Relationship between seminal plasma zinc and seminal plasma magnesium in case group  \( n = 72 \).
4.4.2. Copper correlations

4.4.2.1. Copper and seminal parameters

Linear regression analysis between copper in seminal plasma of infertile men and seminal parameters, no significant correlations (P>0.05) were found as shown in Table (7).

Table (7): Linear regression analysis between seminal plasma copper and seminal parameters in case group  n = 72

<table>
<thead>
<tr>
<th></th>
<th>β (slope)</th>
<th>r</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume</td>
<td>-21.92</td>
<td>-0.110</td>
<td>0.359 NS</td>
</tr>
<tr>
<td>Count</td>
<td>0.717</td>
<td>0.072</td>
<td>0.546 NS</td>
</tr>
<tr>
<td>Forward motility</td>
<td>-2.22</td>
<td>-0.143</td>
<td>0.230 NS</td>
</tr>
<tr>
<td>Weak motile</td>
<td>-0.998</td>
<td>-0.048</td>
<td>0.686 NS</td>
</tr>
<tr>
<td>Non-motile</td>
<td>1.596</td>
<td>0.136</td>
<td>0.255 NS</td>
</tr>
</tbody>
</table>

NS  non-significant (P>0.05)

4.4.2.2. Copper and serum endocrine parameters

Linear regression analysis between copper seminal plasma of infertile men and the endocrine parameters in serum of infertile men, no significant correlations (P>0.05) were found as shown in Table (8).

Table (8): linear regression analysis between seminal plasma copper and endocrine parameters in case group  n = 72

<table>
<thead>
<tr>
<th></th>
<th>β(slope)</th>
<th>r</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LH</td>
<td>4.58</td>
<td>0.031</td>
<td>0.799 NS</td>
</tr>
<tr>
<td>FSH</td>
<td>-43.66</td>
<td>-0.123</td>
<td>0.304 NS</td>
</tr>
<tr>
<td>Testosterone</td>
<td>0.034</td>
<td>7.07</td>
<td>0.779 NS</td>
</tr>
</tbody>
</table>

NS  non-significant (P>0.05)
4.4.2.3. Copper and other seminal plasma trace elements

Linear regression analysis between copper and other trace elements in seminal plasma of infertile men, no significant correlation found (P>0.05) as shown in Table (9)

Table (9) : linear regression analysis between seminal plasma copper and other trace elements in seminal plasma in case group n = 72

<table>
<thead>
<tr>
<th>Element</th>
<th>β (slope)</th>
<th>r</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zinc (mg/L)</td>
<td>0.828</td>
<td>0.143</td>
<td>0.230  NS</td>
</tr>
<tr>
<td>Magnesium (mg/L)</td>
<td>1.378</td>
<td>0.230</td>
<td>0.052  NS</td>
</tr>
<tr>
<td>Iron (µg/L)</td>
<td>-2.79</td>
<td>-0.013</td>
<td>0.088  NS</td>
</tr>
</tbody>
</table>

NS non-significant (P>0.05)
4.4.3. Iron correlations

4.4.3.1. Iron and seminal parameters

Linear regression analysis between iron seminal plasma and semen parameters in case group, positive correlation with count (P<0.05) and no correlation with the other semen parameters (P>0.05) as shown in Table (10).

Table (10): linear regression analysis between seminal plasma iron and seminal parameters in case group n = 72

<table>
<thead>
<tr>
<th></th>
<th>β (slope)</th>
<th>r</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume</td>
<td>27.3</td>
<td>0.105</td>
<td>0.380 NS</td>
</tr>
<tr>
<td>Count</td>
<td>3.826</td>
<td>0.290</td>
<td>0.014*</td>
</tr>
<tr>
<td>Forward motility</td>
<td>-1.912</td>
<td>-0.092</td>
<td>0.440 NS</td>
</tr>
<tr>
<td>Weak motile</td>
<td>4.26</td>
<td>0.155</td>
<td>0.193 NS</td>
</tr>
<tr>
<td>Non-motile</td>
<td>-0.291</td>
<td>-0.019</td>
<td>0.877 NS</td>
</tr>
</tbody>
</table>

*Correlation is significant at p<0.05 level

β slope of linear regression

r Pearson correlation coefficient

NS non-significant
Figure (7): Relationship between seminal plasma iron and sperm count in case group n = 72.

