Leptin status and some biochemical parameters in germ cell aplasia among infertile men in Gaza Strip

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Declaration

I hereby declare that this submission is my own work and, to the best of my knowledge and belief, if contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree of the university or other institute, except where due acknowledgement has been made in the text.

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Leptin status and some biochemical parameters in germ cell aplasia among infertile men in Gaza Strip

Abstract

Background: Germ cell aplasia is a type of azoospermia which contains only Sertoli cells and no other cells involved in spermatogenesis. Recently leptin has been suggested to be linked to regulation of reproduction including spermatogenesis, controlling gonadotropins, testosterone secretion and directing the spermatocytes to full development to spermatids.

Objective: To assess leptin status and some biochemical parameters in germ cell aplasia among infertile men in Gaza Strip.

Design: Case control study.

Setting: Specialized Medical Centers in Gaza Strip.

Study Subjects: A total of 78 infertile men with germ cell aplasia were interviewed to fill a questionnaire. Hormonal analysis (serum leptin, LH, FSH and testosterone) and biochemical analysis (serum cholesterol, triglyceride, HDL-C and LDL-C) were analyzed. Data analyzed using SPSS statistical package version 18. Seventy eight apparently healthy fertile men were served as controls.

Results: The mean ages of controls and patients were 31.7 ± 3.9 and 32.8 ± 6.4 years old, respectively. Sexual history showed that problems with erection and ejaculation, and gonadal infection were significantly associated with infertility (P=0.000). Medical history revealed that Orchitis, trauma, cryptorchidism, varicocele and hormonal problems are associated with male infertility (P=0.000). In addition, exposure to thermal and chemical conditions were significantly related to infertility (P=0.000 and P=0.003, respectively). treatment of the antifungal Ketoconazole and the steroid were also significantly associated with infertility (P=0.000). The number of obese patients were 28 (35.9) whereas for controls were 6 (7.7%) with \( \chi^2 = 19.338 \), P=0.000 indicating there was strong association between infertility and obesity. Serum leptin was significantly increased in patients compared to controls with percentage difference of 52.4% (21.2 ± 26.1 vs. 12.4 ± 7.6 ng/ml, t=2.019, P=0.047). In contrast, testosterone was significantly
decreased in patients compared to controls with percentage difference of 45.5% (3.9 ± 2.8 vs. 6.2 ± 1.7 ng/ml, t=4.529, P=0.000). Serum LH and FSH were increased in patients compared to controls showing percentage differences of 100.6% and 130.5%, respectively (12.4 ± 6.7 vs. 4.1 ± 1.8 mIU/ml, t=7.460, P=0.000 and 25.7 ± 16.7 vs. 5.4 ± 2.6 mIU/ml, t=7.491, P=0.000, respectively). Serum cholesterol, triglycerides and low density lipoprotein (LDL-C) were significantly higher in patients compared to controls registering % differences of 28.8, 47.3 and 43.6, respectively (206.1 ± 45.5, 211.1 ± 129.0 and 123.4 ± 45.2 mg/dl vs. 154.2 ± 33.2, 130.3 ± 70.3 and 79.2 ± 33.6 mg/dl, P=0.000). In contrast, high density lipoprotein (HDL-C) was significantly lower in patient than in controls (40.4 ± 7.6 vs. 49.0 ± 4.0 mg/dl, % difference=19.2 and P=0.000). Leptin was negatively correlated with testosterone (r=-0.223 and P=0.049) and positively correlated with LDL-C and body mass index (BMI) (r=0.222, r=0.368 and P=0.05, P=0.001, respectively).

**Conclusions:** Leptin play an important role in regulation of testicular function among patients with germ cell aplasia by direct action on testes by binding to specific receptor on leydig cell leading to inhibition of testosterone secretion and indirect action where it bind to specific receptor in hypothalamus and induce secretion gonadotropin releasing hormone.

**Key words:** Azoospermia, germ cell aplasia, leptin, infertile men, biochemical parameters, Gaza Strip.
مستوى هرمون الليبتين وبعض المعايير البيوكيميائية في ضمور الخلايا الجرثومية المنتجة للحيوانات المنوية لدى الرجل المصابين بالعقم في قطاع غزة

الملخص

ضمور الخلايا الجرثومية في الأندنب المنوية (Germ cell aplasia) هو نوع من أنواع العقم يحتوي فقط على خلايا المساعدة (Sertoli cells) ولا يوجد فيه أي نوع من خلايا المنتجة للحيوانات المنوية. ووجد حديثا أن الليبتين له علاقة في تنظيم التناسل حيث أنه يؤثر في إنتاج الحيوانات المنوية، وفي التحكم في الهرمونات الجنسية مثل هرمون ملوتن (LH)، وهرمون منه للجريب (FSH)، وهرمون التستيرون. كما أنه يساعد في توجيه الخلايا الجرثومية للتطور إلى النقطة الأورومية (spermatid).

الهدف:

هدف الدراسة إلى تقييم وجود هرمون الليبتين وبعض المعايير البيوكيميائية وتأثيرها لدى الرجال المصابين بالعقم في قطاع غزة.

المنهجية:

دراسة إحصائية مقارنة.

مكان الدراسة:

المركز الطبي المتخصص في علاج العقم في قطاع غزة.

شخصيات الدراسة:

أجريت هذه الدراسة على مجموعه من (78) رجلا مصابا بالعقم ويعانون من ضمور الخلايا الجرثومية في الأندنب المنوية في الخصية وذلك بالمقارنة مع (78) رجلا لديهم القدرة على الإنجاب (العينة الضابطة)، حيث تم إجراء مقابلة لتعريف الاستبيان وأخذ عينة دم لإجراء الفحوصات.
النتائج:

متوسط عمر العينة التجريبية والعينة الضابطة (32.8 ± 3.9) سنة على التوالي، فالتأريخ الجسمي للمرضى أظهر أن المشاكل المتعلقة بالانصباب والوذف والتهابات الغدد التناسلية مرتبطة بشكل كبير بالخصوبة، والتاريخ الطبي للمريض قبآ أن التهابات الخصية، الصدمات، اختفاء الخصائص، ودوالي الخصية هي عوامل خطر مرتبطة بالعقم عند الرجال، بالإضافة إلى ذلك أن التعرض للحرارة والمواد الكيميائية تشكل خطراً كبيراً بالإصابة بالعقم.

كذلك بنت الدراسة أن المرضى الذين يتلوتون الكيتوكورتيزول المضاد للالتهابات والستيرويد له ارتباط كبير بالعقم. وتبين أيضا أن هناك فوق ذات دلالات إحصائية في المرضى الذين كانوا يعانون السمنة بالمقارنة بالعينة الضابطة حيث كانت نسبة المرضى الذين لديهم سمنة من العينة الضابطة والعينة المرضية 28 (7.7%) و 6 (35.9%), ارتفاع معدل هرمون اللبتين عند المرضى أعطى دلائل إحصائية واضحة بالمقارنة مع العينة الضابطة، على العكس من هرمون التستيرون الذي سجل انخفاضاً ملحوظاً ذو دلالات إحصائية بالمقارنة مع العينة الضابطة، وعند إجراء الفحص لهورمون منه للجريب و هورمون ملونتين تبين أن كلاهما سجل ارتفاعاً ملحوظاً عند المرضى بالمقارنة مع العينة الضابطة، وكذلك تبين من خلال الدراسة أن ارتفاع الكولسترول والشحوم الثلاثية والكولسترول منخفض الكثافة عند المرضي بالمقارنة مع العينة الضابطة، على عكس الكولسترول مرتفع الكثافة الذي سجل انخفاضاً بالمقارنة مع العينة الضابطة، وتوصلت الدراسة إلى أن مستوى هورمون اللبتين يرتبط ارتباطاً عكسياً مع مستوى هرمون التستيرونز وطردياً مع الكولسترول منخفض الكثافة وكذلك مع مؤشر كتلة الجسم.

تخص الدراسة إلى:

أن هرمون اللبتين يلعب دوراً مهماً في تنظيم وظيفة الخصية وله تأثير كبير على المرضى الذين يعانون من ضمور الخلايا الجرثومية داخل الأحيانين المنوية لدى الرجال في قطاع غزة حيث أن هرمون عمل على كبح هرمون التستيرونز.

الكلمات المفتاحية:

العقم، ضمور الخلايا الجرثومية في الأحيانين المنوية في الخصية، اللبتين، الرجال المصابون بالعقم، المعایير البيوكيميائية، قطاع غزة.
Dedication

This work is dedicated to:
To the dearest person to my heart my father
To the purest heart My mother
and my family members
The dearest to me; my wife, children : Nur, Mohammed,
Hadel, Toga and Jana
brothers and sisters

Dedication is almost expressed to the Palestinian people who
have suffered and will be struggling with the persistence to
have a free Palestine.
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Chapter 1

Introduction

1.1 overview

The testes are the main male reproductive organ and they are very important for the normal functioning of the male reproductive system. They consist of convoluted seminiferous tubules embedded in the interstitium that contains a mixture of blood and lymph vessels, nerves, fibroblast cell, macrophages and Leydig cells. The epithelium of seminiferous tubules consists of continually dividing germ cells that produce sperm and supporting Sertoli cells (Krohmer, 2004).

The regulation of the testis function is mediated primarily by two pituitary hormones under control of GnRH (Gonadotropin releasing hormone) from the hypothalamus: Luteinizing hormone (LH) and follicular stimulating hormone (FSH). LH stimulates Leydig cells to produce testosterone and FSH acts on Sertoli cells to stimulate spermatogenesis (Weinbauer et al., 2001).

The causes of male infertility generally fall into three categories: pre-testicular, testicular and post-testicular causes. The pre-testicular causes are extragonadal endocrine disorders, such as those originating in the pituitary or the adrenals, which have an adverse effect on spermatogenesis. The testicular causes of infertility are conditions in which the primary defects reside in the testes such as germ cell aplasia. The post-testicular causes of infertility consist mainly of obstructions of the ducts leading away from the testes (Link et al., 1979 and McElreavey and Fellous, 1999).

Azoospermia is defined as the total absence of spermatozoa in the ejaculate. A classification of azoospermia can be based on obstructive and nonobstructive forms (Schill et al., 2006). Histologically, germ cell aplasia or Sertoli cell only (SCO) syndrome is considered as one form of nonobstructive azoospermia. Germ cell aplasia is a histopathologic phenotype that was first described by Del Castillo et al. (1947). In complete germ cell aplasia the tubules are reduced in diameter and contain only Sertoli cells but no other cells involved in spermatogenesis. Germ cell aplasia can also be focal with a
variable percentage of tubules containing germ cells, but in these tubules spermatogenesis is often limited in both quantitative and qualitative terms (Silber et al., 1995), and such cases should be referred to as hypospermatogenesis.

In many instances non-obstructive azoospermia was related to the history of clinical unilateral or bilateral varicocele (Poulakis et al., 2006, Inci et al., 2009). There is no clear evidence that certain work environments affect fertility, it is generally recommended that couples trying to become pregnant avoid exposure to any possible harmful chemicals, pesticides, heavy metals, toxic chemicals and radiation may affect the quality and quantity of sperm produced (Bezold et al., 2000).

