Clinical And Hormonal Study In Men With Infertility

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By

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بِسْمِ اللهِ الرَّحْمنِ الرَّحِيمِ

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Dedication

To memory of my father….
For his ideal fatherhood
To my kind mother…
For making impossible wishes come true
To my dearest brothers and sisters…
For their limitless support
To my sisters' kids Dania , Zainab & Mostafa

Ahmed
2009

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LIST OF ABBREVIATIONS

ABP	**Androgen binding protein**
ALH	**Amplitude of lateral head displacement**
AR	**Androgen receptor**
ART	**Assisted reproductive techniques**
ASA	**Antisperm antibodies**
BTB	**Blood-testis barrier**
°C	**Centigrade**
C.I.	**Colour index**
cAMP	**Cyclic adenosine monophosphate**
CASA	**Computer-assisted semen analysis**
CBAVD	**Congenital bilateral absence of vas deferens**
CFTR	**Cystic fibrosis transmembrane conductance regulator**
cGMP	**Cyclic guanosine monophosphate**
cm	**Centimeter**
DHT	**Dihydrotestosterone**
dl	**Deciliter**
DNA	**Deoxyribonucleic acid**
E	**Estradiol**
EDTA	**Ethylenediamine tetraacetic acid**
ESR	**Erythrocytes sedimentation rate**
Fig	**Figure**
FSH	**Follicle-stimulating hormone**
FSH-Rs	**Follicle-stimulating hormone receptors**
g	**Gram**
g/l	**Gram per liter**
G21	**Gauge 21**
GnRH	**Gonadotropin releasing hormone**
IBT	**Immunobead test**
ICSI	**Intracytoplasmic sperm injection**
IE	**Internationale Einheit (Dutch,means International unit)**
IgA	**Immunoglobulin A**
IgG	**Immunoglobulin G**
IgM	**Immunoglobulin M**

IL	**Interleukin**
IUI	**Intrauterine insemination**
IVF	***In vitro* fertilization**
LH	**Luteinizing hormone**
LIN	**Linearity**
MAR	**Mixed anti-globulin reaction**
MESA	**Microsurgical epididymal sperm aspiration**
mg	**Milligram**
mg/ml	**Milligram per milliliter**
min	**Minutes**
mIU	**Milli international unit**
ml	**Milliliter**
mm	**Millimeter**
mmol	**Millimole**
n	**Number**
N.B.	**Note**
ng	**Nanogram**
NL	**Normal**
nmol	**Nanomole**
NO	**Nitrous oxide**
No.	**Number**
NOA	**Non-obstructive azoospermia**
OAT	**Oligoasthenoteratozoospermia**
OR	**Odds ratio**
PCV	**Packed cell volume**
pmol	**Picomole**
PRL	**Prolactin**
RBCs	**Red blood cells**
RNA	**Ribonucleic acid**
SCI	**Spinal cord injury**
SCSA	**Sperm chromatin structure assays**
SD	**Standard Deviation**
SFA	**Seminal fluid analysis**
SHBG	**Sex-hormone binding globulin**
SPSS	**Statistical package for social sciences**
T	**Testosterone**
T_3	**Triiodothyronine**
T_4	**Thyroxin**
TGF	**Transforming growth factor beta-1**

TT	Total testosterone
WBCs	White blood cells
µl	Microliter
µm	Micrometer
µm/s	Micromiter per second
(+ve)	Positive
(-ve)	Negative

Summary

The aims of this case-control study were to identify the clinical risk factors for male infertility and to explore seminal fluid abnormalities in Iraqi men with infertility as well as in fertile control .Also to demonstrate the types of serum hormones (Follicle Stimulating Hormone, Luteinizing Hormone,Testosterone and Prolactin) variability in the study groups and their correlations with seminal parameters.Furtheremore, to investigate some hematological changes in patients and control.

Eighty one Iraqi men with infertility (patients) and thirty fertile men (control) fulfilled the selection criteria. A well structured questionnaire was designed for this study.Full ,detailed and relevant history and physical examination was performed for every patient and control.Seminal fluid analysis (SFA) was performed according to the latest published WHO method and guidelines.The patients were subdivided (by their seminal fluid analysis results) into azoospermic,oligoasthenozoospermic and oligozoospermic groups.Serum levels of the hormones (Testosterone, FSH,LH and Prolactin) were measured for patients and control using enzyme-linked immunosorbent assay (ELIZA) method.Packed cell volume (PCV), total WBCs count and erythrocytes sedimentation rate (ESR) were measured as well.Clinical risk factors,seminal fluid analysis parameters mean levels and serum hormones levels and haematological parameters were compared for patients and control.