4.4.3.2. Iron and serum endocrine parameters

Linear regression analysis between seminal plasma iron and serum endocrine parameters in case group, inverse correlation between iron and FSH (P<0.01), while no correlation with LH and testosterone (P>0.05) as shown in Table (11).

Table (11): Linear regression analysis between iron in seminal plasma and serum endocrine parameters in case group n = 72

<table>
<thead>
<tr>
<th>Parameter</th>
<th>β (slope)</th>
<th>r</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LH</td>
<td>15.2</td>
<td>0.077</td>
<td>0.522 NS</td>
</tr>
<tr>
<td>FSH</td>
<td>-165.2</td>
<td>-0.349</td>
<td>0.003*</td>
</tr>
<tr>
<td>Testosterone</td>
<td>-58.1</td>
<td>-0.207</td>
<td>0.081 NS</td>
</tr>
</tbody>
</table>

*Correlation is significant at p<0.05 level

β slope of linear regression
r Pearson correlation coefficient
NS non-significant
Figure (8): Relationship between seminal plasma iron and serum FSH in case group \( n = 72 \).
4.4.4. Magnesium correlations

4.4.4.1. Magnesium and seminal parameters

Linear regression analysis between magnesium seminal plasma of infertile men and seminal parameters were studied. There were an inverse correlation between seminal plasma magnesium and semen volume (P<0.001), positive correlation with semen count (P<0.01), while there was no significant correlation with other semen parameters was found (P>0.05) as shown in Table (12).

**Table (12):** linear regression analysis between seminal plasma magnesium and seminal parameters in case group  n = 72

<table>
<thead>
<tr>
<th></th>
<th>β ( slope)</th>
<th>r</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume</td>
<td>-12.04</td>
<td>-0.369</td>
<td>0.001**</td>
</tr>
<tr>
<td>Count</td>
<td>0.484</td>
<td>0.293</td>
<td>0.013*</td>
</tr>
<tr>
<td>Forward motility</td>
<td>-0.290</td>
<td>-0.112</td>
<td>0.349 NS</td>
</tr>
<tr>
<td>Weak motile</td>
<td>0.268</td>
<td>0.078</td>
<td>0.515 NS</td>
</tr>
<tr>
<td>Non-motile</td>
<td>7.86</td>
<td>0.040</td>
<td>0.738 NS</td>
</tr>
</tbody>
</table>

*Correlation is significant at p<0.05 level

β  slope of linear regression

r  Pearson correlation coefficient

NS  non-significant
**Figure (9):** Relationship between seminal plasma magnesium and semen volume in case group  \( n = 72 \)

**Figure (10):** Relationship between seminal plasma magnesium and sperm count in case group  \( n = 72 \).
4.4.4.2. Magnesium and serum endocrine parameters

Linear regression analysis between seminal plasma magnesium and serum endocrine parameters in the case group showed a positive correlation between seminal plasma magnesium with LH and testosterone (P<0.001) and (P<0.01) respectively and no correlation found with FSH level (P>0.05) as shown in Table (13).

Table (13): Linear regression analysis between seminal plasma magnesium and serum endocrine parameters in case group  

<table>
<thead>
<tr>
<th></th>
<th>β(slope)</th>
<th>r</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LH</td>
<td>9.577</td>
<td>0.383</td>
<td>0.001**</td>
</tr>
<tr>
<td>FSH</td>
<td>6.55</td>
<td>0.110</td>
<td>0.355   NS</td>
</tr>
<tr>
<td>Testosterone</td>
<td>11.37</td>
<td>0.324</td>
<td>0.006*</td>
</tr>
</tbody>
</table>

*Correlation is significant at p<0.05 level  
β  slope of linear regression  
r  Pearson correlation coefficient  
NS  non-significant
Figure (11): Relationship between seminal plasma magnesium and serum LH levels in case group  n = 72.