Leptin is a small peptide hormone (16-kDa protein) that is produced in adipose tissue. The circulating leptin concentration therefore directly reflects the amount of body fat (Merabet et al., 1997 and Wolf et al., 2001). Leptin was identified through positional cloning of the obese (ob) gene, which is mutated in the massively obese ob/ob mouse, and it has a pivotal role in regulating food intake and energy expenditure (Zhang, 1994). It binds to the so-called long receptor (Ob-Rb) in the hypothalamus and regulates food intake through the release of other neurotransmitters (Nussey and Whiteheds., 2001 and Malendowicz, et al., 2006).

Leptin is also expressed in the seminiferous tubules and in seminal plasma (Glander et al., 2002), but its cellular origin in these contexts is not exactly defined. Several studies support the role of serum leptin in the regulation of gonadal functions in men (Steinman, et al., 2001) indirectly via the central neuroendocrine system and directly via peripheral tissue membrane receptors (Tena-Sempere et al., 1999). Indirect action of leptin through hypothalamic-pituitary-testicular system involves not only stimulatory, but also inhibitory, effects. Caprio et al. (1999) hypothesized that the net effect of leptin upon male reproductive function may depend on the circulating level of the molecule. Thus, predominant stimulatory effects, primarily at the hypothalamus, are observed at physiological leptin levels above a minimal threshold. In contrast, direct inhibitory actions at the testicular level may take place in the presence of a significantly elevated leptin concentration, as detected in obesity (Tena-Sempere et al., 2002).
Although Germ cell aplasia (nonobstructive form of azoospermia) do exist in Gaza Strip, there is under-diagnosis and under-reporting of the disease. The only study emerged in this topic was focused on the histological aspects of the disease (Taha, 2010). However, data on hormonal aspects of germ cell aplasia including its relation to leptin hormone were limited. This will be the first study to assess leptin status and some biochemical parameters in azoospermic men in Gaza Strip.

1.2 Objectives
The general objective of the present study was to assess leptin status and some biochemical parameters in germ cell aplasia among infertile men in Gaza Strip. The Specific objectives were:
1. To identify factors associated with germ cell aplasia.
2. To assess body mass index (BMI) in patients versus controls.
3. To determine leptin levels in the study population.
4. To measure FSH, LH, testosterone levels in patients and controls.
5. To estimate cholesterol, triglyceride, high density lipoprotein (HDL) and low density lipoprotein (LDL) in the study population.
6. To investigate the possible relationships between leptin and the previous studied parameters.

1.3 Significance
1. In Gaza Strip, one study was carried out on germ cell aplasia without speculation the role of leptin hormone in the disease (Taha, 2010). This will be the first study to assess leptin status and relate it to male reproductive hormones and some other biochemical parameters among infertile men with germ cell aplasia in Gaza Strip.
2. Understanding the role of leptin in germ cell aplasia may be helpful in the treatment of the disease.
3. It is important to give a detailed picture on factors associated with germ cell aplasia in Gaza Strip which may be helpful in future awareness of patients.
Chapter 2

Literature review

2.1 Structure of testis

The testes are a pair of oval-shaped glands that are suspended in the scrotum. The testis consists of convoluted seminiferous tubules embedded in a connective tissue matrix that called interstitium. The interstitium contains mixture of blood and lymph vessels, nerves, fibroblast cell, macrophages and Leydig cells (Figure 2.1). The epithelium of seminiferous tubules consists of continually dividing germ cells (spermatogonia) that produce sperm cells and supporting Sertoli cells (Ohl et al., 1996).

Figure 2.1 cross-section of the testis showing sperm producing tube (seminiferous tubule) and leydig cell
2.2 Sperm formation (spermatogenesis)

Spermatogenesis represents the entire process of germ cell development within the seminiferous epithelium of the adult testis. It can be divided into four phases:

2.2.1 Proliferation and differentiation of spermatogonia

Spermatogonia are the diploid (2n2C) stem cells of spermatogenesis, and can be divided into type A and type B (Figure 2.2). In primates type A spermatogonia are further divided into A pale (Ap) and A dark (Ad) according to their differing nuclear appearance. In non-human primates Ad spermatogonia have no or only weak proliferative activity (Schlatt and Weinbauer, 1994), whereas S-phase-specific Ki-67 immunoreactivity indicating mitotic activity was found in both Ap and Ad spermatogonia (Steger et al., 1998) in the human. Type B spermatogonia are able to differentiate and enter the process of meiosis. Due to incomplete cytokinesis, type B spermatogonia remain interconnected after the last mitotic division by intercellular bridges forming cellular clones, which allow synchrony of germ cell maturation. These intercellular bridges persist until late spermiogenesis. In spermatogonia, genomic imprinting for parent-of-origin dependent regulation of gene expression via DNA methylation takes place and is finished before the first meiotic division (Kierszenbaum, 2002).
2.2.2 Spermatocytes/Meiosis

A. Primary spermatocytes

Meiosis starts with DNA synthesis of type B spermatogonia which lose contact with the basal lamina. After completion of DNA synthesis, each chromosome consists of two chromatids (C). These cells are named primary spermatocytes, and the DNA content is duplicate (2n4C). Primary spermatocytes undergo the first meiotic division. The prophase of the first meiotic division takes about 1–3 weeks and is divided into several stages: the leptotene, zygotene, pachytene and diplotene stages. The leptotene stage is characterized by DNA condensation. In the zygotene stage, condensation of chromosomes proceeds, and pairing of homologous chromosomes takes place. In the pachytene stage, pairing of chromosomes leads to a “crossover” between nonsister chromatids and exchange of genetic materials forming chiasmata. In the diplotene stage, chromosomes separate with the exception of the chiasmata sites. The chromosomes shorten and the four separate chromatids become visible. Finally, the nuclear membrane disappears and
chromosomes are subsequently arranged in the metaphase plate. After formation of the spindle apparatus, chromosomes move to opposite poles, but, in contrast to mitotic division, chromatids remain interconnected. Thus the number of chromosomes in resulting secondary spermatocytes is haploid, but the DNA content is still diploid (1n2C).

**B. Secondary spermatocytes**

Secondary spermatocytes undergo the second meiotic division after a short interphase of about 6 h in the human without DNA synthesis. By this division, chromatids are finally separated leading to round spermatids with a haploid n.

**2.2.3 Spermatids/Spermiogenesis**

The transformation of conventional round cell spermatids into spermatozoa with the capacity for motility and fertilization of an egg includes a complex sequence of events: (1) formation of the acrosome, (2) condensation of the nucleus, (3) development of the sperm tail, (4) reorganization of cellular organelles such as mitochondria and centrioles and (5) reduction of the cytoplasm. The synthesis of many acrosome-specific proteolytic enzymes starts as early as in pachytene spermatocytes. These proteins, such as proacrosin, are packed vesicles derive from the trans-Golgi complexes. They start to fuse and the growing acrosome forms a caplike structure that covers about 30–50% of the nuclear surface (Bermúdez et al., 1994). Nuclear condensation in the human is due to replacement of about 85% of the DNA-associated lysine rich histones by transition proteins, and finally by arginine-rich protamines (Figure 2.3). The fertilization capacity of spermatozoa depends on the protamine content being adequate and the ratio of the two protamines PRM1 and PRM2 being correct (Steger et al., 2003). The formation of the tail (flagellum) starts early in spermiogenesis. The axoneme shows the typical “9+2” structure of microtubules. This is derive from one of the pair of centrioles. These centrioles are placed in a nuclear fossa opposite the acrosome. The distal centriole gives rise to the flagellum. The other structures of the flagellum, the fibrous sheet and outer dense fibres are developed when spermiogenesis takes place. Mitochondria from the periphery of the spermatid aggregate around the proximal part of the flagellum in a helical manner forming the latter’s mid-piece. At the end, the spermatid’s
cytoplasm is shed by active involvement of the adjacent Sertoli cell, and this “residual body” is phagocytosed by Sertoli cells. The release of fully haploid germ cell within the seminiferous epithelium is termed the “spermatid” (round, elongating, elongated). The haploid germ cell after spermiation is a “spermatozoon” (sperm).

Figure 2.3. Schematic presentation of histone to protamine exchange

2.2.4 Spermatozoon
The length of the human spermatozoon measures about 60 μm. The flat and oval head (diameter: 3 μm, length: 5 μm) consists of the acrosome and the extremely condensed nucleus. The acrosome covers the head surface, and contains numerous proteolytic enzymes, i.e. hyaluronidase, collagenase, neuraminidase, phospholipase A, acrosin and others. The release of these enzymes, the so-called acrosome reaction, enables the spermatozoon to penetrate the “corona radiata” of follicle cells and the zona pellucida of the egg. The flagellum measures about 55 μm in length (Figure 2.4). It possesses the central axoneme and is divided into:
■ The neck/connecting piece (1 μm): It contains the basal and striated bodies and is the point of articulation between the sperm head and the flagellum.

■ The mid-piece (6 μm): It contains the mitochondria and the nine doublets of microtubules, which are associated with outer dense fibres, each consisting of at least 14 polypeptides with a molecular mass ranging from 11 to 87 kDa (Henkel et al., 1994). Outer dense fibres are believed to maintain the passive elastic structure for flagellar bending and also to protect it from shearing forces during epididymal transit and ejaculation (Baltz et al., 1990). (Hinsch et al., 2004) detected the voltage dependent anion-sensitive channels VDAC2 and VDAC3 in bovine outer dense fibres, indicating their functional role in the regulation of sperm motion or sperm structural integrity.

■ The principal piece (45 μm). In addition to the outer dense fibres, the flagellum contains a fibrous sheet.

■ The end-piece (5 μm) only contains microtubules. Spermatozoa acquire motility during epididymal passage and their competence for fertilization during the passage of the female genital tract (capacitation).
Figure 2.4. Schematic drawing of the human spermatozoon section showing 1 head with acrosome, 2 neck, 3 mid-piece, 4 principal piece, and 5 end-piece. b–f Flagellar cross sections through the b, c mid-piece, d, e principal piece, and f end piece

2.3 Sperm transport

The sperm travel from the seminiferous tubules to the rete testis located in the mediastinum testis, to the efferent ducts, and then to the epididymis where newly-created sperm cells mature (Figure 2.5). The sperm move into the vas deferens, and are eventually expelled through the urethra and out of the urethral orifice through muscular contractions (Ellis et al., 1978).
2.4 Azoospermia

Azoospermia is defined as complete absence of any spermatozoa in semen. A classification of azoospermia can be based on obstructive and nonobstructive forms (Schill et al., 2006). One form of nonobstructive azoospermia is germ cell aplasia or Sertoli cell only syndrome.

2.4.1 Germ cell aplasia

Germ cell aplasia is a histopathologic phenotype that was first described by Del Castillo et al., (1947). In complete germ cell aplasia the tubules are reduced in diameter, and contain only Sertoli cells but no other cells involved in spermatogenesis. Germ cell aplasia can also be focal with a variable percentage of tubules containing germ cells, but in these tubules spermatogenesis is often limited in both quantitative and qualitative terms (Silber et al., 1995), and such cases should be referred to as hypospermatogenesis. In congenital germ cell aplasia the primordial germ cells do not migrate from the yolk sac into the future gonads or do not survive in the epithelium of the seminiferous tubule. Chromosomal abnormalities, especially microdeletions of the Y chromosome, are important genetic causes for
complete germ cell aplasia. Anti-neoplastic therapy with radiation or chemotherapy may cause complete loss of germ cells. Other reasons include viral infections of the testes such as mumps orchitis. Germ cell aplasia can occur in maldescended testes (Foresta et al., 1998).