Some clinical risk factors for male infertility appeared to be more prevalent in infertile men than in control.These include history of wife's abortion,decreased libido,low socio-economic status,low use of contraceptives,family history of infertility, chronic diseases, varicocele,scrotal surgery,testicular biopsy,smoking,excessive heat exposure and atrophied testes.Iraqi infertile men showed lower values for SFA parameters than did the control.Patients with azoospermia showed the most remarkable hormonal abnormalities especially in the levels of serum FSH and Testosterone. Patients groups also demonstrated multiple abnormalities in seminal fluid parameters. There was significant differences in the serum sex hormones levels between the patients and control groups and among the

infertile men groups.Besides, significant correlation between serum follicle stimulating hormone and testosterone and sperm concentration was observed.Significant differences were obtained between patients and control regarding the hematological parameters studied but the majority of values were within normal ranges.

Iraqi infertile men have certain clinical , seminal and hormonal abnormalities which are different from fertile control.Moreover, the differences are present between patients groups themselves and these abnormalities are multi-components.It is important that these abnormalities should be taken in account when the clinician is going to manage infertile men.Besides,all semen analyses should be performed according to the latest published WHO guidelines. Furthermore, measurements of serum hormones (FSH,LH and Testosterone) should be preserved for infertile men with azoospermia, moderate to sever oligozoospermia (sperm concentration <10 million/ml) or when there is clinical indication and should not be done routinely. Serum follicle stimulating hormone and testosterone are the best two hormones for initial hormonal evaluation of infertile men.Finally, further studies are needed to consolidate the evidence provided by this study.

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CHAPTER ONE

INTRODUCTION

Infertility is one of commonest disorders to afflict young men and women.It is defined as the inability to conceive after 12 months of regular unprotected intercourse (**Gracia, *et al.* , 2005**). It affects 10–15 percent of all couples (**Gracia, *et al.* , 2005**) . The causes of infertility can be divided into four major categories: 1: the female factor; 2: the male factor; 3: combined factors and 4: unexplained infertility (**Forti & Krausz ,1998**).

In roughly half of cases, a male factor is identified, while an occult male factor may be involved in 15–24 percent of cases in which no etiology is uncovered ("unexplained" infertility).Using this approach (medical history and physical examination with testicular volume assessment, semen analysis, and hormone measurement), a definite diagnosis of the cause of male infertility can be obtained in approximately 70% of cases **(Forti & Krausz ,1998).**

Understanding the main determinants of male fertility would allow us to advance our knowledge of male reproductive function and lifetime reproductive strategies, and also to design appropriate tests to evaluate males and semen samples collected from them. Sperm evaluation has moved away from subjective analysis to more objective means of assessing cell morphology, dimensions, functions, and the underlying mechanisms that ultimately lead to successful fertilization and generation of viable offspring The identification of sperm parameters important for fertility also requires advances on both the development of laboratory tests for sperm assessment and the definition of the best conditions for fertility evaluation**.**Sperm assessment methods can measure general traits such as concentration, motility, morphology, organelle integrity, and, more recently, DNA integrity. Few tests for semen evaluation have incorporated molecular analyses of sperm function and few have attempted to link this information with fertility**.** In the future it will be important to identify cellular and molecular markers that reveal various aspects of the potential of sperm to undergo functional changes **(Roldan , 2007).**

In the management of the infertile male, it is important to understand how the common endocrinopathies may cause testicular and sexual dysfunction. Normal function of the gonads is dependent upon an adequate production of the trophic hormones by the anterior pituitary gland. Thus, normal activity of the gonads can be interrupted by reduced gonadotropins secretion but may also be altered by abnormal gonadal responses to gonadotropins as well as by the interaction of other nongonadal endocrine abnormalities on the hypothalamic–pituitary–gonadal axis **(Jequier, 2000).**

<u>The aims of this study were:</u>

1. Assessment of the prevalence of clinical and laboratory etiological and risk factors for male infertility in men with infertility (attending Babylon Hospital Infertility Center) and controls in Hilla city.
2. Identification of seminal fluid analysis abnormalities in the study groups (patients & controls).
3. Demonstration of the patterns of serum hormones (FSH,LH, Testosterone and Prolactin) abnormalities in the study groups.
4. Measurement of the degree of correlation between serum hormones (FSH,LH, Testosterone and prolactin) and sperms count and motility in the study groups.
5. Estimation of the strength of correlation between age and serum hormones (FSH,LH, Testosterone and prolactin) in the study groups.
6. Quantification of PCV, Total WBCs count, and ESR abnormalities in the study groups.