Figure (12): Relationship between seminal plasma magnesium and serum testosterone in case group  n = 72.
4.4.4.3. Magnesium and other seminal plasma trace elements

Linear regression analysis between magnesium seminal plasma and other seminal plasma trace elements showed a positive correlation with zinc ($P<0.001$) and no correlation with copper and iron ($P>0.05$) as shown in Table (14).

Table (14): Linear regression analysis between magnesium and other trace elements in seminal plasma in case group $n = 72$

<table>
<thead>
<tr>
<th></th>
<th>$\beta$(slope)</th>
<th>$r$</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zinc (mg/L)</td>
<td>0.772</td>
<td>0.744</td>
<td>0.001***</td>
</tr>
<tr>
<td>Copper (µg/L)</td>
<td>1.378</td>
<td>0.230</td>
<td>0.052</td>
</tr>
<tr>
<td>Iron (µg/L)</td>
<td>8.044</td>
<td>0.003</td>
<td>0.978</td>
</tr>
</tbody>
</table>

*Correlation is significant at $p<0.05$ level

$\beta$ slope of linear regression

$r$ Pearson correlation coefficient

NS non-significant
5.1. Zinc

Abnormalities associated with trace elements can be due to specific deficiency from dietary inadequacies and imbalances, or abnormality secondary to other diseases. Both kinds of abnormality can be diagnosed by analysis of trace elements in body fluids or other tissues. However secondary changes, which occur as a result of diseases, are not exactly understood.

Our study suggests that zinc is an essential trace element for male infertility. This supports early studies which conclude that zinc is an essential trace element required for normal spermatogenesis and steroidogenesis and zinc deficiency considered as one of the factors responsible for decreased testicular function in infertile male subjects (37).

5.1.1. Zinc reduces seminal volume

Our results indicate that both zinc and magnesium co-exist either intracellularly or extracellularly as shown by the positive correlation between them. Since magnesium is the second major intracellular cation to potassium, the positive correlation found with zinc indicates that zinc is important intracellularly (i.e. in reproductive cells).

Early studies have shown that increase in cAMP due to hormonal changes enhances that outlet of magnesium and its increase extracellularly. It is well known that FSH and LH act intracellularly by increasing the levels of cAMP. Therefore it is expected that FSH and LH promotes the export of magnesium, potassium and consequently zinc (64).
Our study has indicated that increase in seminal plasma magnesium or zinc reduces seminal plasma volume. Since deficiency in magnesium is considered a deficiency in potassium, it is expected that the outlet of either magnesium or zinc will lead to reduction in potassium therefore to loss of water in the seminal plasma therefore leading to reduction in the seminal plasma volume.

Our results agrees with reports which found inverse association between zinc concentration in seminal plasma and semen volume more than 5ml (P=0.034) and a positive correlation with hypoviscosity of semen and conclude that zinc and calcium and physical analysis of ejaculate was also found to be clinically useful for evaluating the secretary activity of the seminal vesicles and prostate; abnormal coagulation, liquefaction, volume, viscosity and pH strongly suggest gland dysfunction (4).

**Figure (13):** proposed illustration showing that FSH and LH lower the levels of seminal fluid volume by exporting zinc and magnesium extracellularly.
5.1.2. Zinc enhances spermatogenesis

Our results supports the hypothesis that zinc is essential for spermatogenesis and steroidogenesis, as shown by the positive correlation between the zinc concentration and the semen count and serum testosterone.

Our results agrees with other studies which have found a positive association between seminal plasma zinc concentration with sperm count \((r=0.33, p <0.05)\) and with sperm motility, positive association with serum testosterone levels of the case group (27).

Another studies which concordant with our study has shown that seminal plasma zinc concentration was significantly correlated with sperm density (7,1,112).

Zinc is a trace element essential for normal functioning of the male reproductive system. Numerous biochemical mechanisms are zinc dependent, including more than 200 enzymes in the body (25).