2.4.2 Hormonal levels in azoospermia
Endocrine malfunctions are more prevalent in infertile men than in the general population, but still quite uncommon. Hormonal screening can be limited to determining FSH, LH and testosterone levels. In men diagnosed with azoospermia or extreme oligozoospermia, it is important to distinguish between obstructive and nonobstructive causes. A criterion with reasonable predictive value for obstruction is a normal FSH with bilaterally a normal testicular volume. However, 29% of men with a normal FSH appear to have a defective spermatogenesis (Jungwirth et al., 2004).

2.4.2.1 Primary testicular failure
Primary testicular Failure is characterized by low testosterone level caused by a deficiency or absence of Leydig cell function, impairment of spermatogenesis, and elevated LH and FSH concentrations (Bhasin et al., 2006).

2.4.2.2 Hypergonadotrophic hypogonadism (Elevated FSH/LH)
Hypergonadotrophic hypogonadism is a primary testicular development disorder with an elevated production of gonadotrophins. It is an isolated failure of spermatogenesis and generally not caused by a disruption of the endocrine system. The main causes are congenital (Klinefelter syndrome, anorchia, enzyme defects in the androgen synthesis and cryptorchidism) and acquired: after orchitis, testicular torsion, castration, cytotoxic therapy (Carani et al., 1999).

2.4.2.3 Hypogonadotrophic hypogonadism (Deficient FSH/LH)
The main causes of low levels of gonadotrophins due to a dysfunction of the pituitary gland or hypothalamus are: 1) Congenital: isolated arrest of FSH and LH secretion (Kallmann’s syndrome, accompanied by anosmia), isolated arrest of LH secretion (fertile eunuch), idiopathic hypopituitarism and delayed puberty, and 2) Acquired: generally as an expression of a more complex disorder of the pituitary gland or hypothalamus, or iatrogenic GnRH agonists and anti-androgens. In case hypogonadotrophic hypogonadism is suspected, the
medical examination should include an magnetic resonance imaging MRI scan of the pituitary gland (Yong et al., 1997)

2.5 leptin

2.5.1 Definition and site of secretion

Leptin was identified through positional cloning of the obese (ob) gene, which is mutated in the massively obese ob/ob mouse, and it has a pivotal role in regulating food intake and energy expenditure (Zhang, 1994). Leptin is a small peptide hormone (16-kDa Protein) that is mainly produced in adipose tissue (Figure 2.6). Leptin is also found in human and rodent Sertoli cells, Leydig cells, seminiferous tubules and germ cells (Soyupek et al., 2005; Ishikawa et al., 2007 and Herrid et al., 2008).

Figure 2.6. Structure of leptin
2.5.2 Mechanism of leptin action

Leptin binding induces dimerization of the leptin receptor, activating a kinase molecule (JAK-2) which phosphorylates tyrosine(Y) residues on the receptor. This creates phosphotyrosine docking sites on for Signal Transducers and Activators of Transcription (STAT) proteins (Figure 2.7). These STAT proteins then dimerize and return to the nucleus to control expression of response genes (Friedmann, 2000).

![Figure 2.7. Mechanism of action of leptin](image)

Genes expression in response to leptin action causes an increase in proopiomelanocortin (POMC). POMC is a prohormone which means that this protein is processed into smaller pieces which each act as individual hormones. One of the products of POMC is α-MSH which decreases hunger. The enzyme that processes POMC into α-MSH is called proenzyme convertase 1 (PC-1). α-MSH activates a receptor on cell surfaces in the hypothalamus called type 4 melanocortin receptor (MC4R) (Figure 2.8). Activation of this receptor is like flipping a switch, telling the cell to inhibit appetite (Janeckova, 2001 and Flier, 2004).
2.5.3. Leptin action on testes
Leptin modulation of testicular function involves both direct and indirect actions:

2.5.3.1 Direct action of leptin on testes
Leptin is found in human and rodent Sertoli cells, Leydig cells, seminiferous tubules and germ cells (Ishikawa et al., 2007 and Herrid et al., 2008). This testicular leptin, in addition to peripheral leptin which is able to cross the testis–blood barrier can act directly on leptin receptor (Ob-R) of Leydig cells (Ishikawa et al., 2007) as a negative regulator of testicular steroidogenesis i.e. exerts inhibitory effect.

2.5.3.2 Indirect action of leptin on testes (Leptin modulation of hypothalamic–pituitary–testis axis)
Leptin provides a physiological link between energy expenditure and reproduction by stimulating expression of Kisspeptin (KiSS), located in the hypothalamic arcuate nucleus (Figure 2.9). KiSS neurons stimulate gonadotropin-releasing hormone (GnRH) release and trigger the gonadotropin cascade (Follicle-stimulating hormone and Luteinizing hormone) and, in turn, testicular steroidogenesis and spermatogenesis (Gottsch et al., 2004).
2.5.4 Degradation of leptin
The half-life of leptin is 9.4±3.0 min, and the leptin production rate was 3.6±1.2ng/100g fat/min (Zeng et al, 1997). The human kidney Plays a substantial role in leptin removal from plasma by enterilization and degrading the peptide (Chabova et al.,1999). Renal leptin uptake could account for ~80% of all leptin removal from plasma (Meyer et al, 1997).
2.5.5 Leptin and fertility

In order to investigate the regulation of testicular functions by leptin von Sobbe et al. (2003) analyzed leptin concentrations in the semen of men with different andrological diseases. It was demonstrated that semen leptin concentrations were inversely correlated with serum testosterone levels and directly with serum leptin concentrations. Furthermore, semen leptin concentrations display only a fraction of serum leptin levels. Semen leptin levels of patients with azoospermia due to hypergonadotrophic hypogonadism, associated with increased follicle-stimulating hormone levels and of high-grade oligozoospermia, were significantly elevated (1.19 ± 0.46 and 1.09 ± 0.54 \( \mu g \) l\(^{-1} \), respectively), while semen levels of leptin in patients with obstructive azoospermia (0.54 ± 0.41 \( \mu g \) l\(^{-1} \)) and low-grade oligozoospermia (0.54 ± 0.34 \( \mu g \) ml\(^{-1} \)) were comparable with those of normozoospermic men (0.21 ± 0.21 \( \mu g \) l\(^{-1} \)).

Differences in leptin levels between different groups of male patients presenting with infertility problems and possible correlations between leptin levels and clinical, spermiological, histological and hormonal characteristics were examined (Zorn et al., 2007). Two hundred and ten male partners from infertile couples were included in the study. Based on clinical examination, spermiogram and testicular histology results, patients were divided into four groups: 42 men with non-obstructive azoospermia, 15 men with obstructive azoospermia, 68 men with oligoasthenoteratozoospermia and 85 men with normozoospermia. Serum levels of follicle-stimulating hormone, luteinizing hormone, inhibin B, testosterone, sex hormone binding globulin (SHBG) and leptin were measured. After adjustment for body mass index, there was a negative correlation between serum levels of leptin and inhibin B, total testosterone and SHBG (r=-0.189, P=0.009, r=-0.250, P=0.001 and r=-0.221, P=0.003 respectively) but there was no correlation between leptin and classical sperm characteristics.

Ishikawa et al. (2007) investigated the relationships between the expression of leptin, leptin receptor in the testis, spermatogenesis and testosterone
concentration in infertile men. Testicular tissue samples were collected from the
testes of five fertile volunteers, eight patients with obstructive azoospermia, six
patients with Sertoli cell-only syndrome and 32 oligospermic patients with
varicocele testis. In testicular tissue, leptin and leptin receptor were identified by
staining with polyclonal antibodies. Serum follicle stimulating hormone,
luteinizing hormone and testosterone were determined by chemiluminescence
assays. Leptin was expressed on germ cells, mainly on spermatocytes. The
ratio of immunostained germ cells to total germ cells was inversely correlated
with the concentration of testosterone \((r=-0.32, P=0.01)\), sperm concentration
\((r=-0.51, P=0.002)\) and Johnsen's score \((r=-0.44, P=0.005)\). In contrast, leptin
receptor immunostained cells were found in the interstitium, primarily in Leydig
cells. Leptin receptor expression on Leydig cells was inversely correlated with
serum testosterone concentration \((r=-0.50, P< 0.001)\). The dysfunction of
spermatogenesis is associated with an increase in leptin and leptin receptor
expression in the testis.

Hanafy et al., (2007) investigated 80 men; fertile normozoospermia as a control
and infertile oligozoospermia. The patients underwent estimation of body weight
(kg), height (cm), calculation of body mass index (BMI), semen analysis, serum
leptin and testosterone hormones. Mean body weight was significantly higher in
infertile oligozoospermia compared with controls. Mean height, BMI and serum
testosterone levels showed non significant differences between the two groups.
Infertile oligozoospermia had significantly higher mean serum leptin level than
controls \((mean +/- SD; 6.88 +/- 8.65, 16.3 +/- 13.98 \text{ ng ml}^{-1}), P< 0.01)\). Serum
leptin demonstrated significant positive correlation with age, body weight, BMI
and significant inverse correlation with serum testosterone. It had nonsignificant
correlation with the height and sperm concentration.

Hofny et al. (2010) studied the changes in semen parameters, gonadotropic
and sex hormones, and serum leptin in 42 obese fertile and 80 infertile
oligozoospermic men. Obese oligozoospermic patients had significant increase
in mean BMI, serum FSH, LH, estradiol, prolactin, and leptin compared with
obese fertile controls. The BMI had significant positive correlation with abnormal sperm morphology, LH, serum leptin and significant negative correlation with sperm concentration, sperm motility and serum testosterone. Serum leptin demonstrated significant positive correlation with patients' age, abnormal sperm morphology, serum FSH, LH and prolactin and significant negative correlation with sperm concentration, sperm motility, and serum testosterone.
Chapter 3

Subjects and Methodology

3.1 Study design
The present study is a case control one.

3.2 Target Population
The target population was infertile men with azoospermia, germ cell aplasia.

3.3 Sampling and sample size
Samples were collected from men who visiting the Specialized Medical Centers in Gaza Strip with the history of at last 3 years duration of infertility and according to histological reports (Babu et al., 2004). The sperm count of infertile men was performed confirming azoospermia based on the previous criteria. The number of patients (sample size) was 78 men with azoospermia, germ cell aplasia where mean ages of patients were 32.8 ± 6.4. A total number of 78 controls were selected at the basis of being married with at least one child and sperm count >20 million sperm/ml from men who visit laboratories where mean ages of controls were 31.7 ± 3.9.

3.4 Ethical consideration
The necessary approval to conduct this study was obtained from Helsinki committee in Gaza Strip (Annex 1). Coordination with Al-Basma Medical Center was fulfilled (Annex 2).

3.5 Data collection
3.5.1 Questionnaire interview
A face to face interview was used for filling the questionnaire which is designated for matching the study needs. The questionnaire (Annex 3) was
based on male infertility patient questionnaire urologic clinics of North Alabama, and on that used in a similar study with some modifications (Taha, 2010). During the study the interviewer explain to the patient confused questions that were not clear to them. Most questions were the yes/no questions, which offer a dichotomous choice (Backstrom and Hursh-César, 1981). The questionnaire was validated by four experts in the fields of andrology, urology, obstetrics and gynecology. The questionnaire was piloted with 10 patients, and modified as necessary to improve reliability. The questionnaire included questions on the personal data (age, education, occupation and family history), sexual history, medical history, exposure history, and medications.