CHAPTER TWO
LITERATURE REVIEW

2.1 Anatomy of male genital tract

The structures of the male reproductive system can be categorized on a functional basis as follows:

1. Primary sex organs: The primary sex organs are called gonads; specifically, the testes in the male. Gonads produce the gametes, or spermatozoa, and produce and secrete sex hormones**(Graff , 2001).**

2. Secondary sex organs: The three categories of secondary sex organs are the sperm-transporting ducts (that transport sperm include the epididymis, ductus deferentia (vas deferens), ejaculatory ducts, and urethra), the accessory glands (the seminal vesicles, the prostate, and the bulbourethral glands) and the copulatory organ (the penis, which contains erectile tissue).The scrotum is a pouch of skin that encloses and protects the testes**(Fig.2.1) (Graff , 2001).**

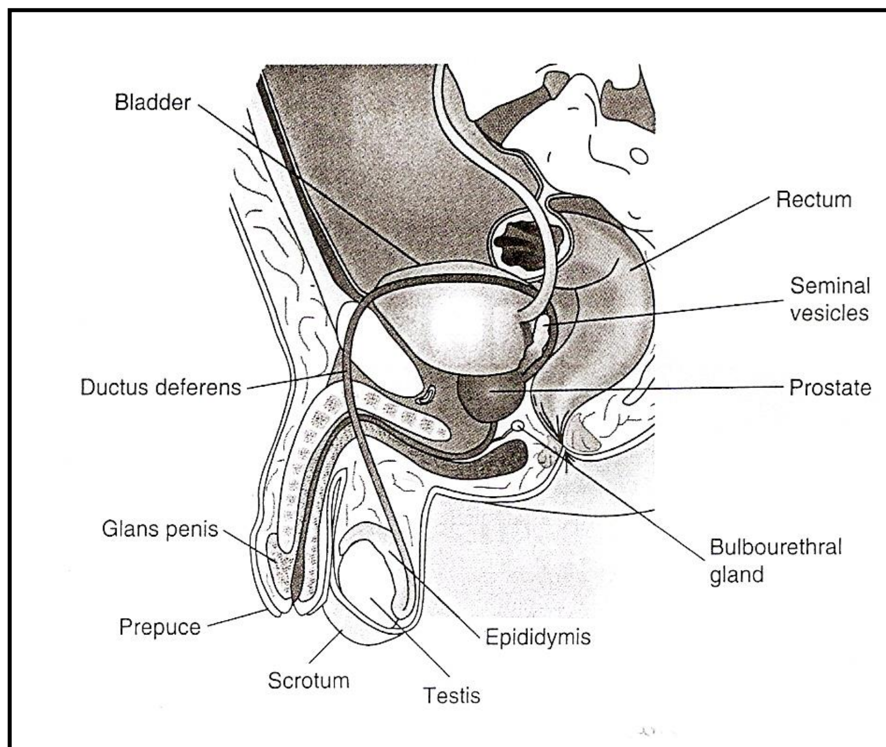
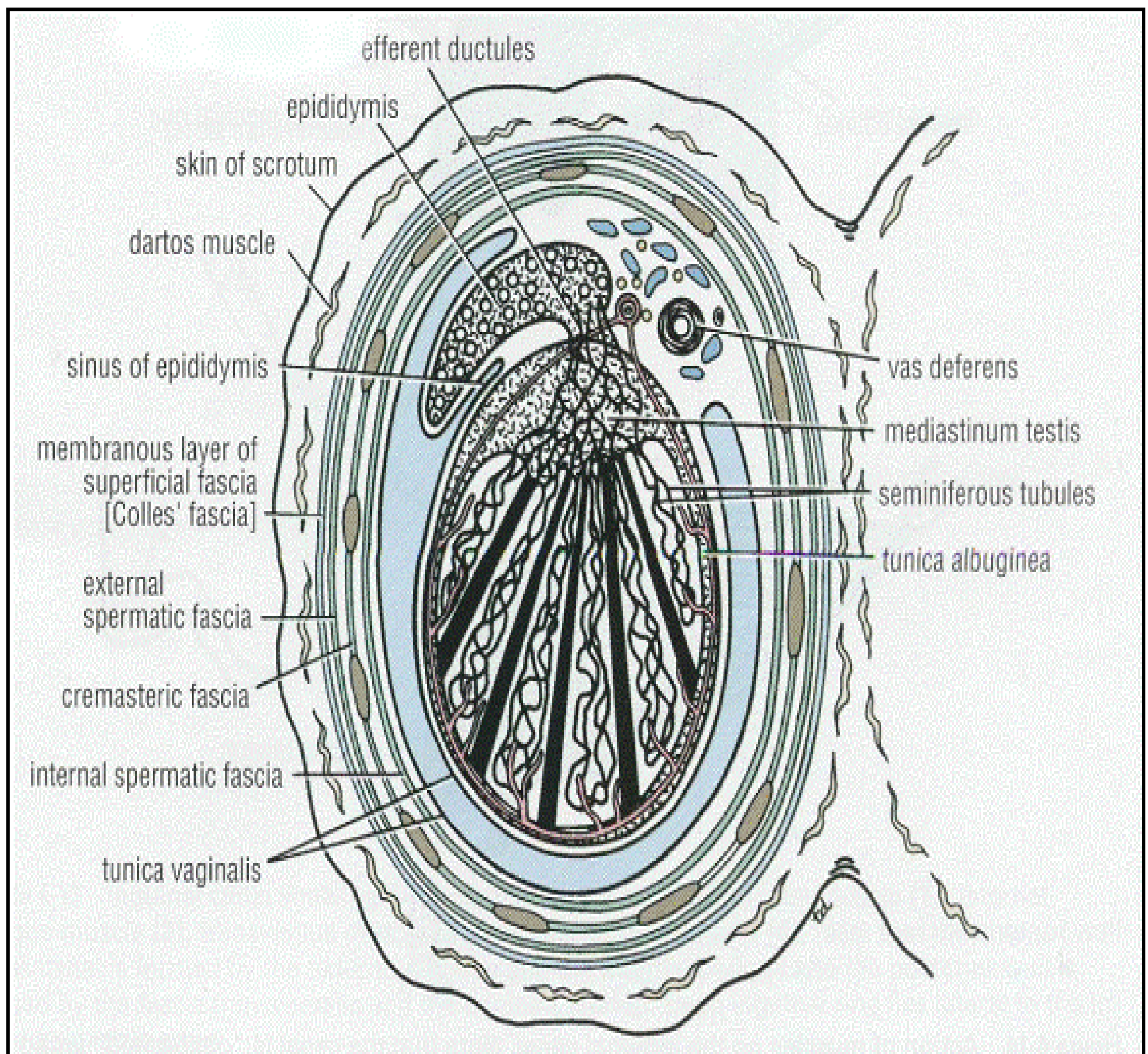