Zinc deficiency is associated with decreased testosterone levels and sperm count, an adequate amount of zinc ensures proper semen motility and production. Zinc levels are generally lower in infertile men with diminished sperm count, and several studies found supplemental zinc may prove helpful in treating male infertility (57).

In one trial, the effect of zinc supplementation on testosterone, dihydrotestosterone and sperm count was studied. Thirty seven patients with idiopathic subfertility of more than five years duration and diminished sperm count received twenty four milligrams of elemental zinc from zinc sulfate for forty five to fifty days. The results were dramatic in the twenty two subjects with initially low testosterone levels; a significant increase in testosterone levels and sperm count (from eight to twenty millions/ml) was noted, along with nine resulting pregnancies (66).
5.2. Copper

Our results demonstrate the importance of copper in infertility as shown by seminal plasma low levels of copper in infertile group. However there were no correlation between copper in seminal plasma and any of semen parameters or endocrine parameters. These results contrasted with other results and agree with other studies (46,107).

The role of copper in the sperm is unclear, but it seems to be related to spermatozoan motility. Copper may also act at the pituitary receptors which control the release of LH (94). However copper seminal fluid levels were found to fall in cases of azoospermia and to increase in oligo- and asthenozoospermia, but the findings of different authors are somewhat contradictory (106,93).

Although most of the studies have shown no change in the level of copper in the seminal plasma of the infertile group compared with the controls (71,107,96), other studies has shown a decrease (45), or an increase in the seminal plasma copper levels (40,93). In accordance with our results some authors do not report any correlation between the seminal level of copper and the number of motility of the gametes (94); with sperm count (2).

However in other studies a significant correlation was found between copper concentration in semen, sperm concentrations, percentage progression motility and normal morphology (46) and the decrease in the concentration of sperms (106).

Both zinc and copper are necessary for both the structure and activity of SOD, which is closely related to sperm function (113).

Despite the absence in our study of any correlation between the decreased copper levels and any of the studied seminal parameters, a decrease in the zinc and copper levels increased production of free radicals that will impair the synthesis and/or augment the consumption of SOD (113), which will increase the levels of
ROS. Increased levels of ROS production has been found to lower the quality of sperms, by increasing the number of immature spermatozoa (29,88).

5.3. Iron

5.3.1. Iron Improves fertility

The mean value of seminal plasma concentration of iron is higher in the case group than the mean concentration of the control (411.5±270), and (329±223) respectively. This supports the model described by Griswold (1998) ,(Fig-2), which describes the entry of iron in sertoli cells via transferrin which then delivered to the germ cells and stored as ferritin or, which is needed in the meiotic stages of spermatocytes. The difference in seminal plasma levels of iron between the infertile and control groups may be due to inability to transport iron inside the testis, in sertoli and/or leydig cells in infertiles, may be due to defect in transferrin (iron transporter). Therefore, further mutational screening studies of the transferrin receptor need to be carried out in the infertile group (30).

5.3.2. Iron is stored in sertoli cells

Our study indicates that seminal plasma iron levels were positively correlated with sperm count and inversely correlated with FSH levels. This indicates that iron is essential for spermatogenesis. This also supports the hypothesis that FSH may promote the entrance of iron into sertoli cells (30).

Accumulation of iron in the testis is the accumulation of Ferritin where iron is safely stored within leydig cells; iron is bound in the center of ferritin molecule. Unlike the liver, the gene expression of transferrin and its membrane receptor in sertoli cells is not controlled by iron concentration, but by FSH (10) and other endocrine factors such as insulin like growth factors, epidermal growth factors and platelet derived growth factor (41). Iron is bound to transferrin as a transport protein, the iron-transferrin complex is internalized through a transferrin membrane receptor
and after internalization, dissociates then iron to cellular iron storage protein until further use (36).