3.5.2 Body mass index
Body mass index was calculated as the ratio of body weight in Kg / height in square meter. The subjects were asked to remove shoes and heavy clothes before measurement of weight and height. The balance (Camry, Italy) was used for measuring weight in kilograms and stadiometer (Henglida, China) for measuring height in centimeters. People with BMI less than 18.5 were considered to have under weight, people with BMI=18.5–24.9 were considered to have normal weight, people with BMI=25.0–29.9 were classified overweight, people with BMI ≥ 30.0 were considered obese (WHO, 2000).

3.5.3 Blood sampling and Processing
Venous blood sample (8 ml) was drawn by the researcher himself who had a Bachelor Degree in Medical Technology into vacutainer tubes from each patient. Blood left for a while without anticoagulant to allow blood to clot. Then, serum samples were obtained by centrifugation at room temperature at 3000 rpm for 10 minutes for biochemical analysis.

3.5.4 Hormonal analysis
3.5.4.1 Determination of serum leptin
Determination of human serum leptin level was carried out by competitive enzyme immunoassay (Diagnostic System Laboratories (DSL). USA) technique.
**Principle**
The DSL-10-23100 ACTIVE Human Leptin ELISA is an enzymatically amplified "two step" sandwich-type immunoassay. In the assay, standards, controls and unknown serum or plasma samples were incubated in microtitration wells, which have been coated with anti-human leptin antibody. After incubation and washing, the wells were treated with another anti-human leptin detection antibody labeled with the enzyme horseradish peroxidase (HRP). After a second incubation and washing step, the wells were incubated with the substrate tetramethylbenzidine (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 nm. The absorbance measured was directly proportional to the concentration of human leptin present. A set of human leptin standards was used to plot a standard curve of absorbance versus human leptin concentration from which the human leptin concentrations in the sample can be calculated.
The assay procedure sheets were available with the kit, the application of assay procedure mentioned below.

**Assay procedure**
Annabel all specimens and reagents to reach room temperature (~25°C) and mix thoroughly by gentle inversion before use. Standards, controls and samples should be assayed in duplicate.
1. The microtitration strips were marked to be used.
2. Twenty five microtiteres of the standards, controls and samples were pipetted into the appropriate wells.
3. One hundred microtiteres of the Assay Buffer E were added to each well using a semi-automatic dispenser.
4. Incubate the wells, shaking at a fast speed (500-700 rpm) on an orbital microplate shaker, at room temperature (~25 °C) for 2 hours.
5. Aspirate and wash each well 5 times with the Wash Solution using an automatic microplate washer. Blot dry by inverting plate on absorbent material.
6. The Antibody-Enzyme Conjugate Solution was prepared by diluting the Antibody- Enzyme Conjugate Concentrate in the Assay Buffer.
7. One hundred microtiters of the Antibody-Enzyme Conjugate Solution was added to each well using a semi-automatic dispenser.
8. The wells were incubated, shaked at a fast speed (500-700 rpm) on an orbital microplate shaker, at room temperature (~25 °C) for 1 hour.
9. Aspirate and wash each well 5 times with the Wash Solution using an automatic microplate washer. Blot dry by inverting plate on absorbent material.
10. One hundred microtiters of the TMB Chromogen Solution was added to each well using a semi-automatic dispenser.
11. Incubate the wells, shaking at a fast speed (500-700 rpm) on an orbital microplate shaker, at room temperature (~25°C) for 10 minutes. Avoid exposure to direct sunlight.
12. One hundred microtiters of the Stopping Solution (0.2M sulfuric acid) was added to each well using a semi-automatic dispenser.
13. The absorbance of the solution in the wells was read within 30 minutes, using a microplate reader set to 450 nm.

**Calculation**
A. The mean absorbance for each standard, control and samples were calculated.
B. Plot the log of the human leptin concentrations in ng/mL along the x-axis versus the mean absorbance readings for each of the standards along the y-axis versus, using a linear curve-fit. Alternatively, the data can be plotted linear vs. linear and a smoothed spine curve-fit can be used.
C. Determine the human leptin concentrations of the controls and samples from the standard curve by matching their mean absorbance readings with the corresponding human leptin concentrations.

### 3.5.4.2 Determination of serum testosterone
Testosterone hormone level was determined according to the method of Tiez, (1986) using ELISA TECO kit for testosterone.
Principle
The testosterone ELISA Kit is a solid phase enzyme-linked immunosorbent assay (ELISA), based on the principle of competitive binding. The microtiter wells are coated with an antibody directed towards an unique antigenic site on the testosterone molecule. Endogenous testosterone of a patient sample competes with a testosterone horseradish peroxidase conjugate for binding to the coated antibody. After incubation the unbound conjugate is washed off. The amount of bound peroxidase conjugate is reverse proportional to the concentration of testosterone in the sample. After addition of the substrate solution, the intensity of colour developed is reverse proportional to the concentration of testosterone in the patient sample.

Kit components
1. Microtiter wells, 12x8 (break apart) Strips, 96 wells coated with mouse monoclonal anti-testosterone antibody
2. Standard (Standard 0-6), 7 vials, 1 ml, ready to use concentrations: 0 - 0.2 - 0.5 – 1 – 2 – 6 - 16 ng/ml Conversion: 1 ng/ml = 3.467 nmol/l
3. Enzyme conjugate, 1 vial, 25 ml, ready to use testosterone conjugated to horseradish peroxidase
4. Substrate solution, 1 vial, 25 ml,
5. Stop Solution, 1 vial, 14 ml, ready to use contains 0.5M H2SO4 avoid contact with the stop solution. It may cause skin irritations and burns.
6. Wash Solution, 1 vial, 30 ml (40X concentrated)
Note: Additional standard 0 for sample dilution is available on request.

Assay procedure
All samples and reagents were allowed to reach at room temperature (~25°C). Reagents mixed by gentle inversion before use. Standards, controls and samples assayed in duplicate.
1. Microtitration Strip was marked to be used.
2. Twenty-five µL of the standards, controls and samples were added into each appropriate well.
3. Two hundred µL of Conjugate Reagent were added into each well using a precision pipette.
4. The wells were mixed for 10 seconds.
5. The wells were incubated for 60 minute at room temperature (~25°C).
6. Each well was aspirated and washed 3 times by added 400 µL of working Wash Solution.
7. Two hundred µL of substrate solution were added into each well using a precision pipette and gently mixed for 10 seconds.
8. The wells were incubated in the dark for 15 minute at room temperature (~25°C).
9. One hundred µL of Stop Solution were added into each well using a precision pipette and mixed for 10-20 seconds.
10. The absorbances of the solution in each well were read at 450 nm.

**Calculation**
The absorbance for each standard, control, or samples were obtained, and then the stander curve prepared by plotted the absorbance readings for each of the standards along the Y-axis versus standard concentrations in ng/mL along the X-axis, the mean absorbance values for each sample were determined the corresponding concentration of testosterone in ng/mL from the standard curve (Figure 3.1).

![Figure 3.1. Standard curve for testosterone hormone](image-url)
Normal reference values of testosterone for adult male: 2.0-7.0 ng/mL.

3.5.4.3 Determination of serum luteinizing hormone
Luteinizing hormone level was determined according to Lenton et al., (1982) method using ELISA TECO kit for LH.

Principle
The essential reagents required for an immunoenzymometric assay include excess amount of antibodies (both enzyme conjugated and immobilized) with high affinity, high specificity and contain different epitopes with distinct recognition and native antigen. In this assay procedure, the immobilization takes place at the surface of a microplate well through the interaction of streptavidin coated on the well and exogenously added biotinylated monoclonal anti-LH antibody. Upon mixing, a reaction results between the native antigen contained in serum, the monoclonal biotinylated antibody and the enzyme-labeled antibody, without competition or steric hindrance, to from a soluble sandwich complex. Simultaneously, the complex is deposited to the well through the high affinity reaction of streptavidin and biotinylated antibody. After equilibrium is attained, the antibody-bound fraction is separated from unbound antigen by decantation or aspiration. The enzyme activity in the antibody-bound fraction is directly proportional to the native antigen concentration. By utilizing several different serum references of known antigen value, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

Kit components
One Strip holder containing 96 microtitration wells coated with streptavidin, six LH reference standards with concentrations of approximately (0, 5.0, 25, 50, 100 and 200 mIU/mL). Enzyme Conjugate, TMB Chromogen Solution, Stop Solution and Wash Solution concentrate.
**Assay Procedure**

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

1. Microtitration Strip was marked to be used.
2. Fifty µL of the standards, controls and samples were added into each appropriate well.
3. One hundred µL of Conjugate Reagent were added into each well using a precision pipette and then mixed for 30 seconds.
4. The wells were incubated for 60 minutes at room temperature (~25°C).
5. Each well was aspirated and washed 3 times by added 300 µL of working Wash Solution.
6. One hundred µL of TMB reagent were added into each well and gently mixed for 10 second.
7. The wells were incubated in the dark for 15 minutes at room temperature (~25°C) without shaking.
8. Fifty µL of Stop Solution were added into each well and gently mixed for 10-20 second.
9. The absorbance for each well was read at 450 nm.

**Calculation**

The absorbance for each standard, control, or samples were obtained, and then the stander curve prepared by plotted the absorbance readings for each of the standards along the Y-axis versus standard concentrations in mIU/mL along the X-axis, the mean absorbance values for each sample were determined the corresponding concentration of LH in mIU/mL from the standard curve (Figure 3.2).
Normal reference values of LH for adult male: 2.0-13.0 mIU/mL.

3.5.4.4 Determination of serum follicle stimulating hormone
Follicle stimulating hormone level was determined according to Vitt et al., (1998) method using ELISA TECO kit for FSH.

Principle
The essential reagents required for an immunoenzymometric assay include excess amount of antibodies (both enzyme conjugated and immobilized) with high affinity, high specificity and contain different epitopes with distinct recognition and native antigen. In this assay procedure, the immobilization takes place at the surface of a microplate well through the interaction of streptavidin coated on the well and exogenously added biotinylated monoclonal anti-FSH antibody. Upon mixing, a reaction results between the native antigen contained in serum, the monoclonal biotinylated antibody and the enzyme-labeled antibody, without competition or steric hindrance, to form a soluble sandwich complex. Simultaneously, the complex is deposited to the well through the high affinity reaction of streptavidin and biotinylated antibody. After
equilibrium is attained, the antibody-bound fraction is separated from unbound antigen by decantation or aspiration. The enzyme activity in the antibody-bound fraction is directly proportional to the native antigen concentration. By utilizing several different serum references of known antigen value, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

**Kit components**
One Strip holder containing 96 microtitration wells coated with streptavidin, six FSH reference standards with concentrations of approximately (0, 5.0, 10, 25, 50 and 100 mIU/mL). Enzyme Conjugate, TMB Chromogen Solution, Stop Solution and Wash Solution concentrate.

**Assay Procedure**
All samples and reagents were allowed to reach at room temperature (~25°C). Reagents mixed by gentle inversion before use. Standards, controls and samples assayed in duplicate.
1. Microtitration Strip was marked to be used.
2. Fifty µL of the standards, controls and samples were added into each appropriate well.
3. One hundred µL of Conjugate reagent were added into each well using a precision pipette and then mixed for 30 seconds.
4. The wells were incubated for 60 minutes at room temperature (~25°C).
5. Each well was aspirated and washed 3 times by added 300 µL of working wash solution.
6. One hundred µL of TMB reagent were added into each well and gently mixed for 10 second.
7. The wells were incubated in the dark for 15 minutes at room temperature (~25°C) without shaking.
8. Fifty µL of Stop Solution were added into each well and gently mixed for 10-20 second.
9. The absorbance for each well was read at 450 nm.
Calculation
The absorbance for each standard, control, or samples were obtained, and then the standard curve prepared by plotted the absorbance readings for each of the standards along the Y-axis versus standard concentrations in mIU/mL along the X-axis. The mean absorbance values for each sample were determined the corresponding concentration of FSH in mIU/mL from the standard curve (Figure 3.4).

![Graph of standard concentration vs absorbance at 450 nm](image)

Figure 3.3. Follicle stimulating hormone standard curve.

Normal reference value of FSH for adult male: 2.5-10.0 mIU/mL.

3.5.4.5 Determination of total cholesterol
Total Cholesterol was determined by enzymatic colorimetric method using Globe diagnostics kit, Italy.
**Principle**

The measurement is based on the following enzymatic reactions:

**CHE**

\[
\text{Cholesterol esters} + \text{H}_2\text{O} \rightarrow \text{Cholesterol} + \text{Fatty acids}
\]

**CHOD**

\[
\text{Cholesterol} + \text{O}_2 \rightarrow \text{Cholest-4-en-3-one} + \text{H}_2\text{O}_2
\]

**POD**

\[
2\text{H}_2\text{O}_2 + \text{hydroxybenzoate} + 4\text{-AmminoantiPyrine} \rightarrow \text{Red complex} + 4\text{H}_2
\]

The intensity of the red complex is proportional to the total cholesterol present in the sample.

**Assay procedure**

About 0.5 ml of serum was transferred to the Mindray BS-120 chemistry autoanalyzer to perform the test according to these parameters:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent (μl)</td>
<td>300</td>
</tr>
<tr>
<td>Serum (μl)</td>
<td>3</td>
</tr>
<tr>
<td>Incubation period (min)</td>
<td>3</td>
</tr>
<tr>
<td>Reaction type</td>
<td>End point</td>
</tr>
<tr>
<td>Wavelength (nm)</td>
<td>510</td>
</tr>
</tbody>
</table>

**Reference range**

Cholesterol values according to a study on a population of adults are the following:
### 3.5.4.6 Determination of triglycerides

Triglycerides were determined by enzymatic colorimetric method using Globe diagnostics kit, Italy.

**Principle**

Glycerol, released from triglycerides after hydrolysis with lipoproteinlipase is transformed by glycerolkinase into glycerol-3-phosphate which is oxidized by glycerolphosphatase oxidase into dihydroxyacetone phosphate and hydrogen peroxide. In presence of peroxidase, the hydrogen peroxide oxidizes the chromogen ESPT (4-aminophenazone/N-ethylmethyleneilin-propan-sulphonate sodic) to form purple quinoneimine whose colour intensity, measured at 510 nm, is proportional to the concentration of triglycerides in the sample.

\[
\text{LPL} \\
\text{Triglycerides} \rightarrow \text{Glycerol} + \text{Fatty acids}
\]

\[
\text{GK} \\
\text{Glycerol} + \text{ATP} \rightarrow \text{Glycerol-3-phosphate} + \text{ADP}
\]

\[
\text{GPO} \\
\text{Glycerol-3-phosphate} + \text{O}_2 \rightarrow \text{Dihydroxyacetone phosphate} + \text{H}_2\text{O}_2
\]

\[
\text{POD} \\
2 \text{H}_2\text{O}_2 + \text{Amminoantipirine} + \text{ESPT} \rightarrow \text{Quinoneimine} + \text{HCl} + 4 \text{H}_2\text{O}
\]
Assay procedure
About 0.5 ml of serum was transferred to the Mindray BS-120 chemistry autoanalyzer to perform the test according to these parameters:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent (µl)</td>
<td>300</td>
</tr>
<tr>
<td>Serum (µl)</td>
<td>3</td>
</tr>
<tr>
<td>Incubation period (min)</td>
<td>3</td>
</tr>
<tr>
<td>Reaction type</td>
<td>End point</td>
</tr>
<tr>
<td>Wavelength (nm)</td>
<td>510</td>
</tr>
</tbody>
</table>

Reference range

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Recommended values</td>
<td>&lt; 200 mg/dL</td>
</tr>
<tr>
<td>Upper limit</td>
<td>200-400 mg/dl</td>
</tr>
<tr>
<td>High values</td>
<td>&gt; 400 mg/dl</td>
</tr>
</tbody>
</table>

3.5.4.7 Determination of high density lipoprotein

High density lipoprotein was determined by liquid HDL precipitant for the determination of HDL Cholesterol using Globe diagnostics kit, Italy.

Principle

The VLDL and LDL-C from serum or plasma are precipitated by phosphotungstate in the presence of magnesium ions. After removed by centrifugation the clear supernatant is containing high density lipoproteins (HDL-C) and used for the determination of it.
**Assay procedure**

1. pipette into conic test tubes:
   - 0.5 ml serum
   - 0.5 ml precipitating reagent
2. Mix gently by inversion, wait 5 minutes and centrifuge at (3000 rpm) for 20 minutes.
3. Recovery the supernatant for the HDL cholesterol determination as follows:
   - About 0.5 ml of supernatant was transferred to the Mindray BS-120 chemistry autoanalyzer to perform the test according to these parameters:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent (μl)</td>
<td>200</td>
</tr>
<tr>
<td>supernatant (μl)</td>
<td>3</td>
</tr>
<tr>
<td>Incubation period (min)</td>
<td>3</td>
</tr>
<tr>
<td>Reaction type</td>
<td>End point</td>
</tr>
<tr>
<td>Wavelength (nm)</td>
<td>510</td>
</tr>
</tbody>
</table>

**Reference value**

Based on the risk for heart diseases the sequent reference ranges are suggested:

<table>
<thead>
<tr>
<th>Low value (high risk)</th>
<th>Medium value (moderate risk)</th>
<th>High value (low risk)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 40 mg/dl</td>
<td>40 - 59 mg/dl</td>
<td>&gt; 60 mg/dl</td>
</tr>
</tbody>
</table>

**3.5.4.8 Determination of Low density lipoprotein cholesterol**

Determination of LDL-C was calculated from the primary measurements using the empirical equation.
**3.6 Statistical analysis**

Data were computer analyzed using SPSS/PC (Statistical Package for the Social Science Inc. Chicago, Illinois USA, version 18.0) statistical package. Simple distribution of the study variables and the cross tabulation were applied. Chi-square ($\chi^2$) was used to identify the significance of the relations, associations, and interactions among nominal various variables. Yates's continuity correction test, $\chi^2_{(corrected)}$, was used when not more than 20% of the cells had an expected frequency of less than five and when the expected numbers were small. The independent sample t-test procedure was used to compare means of quantitative variables by the separated cases into two qualitative groups such as the relationship between patients and controls hormones. Pearson's correlation test between leptin and other studied variables was applied.

**Percentage difference formula:**

Percentage difference equals the absolute value of the change in value, divided by the average of the 2 numbers, all multiplied by 100.

\[
\text{Percent difference} = \left( \frac{|(V1 - V2)|}{((V1 + V2)/2)} \right) * 100.
\]

The results were accepted as statistical significant when the P-value was less than 5% (P<0.05).
Chapter 4

Results

4.1 General characteristics of the study population
Table 1 illustrates the personal data of the study population. Age calcification showed that 34 (43.6%) controls and 28 (35.9%) cases were ≤29 years old. Age group 30-34 years comprised 28 (35.9%) controls and 26 (33.3%) cases. Controls and patients aged >35 years old were 16 (20.5%) and 24 (30.8%), respectively. The difference between controls and patients in term of age distribution was not significant \( \chi^2 = 1.127, P=0.569 \). The mean ages of controls and patients were 31.7 ± 3.9 and 32.8 ± 6.4 years old, respectively. The independent sample t-test also showed no significant difference between mean ages of controls and patients (t=1.129, P=0.262). Analysis of the educational status of the study population showed that 48 (61.5%) controls and 34 (43.6%) patients had a university degree, 15 (19.2%) and 14 (17.9 %) finished secondary school, 13 (16.7%) and 18 (23.1%) had finished preparatory school, 1 (1.3%), 8 (10.3%) had passed primary school, and 1 (1.3%), 4 (5.1%) were illiterate. The difference between various education levels of controls and patients was not significance \( \chi^2\text{ corrected} = 7.377, P=0.117 \). The employed controls and patients were 66 (84.6%) and 52 (66.7%) whereas 12 (15.4%) controls and 26 (33.3%) patients were unemployed. The difference between the two groups was not significant \( \chi^2 = 3.409, P=0.065 \). Regarding family history, 10 (12.8%) controls and 20 (25.6%) patients reported that they have a family history of male infertility \( \chi^2 = 2.036, P=0.151 \).
Table 4.1 General data of the study population.

<table>
<thead>
<tr>
<th>General characteristics</th>
<th>Controls (n=78)</th>
<th>Patients (n=78)</th>
<th>Statistical test</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (Year)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤29</td>
<td>34</td>
<td>28</td>
<td>( \chi^2 = 1.127 )</td>
<td>0.569</td>
</tr>
<tr>
<td>30-34</td>
<td>28</td>
<td>26</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;35</td>
<td>16</td>
<td>24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean±SD</td>
<td>31.7±3.9</td>
<td>32.8±6.4</td>
<td>( t = 1.129 )</td>
<td>0.262</td>
</tr>
<tr>
<td>Education</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>illiterate</td>
<td>1</td>
<td>4</td>
<td>( \chi^2 = 7.377 )</td>
<td>0.117*</td>
</tr>
<tr>
<td>Primary</td>
<td>1</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preparatory</td>
<td>13</td>
<td>18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Secondary school</td>
<td>15</td>
<td>14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>University</td>
<td>48</td>
<td>34</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Occupation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Employed</td>
<td>66</td>
<td>52</td>
<td>( \chi^2 = 3.409 )</td>
<td>0.065</td>
</tr>
<tr>
<td>Un-employed</td>
<td>12</td>
<td>26</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Family history</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>10</td>
<td>20</td>
<td>( \chi^2 = 2.036 )</td>
<td>0.151</td>
</tr>
<tr>
<td>No</td>
<td>68</td>
<td>58</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*P-value of \( \chi^2 \) (corrected) test

4.2 Sexual problems of the study population

The sexual history of the study population is presented in Table 4.2. None of the controls 0 (0.0%) had problems with erection and ejaculation compared to 12 (15.4%) and 22 (28.2%) patients, respectively. The difference between the two groups in terms of problems with erection and ejaculation was significant (\( \chi^2 \) corrected=10.924, \( P=0.000 \) and \( \chi^2 \) corrected=23.336, \( P=0.000 \), respectively). Regarding to gonadal infection, 4 (5.1%) controls had gonadal infection compared to 24 (30.8%) patients (\( \chi^2 \) corrected=15.713, \( P=0.000 \)).
Table 4.2 Sexual history of the study population.