Studies of iron-transport into testis via the transferrin receptor has focused primarily on the sertoli cell function (99), but the leydig cells through its storage of iron via ferritin may also play a role in iron hemostasis and may be a primary source of iron for sertoli transport to developing sperm. Each ferritin molecule can hold a tremendous amount of iron (23% of ferritin dry weight can be iron). Storage of iron in leydig cells also provides an extra layer of protection to germinal cells and still maintains easy availability of iron to sertoli and germ cells.

Although there is limited information on the mechanism of control of the testicular transferrin receptor, it was reported that FSH increase transferrin receptor messenger RNA levels in testis of hypophysectomized rats (82). Elevated FSH can also increase mRNA for transferrin (10). If increased FSH concentrations induce extracellular iron-transport via the transferrin iron exchange system, then this iron must be safely stored.

The lower levels of iron in the control group than infertiles indicates that not only iron is needed within the sertoli cells, but it must be present at lower levels as it could damage sperms by lipid peroxidation. The production of ROS molecules as a result of lipid peroxidation could have an adverse effect on fertility, therefore reduce fertility, excessive iron is destructive to testicular function and spermatogenesis (56), and small testis and reduce sperm production may be related to elevated iron-concentrations, even though the demands of iron may be of greatest importance for sperm production. Most of the ferritin and iron seem to be stored in the leydig cells, and may provide further protection to developing gamete within the tubules surrounded by sertoli cells.

Human males with β-Thalassemia major have decreased pituitary function from iron overload, and serum ferritin is highly correlated with the presence of hypogonadism (77).
Despite the absence of no correlation between seminal plasma levels of iron and LH levels, LH has been found to increase c-Myc in the leydig cells (34); the oncogene c-Myc has a role of coordinating regulating genes controlling intracellular Fe concentrations (111).

5.4. Magnesium

LH increases seminal magnesium levels

The lower levels of magnesium (Mg) in infertile population indicate that magnesium might play a role in male fertility. Despite the lack of direct analytical studies on the role of magnesium in infertility, a number of studies have shown that magnesium is essential for energy requiring cells such as muscle and heart cells (11). It binds to ATP in the mitochondria in great amounts. However when hormones such as adrenaline binds to the cells it increases the levels of cAMP by activating Adenylate cyclase. The increase of cAMP will encourage the release of ATP-Mg from the mitochondria (59). This will disassociate leading to free of ATP and exporting magnesium outside the cells.

LH, which acts on leydig cells during spermatogenesis, has been shown to increase the levels of cAMP (64). Therefore we assume that this will lead the export of magnesium into the seminal fluid. This could explain the direct relation between the increase in LH levels and magnesium levels, which our data has demonstrated. Therefore the increase in magnesium in seminal plasma improves fertility. It is worth mentioning that the increase in seminal plasma means free ATP in spermatocytes and good fertility.

Since magnesium is an intracellular component (84) means that it moves against the water. Since the increase in LH increases magnesium in the seminal plasma, this will lead to lower amount of water in the seminal plasma. This explains the inverse relations between the levels of both magnesium and LH and seminal volume in our studied population.
CHAPTER - 6

Conclusions and Recommendations

6.1. Conclusions

Male infertility is a worldwide problem of multiple causes. The ability to treat such problem is based on good diagnosis of the primary cause. Our study signifies the importance of some trace elements abnormalities in infertiles in Gaza Strip. This implies the importance of them in patient treatment.

Seminal plasma low levels of zinc, magnesium, and copper as well as the increase in iron could be an indication of male infertility.

Seminal plasma levels of zinc, magnesium and iron are directly correlated with sperm count.

There is a direct correlation between the seminal plasma levels of zinc and testosterone.

There is a direct correlation between seminal plasma magnesium and the levels of testosterone and LH.

6.2. Recommendations

Trace elements analysis should be introduced as a method for search of infertility causes.

Further mutational studies of transferrin receptor needed to be carried out in infertile subjects.

Further studies needed to be carried out for lipid peroxidation in human semen.

Standardization potential for semen analysis should be carried out in Gaza laboratories.


CHAPTER 7

REFERENCES


[34] - Hall S.H., Berthelon M.C., Avallet O. and Saez J.M., 1991- Regulation of c-fos, c-jun, jun-B, and c-myc messenger ribonucleic acids by


Appendices

Appendix1: Questionnaire

Age ................. ............yrs.