<table>
<thead>
<tr>
<th>Sexual history</th>
<th>Controls (n=78)</th>
<th>Patients (n=78)</th>
<th>$\chi^2$</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>Problems with erection</td>
<td>Yes</td>
<td>0</td>
<td>12</td>
<td>15.4</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>78</td>
<td>66</td>
<td>84.6</td>
</tr>
<tr>
<td>Problems with ejaculation</td>
<td>Yes</td>
<td>0</td>
<td>22</td>
<td>28.2</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>78</td>
<td>56</td>
<td>71.8</td>
</tr>
<tr>
<td>Gonadal infection</td>
<td>Yes</td>
<td>4</td>
<td>24</td>
<td>30.8</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>74</td>
<td>54</td>
<td>69.2</td>
</tr>
</tbody>
</table>

*P-value of $\chi^2$ (corrected) test

4.3 Medical history of the study population

Table 4.3 provides the medical history of the study population. None of the controls had orchitis, cryptorchidism, varicocele, hydrocele and hormonal problems compared to 26 (33.3%), 20 (25.6%), 18 (23.1%), 2 (2.6%) and 50 (64.1%) patients who had such diseases. The difference between the two groups was statistically significant for orchitis ($\chi^2$ corrected=28.846, $P=0.000$), cryptorchidism ($\chi^2$ corrected=20.704, $P=0.000$), varicocele ($\chi^2$ corrected=18.150, $P=0.000$), hormonal problems ($\chi^2$ corrected=70.671, $P=0.000$). However, no significant difference was found for hydrocele ($\chi^2$ corrected=0.506, $P=0.477$). None of controls and patients were reported to have immune diseases. In addition, controls who had mumps and trauma were 6 (7.7%) and 16 (20.5%) compared to 14 (17.9%) and 44 (56.4%) patients. The difference between two groups was significant for trauma ($\chi^2$ corrected=21.233, $P=0.000$) and not significant for mumps ($\chi^2$ corrected=3.671, $P=0.055$).
Table 4.3 Medical history of the study population.

<table>
<thead>
<tr>
<th>Medical history</th>
<th>Control (n=78)</th>
<th>Patients (n=78)</th>
<th>( \chi^2 )</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>Orchitis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>0</td>
<td>0.0</td>
<td>26</td>
<td>33.3</td>
</tr>
<tr>
<td>No</td>
<td>78</td>
<td>100</td>
<td>52</td>
<td>66.7</td>
</tr>
<tr>
<td>Mumps</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>6</td>
<td>7.7</td>
<td>14</td>
<td>17.9</td>
</tr>
<tr>
<td>No</td>
<td>72</td>
<td>92.3</td>
<td>64</td>
<td>82.1</td>
</tr>
<tr>
<td>Trauma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>16</td>
<td>20.5</td>
<td>44</td>
<td>56.4</td>
</tr>
<tr>
<td>No</td>
<td>62</td>
<td>79.5</td>
<td>34</td>
<td>43.6</td>
</tr>
<tr>
<td>Cryptorchidism</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>0</td>
<td>0.0</td>
<td>20</td>
<td>25.6</td>
</tr>
<tr>
<td>No</td>
<td>78</td>
<td>100</td>
<td>58</td>
<td>74.4</td>
</tr>
<tr>
<td>Varicocele</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>0</td>
<td>0.0</td>
<td>18</td>
<td>23.1</td>
</tr>
<tr>
<td>No</td>
<td>78</td>
<td>100</td>
<td>60</td>
<td>76.9</td>
</tr>
<tr>
<td>Hydrocele</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>0</td>
<td>0.0</td>
<td>2</td>
<td>2.6</td>
</tr>
<tr>
<td>No</td>
<td>78</td>
<td>100</td>
<td>76</td>
<td>97.4</td>
</tr>
<tr>
<td>Immune diseases</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>0</td>
<td>0.0</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>No</td>
<td>78</td>
<td>100</td>
<td>78</td>
<td>100</td>
</tr>
<tr>
<td>Hormonal problems</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>0</td>
<td>0.0</td>
<td>50</td>
<td>64.1</td>
</tr>
<tr>
<td>No</td>
<td>78</td>
<td>100</td>
<td>28</td>
<td>35.9</td>
</tr>
</tbody>
</table>

*P-value of \( \chi^2 \) (corrected) test

4.4 Exposure history of the study population

Exposure history of the study population is presented in Table 4.4. The number of controls exposed to thermal and chemical conditions were 4 (5.1%) and 12 (15.4%) compared to 20 (25.6%) and 28 (35.9%) patients. The differences between two groups for thermal and chemical conditions were significant (\( \chi^2 \) corrected=11.080, \( P=0.000 \) and \( \chi^2 = 8.607, \ P=0.003 \), respectively). The cases of thermal and chemical exposure were reported as a result of working in bakeries, exposure to hot bath, painting, pesticide and cleaning materials. None of controls and patients had radiation therapy or chemotherapy.
Table 4.4 Exposure history of the study population.

<table>
<thead>
<tr>
<th>Exposure history</th>
<th>Controls (n=78)</th>
<th>Patients (n=78)</th>
<th>$\chi^2$</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>Thermal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>4</td>
<td>5.1</td>
<td>20</td>
<td>25.6</td>
</tr>
<tr>
<td>No</td>
<td>74</td>
<td>94.9</td>
<td>58</td>
<td>74.4</td>
</tr>
<tr>
<td>Chemical</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>12</td>
<td>15.4</td>
<td>28</td>
<td>35.9</td>
</tr>
<tr>
<td>No</td>
<td>66</td>
<td>84.6</td>
<td>50</td>
<td>64.1</td>
</tr>
<tr>
<td>Radiation therapy</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>No</td>
<td>78</td>
<td>100</td>
<td>78</td>
<td>100</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>0</td>
<td>0.0</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>No</td>
<td>78</td>
<td>100</td>
<td>78</td>
<td>100</td>
</tr>
</tbody>
</table>

*P-value of $\chi^2$ (corrected) test

4.5 Medication history of the study population.

Table 4.5 gives the medication history of the study population. The controls who reported the treatment with the antifungal Ketoconazole and the steroids were 8 (10.3%) and 0 (0.0%) compared to 26 (33.3%) and 44 (56.4%) patients. The differences between controls and patients were significant ($\chi^2=12.185$, P=0.000 and $\chi^2$ corrected=58.532, P=0.000, respectively). On the other hand, no significant difference between controls and patients was found for histamine H2-receptor antagonist cimetidine ($\chi^2=0.118$, P=0.731). In addition, none of the controls or patients was reported to take the antiandrogen spironolactone and growth hormone.
Table 4.5. Medications of the study population.

<table>
<thead>
<tr>
<th>Medications</th>
<th>Control (n=78)</th>
<th>Patients (n=78)</th>
<th>χ²</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>8</td>
<td>10.3</td>
<td>26</td>
<td>33.3</td>
</tr>
<tr>
<td>No</td>
<td>70</td>
<td>89.7</td>
<td>52</td>
<td>66.7</td>
</tr>
<tr>
<td>Spironolactone</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>0</td>
<td>0.0</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>Yes</td>
<td>78</td>
<td>100</td>
<td>78</td>
<td>100</td>
</tr>
<tr>
<td>Cimetidine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>26</td>
<td>33.3</td>
<td>24</td>
<td>30.8</td>
</tr>
<tr>
<td>No</td>
<td>52</td>
<td>66.7</td>
<td>54</td>
<td>69.2</td>
</tr>
<tr>
<td>Steroids</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>0.0</td>
<td>0.0</td>
<td>44</td>
<td>56.4</td>
</tr>
<tr>
<td>No</td>
<td>78</td>
<td>100</td>
<td>34</td>
<td>43.6</td>
</tr>
<tr>
<td>Growth hormone</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>0</td>
<td>0.0</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>No</td>
<td>78</td>
<td>100</td>
<td>78</td>
<td>100</td>
</tr>
</tbody>
</table>

*P-value of χ² (corrected) test

4.6 Distribution of various classes of BMI among the study population

Table 4.6 shows the distribution of various classes of BMI among the study population. The numbers of underweight, normal, overweight and obese controls were 0 (0%), 30 (38.5%), 42 (53.8%) and 6 (7.7%) whereas in patients the numbers were 0 (0%), 16 (20.5%), 34 (43.6) and 28 (35.9). Chi-Square test showed significant association between underweight versus normal versus overweight versus obese among controls and patients (χ²=19.338, P=0.000).
Table 4.6. Distribution of various classes of BMI among the study population.

<table>
<thead>
<tr>
<th>BMI*</th>
<th>Controls (n=78)</th>
<th>Patients (n=78)</th>
<th>$\chi^2$</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Underweight</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>Normal</td>
<td>30 (38.5)</td>
<td>16 (20.5)</td>
<td>19.338</td>
<td>0.000</td>
</tr>
<tr>
<td>Overweight</td>
<td>42 (53.8)</td>
<td>34 (43.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Obese</td>
<td>6 (7.7)</td>
<td>28 (35.9)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* People with BMI less than 18.5 were considered to have underweight. People with BMI=18.5–24.9 were considered to have normal weight. People with BMI=25.0–29.9 were classified overweight. People with BMI≥30.0 were considered obese (World Health Organization, WHO, 2000).

4.7 Serum leptin of the study population

Mean levels of serum leptin of the study population is presented in Table 4.7. The mean levels of leptin were significantly increased in patients compared to controls with percentage difference of 52.4% (21.2 ± 26.1 vs. 12.4 ± 7.6 ng/ml, t=2.019, P=0.047).

Table 4.7 Leptin levels of the study population.

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Controls (n=78)</th>
<th>Patients (n=78)</th>
<th>% Difference</th>
<th>t</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Leptin</td>
<td>12.4±7.6</td>
<td>21.2±26.1</td>
<td>52.4</td>
<td>2.019</td>
<td>0.047</td>
</tr>
<tr>
<td>Range (min-max)</td>
<td>(2.5-35.8)</td>
<td>(3.5-148.7)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Reference range: 2.5-5.7 ng/ml.
All values are expressed as mean±SD.
P<0.05: significant

4.8 Testosterone, luteinizing hormone and follicle stimulating hormone of the study population

Table 4.8 illustrates testosterone, luteinizing hormone and follicle stimulating hormone of the study population. The mean level of testosterone was significantly decreased in patients compared to controls with percentage difference of 45.5% (3.9 ± 2.8 vs. 6.2 ± 1.7 ng/ml, t=4.529, P=0.000). In
contrast, the mean levels of LH and FSH were increased in patients compared to controls showing percentage differences of 100.6% and 130.5%, respectively (12.4 ± 6.7 vs. 4.1 ± 1.8 mIU/ml, t=7.460, P=0.000 and 25.7 ± 16.7 vs. 5.4 ± 2.6 mIU/ml, t=7.491, P=0.000, respectively).

Table 4.8 Testosterone, luteinizing hormone and follicle stimulating hormone of the study population.

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Controls (n=78)</th>
<th>Patients (n=78)</th>
<th>% Difference</th>
<th>t</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone (ng/ml)</td>
<td>6.2±1.7 (3.3-10.3)</td>
<td>3.9±2.8 (0.95-11.6)</td>
<td>45.5</td>
<td>4.529</td>
<td>0.000</td>
</tr>
<tr>
<td>LH (mIU/ml)*</td>
<td>4.1±1.8 (1.6-9.5)</td>
<td>12.4±6.7 (3.6-31.0)</td>
<td>100.6</td>
<td>7.460</td>
<td>0.000</td>
</tr>
<tr>
<td>FSH (mIU/ml)**</td>
<td>5.4±2.6 (1.6-13.2)</td>
<td>25.7±16.7 (3.0-69.3)</td>
<td>130.5</td>
<td>7.491</td>
<td>0.000</td>
</tr>
</tbody>
</table>

*LH: Luteinizing hormone; **FSH: Follicle stimulating hormone. Reference values: Testosterone 2.0-7.0 ng/ml, Luteinizing hormone 2.0-13.0 mIU/ml and Follicle stimulating hormone 2.5-10.0 mIU/ml.

All values are expressed as mean±SD, P<0.05: significant

4.9 Lipid profile of the study population.

The mean levels of lipid profile including cholesterol, triglycerides, high density lipoprotein cholesterol and low density lipoprotein cholesterol of the study population are shown in Table 4.9. The mean levels of serum cholesterol, triglycerides and LDL-C were significantly higher in patients compared to controls registering % differences of 28.8, 47.3 and 43.6, respectively (206.1 ± 45.5, 211.1 ± 129.0 and 123.4 ± 45.2 mg/dl vs. 154.2 ± 33.2, 130.3 ± 70.3 and 79.2 ± 33.6 mg/dl, P=0.000, P=0.001 and P=0.000, respectively). In contrast, HDL-C was significantly lower in patients than in controls (40.4 ± 7.6 vs. 49.0 ± 4.0 mg/dl, % difference=19.2 and P=0.000).
Table 4.9 Lipid profile of the study population.

<table>
<thead>
<tr>
<th>Lipid profile (mg/dl)</th>
<th>Controls (n=78)</th>
<th>Patients (n=78)</th>
<th>% Difference</th>
<th>t</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cholesterol</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range (min-max)</td>
<td>154.2±33.2</td>
<td>206.1±45.5</td>
<td>28.8</td>
<td>5.757</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>(90-212)</td>
<td>(135-357)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Triglycerides</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range (min-max)</td>
<td>130.3±70.3</td>
<td>211.1±129.0</td>
<td>47.3</td>
<td>3.434</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>(55-478)</td>
<td>(60-560)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>HDL-C</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range (min-max)</td>
<td>49.0±4.0</td>
<td>40.4±7.6</td>
<td>19.2</td>
<td>6.227</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>(40.0-57.0)</td>
<td>(26.5-56.0)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>LDL-C</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range (min-max)</td>
<td>79.2±33.6</td>
<td>123.4±45.2</td>
<td>43.6</td>
<td>4.903</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>(19.6-140.6)</td>
<td>(32.6-275.2)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*HDL-C: High density lipoprotein cholesterol, **LDL-C: Low density lipoprotein cholesterol.
Reference range: cholesterol≤200 mg/dl, triglyceride<200 mg/dl, HDL-C=45-65 mg/dl and LDL-C<150 mg/dl.
All values are expressed as mean±SD, P<0.05: significant

4.10 Leptin vs. some biochemical correlation

4.10.1 Leptin levels in relation to testosterone, LH and FSH
Table 4.10 illustrates the results of Pearson correlation between serum leptin levels and serum testosterone, LH and FSH. Negative significant correlation was found between leptin and testosterone (Figure 4.1, r=-0.223 and P=0.049). on other hand, leptin levels showed no significant correlation with LH and FSH (r=0.112, P=0.329 and r=0.100, P=0.381, respectively).
Table 4.10 Leptin level in relation to testosterone, luteinizing hormone (LH) and follicle stimulating hormone (FSH).

<table>
<thead>
<tr>
<th>Serum parameters</th>
<th>Pearson correlation (r)</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone</td>
<td>-0.223</td>
<td>0.049</td>
</tr>
<tr>
<td>LH</td>
<td>0.112</td>
<td>0.329</td>
</tr>
<tr>
<td>FSH</td>
<td>0.100</td>
<td>0.381</td>
</tr>
</tbody>
</table>

* Correlation is significant the 0.05 level (2-tailed)

![Graph showing correlation between leptin and testosterone](image)

**Figure 4.1** The correlation between leptin levels and testosterone

4.10.2 Leptin levels in relation to lipid profile

Leptin levels in relation to lipid profile including cholesterol, triglycerides, HDL-C and LDL-C is summarized in table 4.11. Pearson correlation test revealed no significant correlation between leptin and cholesterol, triglycerides and HDL-C levels ($r=0.156$, $r=0.064$ and $r=-0.172$, respectively). On the other hand, there was positive significant correlation between leptin level and LDL-C (Figure 4.2, $r=0.222$, $P=0.05$).
Table 4.11 Leptin levels in relation to lipid profile.

<table>
<thead>
<tr>
<th>Lipid Profile</th>
<th>Leptin</th>
<th>Pearson correlation (r)</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>0.156</td>
<td>0.173</td>
<td></td>
</tr>
<tr>
<td>Triglycerides</td>
<td>0.064</td>
<td>0.580</td>
<td></td>
</tr>
<tr>
<td>HDL-C**</td>
<td>-0.172</td>
<td>0.131</td>
<td></td>
</tr>
<tr>
<td>LDL-C***</td>
<td>0.222</td>
<td>0.05</td>
<td></td>
</tr>
</tbody>
</table>

* Correlation is significant the 0.05 level (2-tailed)
**HDL-C: High density lipoprotein cholesterol, ***LDL-C: Low density lipoprotein cholesterol

Figure 4.2 The correlation between leptin levels and low density lipoprotein cholesterol (LDL-C)
4.10.3 Leptin level in relation to body mass index

Table 4.12 and Figure 4.3 point out the correlation between leptin level and BMI. The Pearson correlation test showed positive significant correlation between leptin level and BMI ($r=0.368$, $P=0.001$).

Table 4.12. The correlation between leptin and BMI of the study population.

<table>
<thead>
<tr>
<th>Leptin</th>
<th>Pearson correlation (r)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI</td>
<td>0.368</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Figure 4.3 The correlation between leptin and body mass index (BMI)
Chapter 5

Discussion

Fertility is the natural capability of giving life. Total fertility rate (TFR) - the total number of children a woman would bear during her lifetime if she were to experience the prevailing age-specific fertility rates of women. TFR equals the sum for all age groups of 5 times each Age-specific fertility rate (Garrett, 2001). Worldwide, male infertility contributes to more than half of all cases of childlessness; yet, it is a reproductive health problem that is poorly studied and understood (Inhorn, 2005). It is argued that male infertility may be particularly problematic for Middle Eastern men in their societies; there, both virility and fertility are typically tied to manhood. Thus, male infertility is a potentially emasculating condition, surrounded by secrecy and stigma.

Patterns of male infertility vary greatly among regions and even within regions. A combination of social habits, environmental conditions, and genetics is suspected to contribute to this variation. The male infertility rate is reported to be high in United Kingdom and low in Egypt (Statistics by Country for Male Infertility, 2003). However, in Gaza Strip there are under-reporting or even no real figures on male infertility. The only one study in Gaza Strip was carried out on germ cell aplasia without speculation the role of leptin hormone in the disease (Taha, 2010). Therefore, this will be the first study to assess leptin status and relate it to male reproductive hormones and some other biochemical parameters among infertile men with germ cell aplasia in Gaza Strip.

5.1 General characteristics of the study population

The present study was carried out on 78 patients with germ cell aplasia. The mean age of the patients (32.8 years) was lower than that reported from Nigerian (35.7 years) and Kenyan (36.1 years) studies (Muthuuri, 2005, Geidam et al., 2008). The younger age of our patient sample could be explained on the basis that most men seeking out to have children at younger age immediately after marriage. Male age is not an independent factor to affect fertility as well as
outcome of assisted reproductive techniques (Kumtepe et al., 2003). The finding that only one control and four patients were illiterate do reflect a well educated community. One third of patients were found to be unemployed reflecting the unemployment crisis in Gaza Strip. According to statistical information collected by the Palestinian Central Bureau of Statistics (PCBS, 2010), the percentage distribution of labor force participants aged 15 years and over in the Gaza Strip (43.5 %) was one of highest in the world. There was no significance difference between controls and patients regarding family history indicating that family history is probably not an associated factor with male infertility.

5.2 Sexual history of the study population

Sexual history of the study population showed that 15.4% patients had problems with erection, 28.2% had problems with ejaculation and 30.8% had gonadal infection versus none of the controls had problems with erection and ejaculation and only four controls (5.1%) had gonadal infection. The recorded significant difference between controls and patients implies that such problems play a major role in male infertility at least in our sample. Saleh et al. (2003) reported that 11% of men undergoing infertility evaluation experienced problems with erection. Premature ejaculation was reported to be the most common type of sexual dysfunction in men but the certain cause of that is unknown (Paick et al., 1998). The percentage of premature ejaculation obtained in the present study (28.2%) was more or less in agreement with that documented by laumann in East Asia (29.1%), South and Central America (28.3%), Northern Europe (20.7%), and Southern Europe (21.5%) (Laumann et al., 1999; Nicolosi et al., 2004 and Porst, 2009) and with that from USA (32.5%) (Rowland et al., 2004). However, a study from Iran reported a higher frequency rate of premature ejaculation (43%) with the complaint of infertility (Hassanzadeh et al., 2010). In addition, the result presented here on gonadal infection was close to that reported by Golshani et al., 2006 who revealed that among 88 cases, 35.2% (31 cases) showed gonadal infection. At least one pathogen like E.coli, Saprophyticcus, Group B Streptococci, Entrococci,
Candida sp., Gonococci, Staphylococcus aureus, Klebsiella sp. and Providencia sp. caused such infection.

5.3 Medical history of the study population
As depicted from the medical history of the study population, 33.3%, 25.6%, 23.1% and 64.1% patients had orchitis, cryptochidism, varicocele and hormonal problems, respectively compared to none of the controls. The significant differences between patients and controls imply that such diseases do affect male fertility. Osegbe, 1991 showed that bacterial gonadal infection or orchitis may result in permanent azoospermia or oligospermia in 27 (60%) out of 45 young men and this without question may result in male-related barrenness. Infertility rates were reported as 32% in unilateral cryptorchidism and up to 60% in bilateral cryptorchidism (Lee et al., 1997). In addition, some cases of male infertility were found to be due to anatomical abnormalities such as varicocele (Olayemi, 2010). In many instances non-obstructive azoospermia was related to the history of clinical unilateral or bilateral varicocele (Poulakis et al., 2006 and Inci et al., 2009). Infertile men with non-obstructive azoospermia can have improvement in spermatogenesis and achieve spontaneous pregnancy after repair of clinical varicoceles (Matthews et al., 1998, Weedin et al., 2010). Hormonal problems seem to be the most effective factor affecting male infertility at least in the present study where patients showed significant decrease in testosterone and significant increase in LH and FSH compared to controls. Babu et al. (2004) stated that abnormal spermatogenesis was often associated with altered serum gonadotropins and testosterone. Concerning trauma, 56.4% patients had trauma versus 20.5% controls indicating that trauma is associated with male infertility. This finding is in accordance with that obtained by Cross et al. (1999) who found that 50% of patients had male infertility as a result of trauma. Trauma reported by patients in the present study was mostly refereed to a football match and to the Israeli occupation during the interrogation.