Work ................................ Infertility years ................................

1) Has a doctor ever told you that you had any of the following medical conditions?

   a) Mumps Yes No
   b) Prostate infection (Prostatitis) Yes No
   c) Testicle infection (Orchitis) Yes No
   d) Epidydimis infection (epidydmitis) Yes No
   e) Infection of the seminal vesicles Yes No
   f) Urinary tract infection Yes No
   h) Urithritis or discharge from the penis. Yes No
   i) Chlamydia Yes No
   j) Syphilis Yes No
   k) Gonorrhea Yes No
   l) Genital herpes Yes No

2) Have you ever had any of the following medical procedures?

   a) Vasectomy Yes No
b) Surgery in pelvic area
Yes   No

c) Testicle biopsy
Yes   No

d) Hernia repair
Yes   No

3) Do you take saunas, steam baths or exposed for long time to heat?
Yes   No

4) Have you ever taken any of the following medications/ treatments at least 4 consecutive weeks?
Yes   No
if yes are you currently taking?

a) Antibiotics
Yes   No

b) Non-steroidal anti inflammatory (Ibuprofen)
Yes   No

c) Estrogen
Yes   No

d) Testosterone
Yes   No

e) Radioactive Iodine
Yes   No

f) Steroids
Yes   No

g) Antacids, Maalox
Yes   No

h) Anti-hypertensive or blood pressure medications
Yes   No

f) Seizure medications
Yes   No

5- Are you a smoker?
yes   No

<table>
<thead>
<tr>
<th>Variable</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume</td>
<td>≥ 2 mL</td>
</tr>
<tr>
<td>pH</td>
<td>≥ 7.2</td>
</tr>
<tr>
<td>Sperm concentration</td>
<td>≥20 × 10^6 per mL</td>
</tr>
<tr>
<td>Motility</td>
<td>≥ 50 percent (grade A and B) or 25% or more with progressive motility within 60 minutes of ejaculation.</td>
</tr>
<tr>
<td>Morphology</td>
<td>&gt; 50 percent normal</td>
</tr>
<tr>
<td>White blood cells</td>
<td>&lt; 1 × 10^6 per mL</td>
</tr>
</tbody>
</table>
Appendix 3: Consent Form

الجامعة الإسلامية-غزة
قطاع غزة- ص ب

تُقدِم من أجل الحصول على إجابات لاستبيان خاص بعمل دراسة حول أهمية بعض العناصر النادرة في السائل المنوي ومقارنتها بهرمونات الذكورة في الدم لمرضى الخصوبة.

أقدم لكم نفسي أنا الباحث أحمد حمصف جراد من الجامعة الإسلامية أمل في مساعدتم لنا بالإجابة على أسئلتنا من خلال الاستبيان الذي أعدناه حول عدم الخصوبة وهي المشكلة التي تعانون منها وإعطاء عينة سائل منوي وعينة دم والذي نتعتمد لكم الشفاء العاجل بإذن الله. وأود أن أطمئنك بشأن إجاباتكم سوف تكون في غاية السرية وإن هدفنا هو البحث العلمي وإيجاد السبل للتغلب على هذه المشكلة.

الباحث/ أحمد خميس جراد
الجامعة الإسلامية
برنامج ماجستير التحاليل الطبية
Appendix 4: Helsinki agreement

Palestinian National Authority
Ministry of Health
Helsinki Committee

Date: 14/6/2005

I would like to inform you that the committee has discussed your application about

Significance of Trace Elements in Semen.
Plasma of Infertile Men in Gaza Strip.

In its meeting on June 2005 and decided the Following:

To approve the above mention research study:

Chairperson

Member

Member

Conditions:

- Valid for 2 years from the date of approval to start.
- It is necessary to notify the committee any change in the submitted study protocol.
- The committee appreciate receiving a copy of your final research when it is completed.

Gaza Freem - Telephone: 972-8-3878-66