5.4 Exposure history of the study population
Exposure history of the study population revealed that the number of controls exposed to thermal and chemical conditions were 4 (5.1%) and 12 (15.4%) compared to 20 (25.6%) and 28 (35.9%) patients. The differences between two
groups were significant. The cases of thermal and chemical exposure were reported as a result of working in bakeries, exposure to hot bath, painting, pesticide and cleaning materials. It was concluded that changes in semen characteristics due to elevated body temperature may contribute to heat stress infertility of broiler breeders (Karaca et al., 2002). In addition Shefi and Turek, (2006) listed occupational history including exposure to chemicals and chronic heat exposure (saunas) as components of the male infertility history.

5.5 Medication history of the study population

The medication history of the study population showed that 26 (33.3%) and 44 (56.4) azoospermic patients were administered Ketoconazole and steroids compared to 8 (10.3%) and 0 (0.0%) controls. The significant difference between patients and controls indicates that such medication may affect male fertility. Ketoconazole is an antifungal drug with a broad spectrum of activity that also induces reproductive toxicity in humans and animals through blocking testosterone synthesis (Irsy et al., 1991 and Amin, 2008). It was mentioned that Ketoconazole and steroids administration may have potential adverse effects on male fertility (Shefi and Turek, 2006).

5.6 BMI of the study population

Data presented here showed that 28 (35.9%) of patients were obese compared to 6 (7.7%) controls. This indicates that obesity may be a risk factor of male infertility. Male infertility was reported to be associated with a higher incidence of obesity (Nguyen et al., 2007 and Hammoud et al., 2008). Higher prevalence of oligozoospermia was found in overweight and obese men than in normal weight men with a significant association between sperm count and BMI (Jensen et al., 2004). BMI was also found to be inversely correlated with sperm concentration, sperm motility, and sperm normal morphology (Hofny et al., 2010). An elevated BMI can impair or arrest spermatogenesis by causing an increase in scrotal temperature (Kasturi et al., 2008).

5.7 Hormonal profile of the study population

In the evaluation of male infertility, hormonal evaluation is usually done in patients with severe abnormality in their sperm count (< 5 million/ml), because
endocrinopathy is rare in patients with sperm count above 5 million/ml (Vaidya, 2006). Hormonal profile of the study population showed that the mean level of leptin was significantly increased in patients compared to controls. This result is in agreement with that declared by other authors (Steinman et al., 2001; Hofny et al., 2010 and Ma et al., 2010). Leptin levels showed a negative correlation with percentage of motile spermatozoa (Glander et al., 2002). Excess level of leptin was also shown to have a deleterious effect on sperms, Leydig cells and germ cells which expressed leptin receptors (Caprio et al., 1999; Isidori et al., 1999; El-Hefnawy et al., 2000 and Jope et al., 2003). Ishikawa et al. (2007) reported that spermatogenic dysfunction is associated with increased leptin and leptin receptor expression in the testis. Besides, it is known that reproductive function is affected by leptin through actions at the hypothalamic level (Tena-Sempere et al., 1999, 2001).

In contrast to leptin, the mean level of testosterone was significantly decreased in patients compared to controls. Such decline in testosterone levels may be explained on the basis of hypogonadism which is characterized by a deficiency or absence of Leydig cell function (Allan and McLachlan, 2003). Similar results were obtained by Kostakopoulos et al. (2002) and Ishikawa et al. (2007) who found that the average testosterone concentration in the Sertoli cell only group was significantly lower than normal groups.

The mean levels of LH and FSH obtained in the present study were significantly elevated in patients compared to controls. These results are in accordance with that documented in other studies (Weinbauer and Nieschlag et al., 1995; Yaman et al., 1999; Babu et al., 2004; Geidam et al., 2008 and Nieschlag et al., 2010). In the infertile men, higher concentration of FSH is considered to be a reliable indicator of germinal epithelial damage, and was shown to be associated with azoospermia and severe oligozoospermia (Bergmann et al., 1994). FSH acts directly on the seminiferous tubules whereas LH stimulates spermatogenesis indirectly via testosterone. Elevation of FSH and LH recorded in the present study may explained in the basis of negative feedback regulation exerted by the low level of testosterone.
5.8 Lipid profile of the study population
In the present results there were significant increases in the levels of total cholesterol, triglycerides and LDL-C in patients when compared to controls. In contrast, there was a significant decrease in HDL-C level in patients. Padrón et al., 1989 reported that high levels of cholesterol and/or triglyceride are associated with poor semen quality and higher FSH levels. Correlation studies showed that increased serum VLDL and total triglyceride values were significantly correlated with impaired seminal parameters and spermatogenesis (Ergün et al., 2007). High lipid levels may exert adverse direct effects at the testicular level. However, this point needs further investigation.

5.9 Leptin action
Serum leptin level was negatively correlated with serum testosterone. This finding was in agreement with other studies (Giagulli et al., 1994; Wabitsch et al., 1997; Luukkanen et al., 1998; Soderberg et al., 2001 and Hofny et al., 2010). The increase of serum leptin decreases testosterone synthesis in Leydig cells, thereby inhibiting sperm maturation (Margetic et al., 2002 and Giovambattista et al., 2003). In patients with Sertoli cell only, leptin receptor expression was increased to the greatest extent. Thus, over expression of leptin receptor in Leydig cells leads to inhibition of testosterone production in infertile men (Ishikawa et al., 2007).

As indicated in the present data, there was a significant positive correlation between leptin and LDL-C and BMI. Similar result was declared by Zabut et al., 2007 and by Hofny et al., 2010. In conclusion, leptin acts as a direct inhibitory signal for testicular steroidogenesis, which may be relevant to explain the link between decreased testosterone secretion and hyperleptinemia in obese men. This means that obesity may be a risk factor of male infertility. However this relation needs further research.
Chapter 6

Conclusions and recommendations

6.1 Conclusions

* The mean age of controls was 31.7±3.9 years old whereas that of patients was 32.8±6.4 years.

* The sexual history of the study population showed none of the controls had problems with erection and ejaculation compared to patients. Regarding to gonadal infection, controls had less gonadal infection than patients.

* The medical history revealed that none of the controls had orchitis, cryptorchidism, varicoceles and hormonal problems compared to patients who had such diseases. In addition, controls with trauma were lower compared to patients.

* Exposure history demonstrated that the number of controls exposed to thermal and chemical conditions were lower compared to patients.

* Medication history showed that controls who reported the treatment with the antifungal Ketoconazole and the steroid were lower compared to patients.

* The number of obese controls was less than obese patients.

* Serum leptin was significantly increased in patients compared to controls. In contrast, testosterone was significantly lower in patients. Serum LH and FSH were increased in patients compared to controls.

* Serum cholesterol, triglycerides and LDL-C were significantly higher in patients compared to controls. In contrast, HDL-C was significantly lower in patient than in controls.
* Serum leptin levels showed negative significant correlation with testosterone levels and positive significant correlations with LDL-C and BMI.

### 6.2 Recommendations

1. Research in male infertility needs a special care with psychological aspects of the patients and emphasizing that male infertility is a treatable disease in most cases.
2. Treatment of gonadal infection, orchitis, trauma, cryptorchidism, varicoceles and hormonal disturbances would be useful in treatment of male infertility.
3. Avoiding thermal and chemical exposure and avoiding treatment with Ketoconazole and Steroids are advisable.
4. Ideal weight is recommended to improve male fertility.
5. Leptin may be used as a biomarker for diagnosing germ cell aplasia.
6. Further research focusing on obesity impact on male infertility and on therapeutic strategies of leptin in male infertility are recommended.
7. Avoiding contaminated food with heavy metals, pesticides and others may help in preventing infertility.
Chapter 7

References

Alabama, U.C.O.N. Patient information; Male Infertility Questionnaire (http://wwwucnacom/maleinfertility_uconpdf).


Diagnostic System Laboratories, Inc. Texas, USA. Active Human Leptin ELISA, DSL-10-23100.


Annex 1

Palestinian National Authority
Ministry of Health
Helsinki Committee

Name: Ehab Jaber

I would like to inform you that the committee has discussed your application about:
Leptin status and some biochemical parameters in germ cell aplasia among infertile men in Gaza Strip.

In its meeting on December 2010 and decided the Following:
To approve the above mention research study.

[Signature]
Member

[Signature]
Member

[Signature]
Chairperson

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

السلطة الوطنية الفلسطينية
وزارة الصحة
لجنة هلسنكي

الاسم: إيهاب جابر

أني أعبدك علماً بأن اللجنة قد ناقشت مقترح دراستك حول:

Leptin status and some biochemical parameters in germ cell aplasia among infertile men in Gaza Strip.

و ذلك في جلستها المنعقدة شهر ديسمبر 2010

و قد قررت ما يلي:

- الموافقة على البحث المذكور عليها.

[Signature]
Member

[Signature]
Member

[Signature]
Chairperson

الشروط:
- صالح لمدة 2 سنوات من تاريخ الموافقة.
- من الضروري إبلاغ اللجنة في حالة حدوث أي تغيير في البروتوكول المقبول.
- نقدر إرسال نسخة من بحثك النهائي عند إتمامه.

الاريخ 2010/12/6

الاسم: إيهاب جابر

أني أعبدك علماً بأن اللجنة قد ناقشت مقترح دراستك حول:

Leptin status and some biochemical parameters in germ cell aplasia among infertile men in Gaza Strip.

و ذلك في جلستها المنعقدة شهر ديسمبر 2010

و قد قررت ما يلي:

- الموافقة على البحث المذكور عليها.

[Signature]
Member

[Signature]
Member

[Signature]
Chairperson

الشروط:
- صالح لمدة 2 سنوات من تاريخ الموافقة.
- من الضروري إبلاغ اللجنة في حالة حدوث أي تغيير في البروتوكول المقبول.
- نقدر إرسال نسخة من بحثك النهائي عند إتمامه.

الاريخ 2010/12/6
Annex 2

Leptin status and some biochemical parameters in germ cell aplasia among infertile men in Gaza Strip.

Faculty of Science
The Islamic University - Gaza
# Annex 3

## Germ cell aplasia questionnaire

<table>
<thead>
<tr>
<th>Personal data</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
</tr>
<tr>
<td><strong>Education</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>Occupation</strong></td>
</tr>
<tr>
<td><strong>Do you have any children?</strong></td>
</tr>
<tr>
<td><strong>Family history</strong></td>
</tr>
<tr>
<td><strong>Sexual History</strong></td>
</tr>
<tr>
<td>Do you have any difficulties with your erection?</td>
</tr>
<tr>
<td>Do you have any problems with ejaculations?</td>
</tr>
<tr>
<td>Have you had any infections of your penis, testicles, prostate?</td>
</tr>
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<td><strong>Medical History</strong></td>
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<td>Cryptorchidism</td>
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</tr>
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</tr>
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<tr>
<td>Thermal</td>
</tr>
<tr>
<td>Chemical</td>
</tr>
<tr>
<td>Radiation therapy</td>
</tr>
<tr>
<td>Chemotherapy</td>
</tr>
<tr>
<td><strong>Medications</strong></td>
</tr>
<tr>
<td>ketoconazole</td>
</tr>
<tr>
<td>spironolactone</td>
</tr>
<tr>
<td>cimetidine</td>
</tr>
<tr>
<td>Growth hormone/ Steroids</td>
</tr>
</tbody>
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