Fig.(2.1):The structure of male reproductive system(Porth, *et al.* , 2007).

2.1.1 Testes:

2.1.1.1 Anatomy of testes:

The human testes are two organs of the shape of rotation ellipsoids with diameters of 2.5 × 4 cm **(Middendorff , *et al.* , 2002)** . Testicular size depends on age and stage of sexual development. The testes measure approximately 5 x 3 x 2 cm in the postpuberal male. Each testis weighs between 10 and 14 g. The testes are covered by two layers. The nearest layer to the testis is tunica albuginea. This layer is then covered by the second layer tunica vaginalis which consists of visceral and parietal layers normally separated by a few milliliters of fluid **(Fig.2.2 and Fig.2.3)** (**Dogra ,*et al*, 2003**).



Fig(2.2): Anatomy of testis (Snell, 2000).

2.1.1.2 Histology of the testes:

Each testis is surrounded by a thick capsule of dense connective tissue, the tunica albuginea. The tunica albuginea is thickened in the posterior surface of the testis to form the mediastinum testis, from which fibrous septa penetrate the gland, dividing into about 250 pyramidal compartments called the testicular lobules **(Fig.2.2 and Fig.2.3).** Each lobule is occupied by one to four seminiferous tubules enmeshed in a web of loose connective tissue that is rich in blood and lymphatic vessels, nerves, and interstitial cells, also known as Leydig cells. **(Eroschenko ,2005;Junqueira & Carneiro ,2005)**.

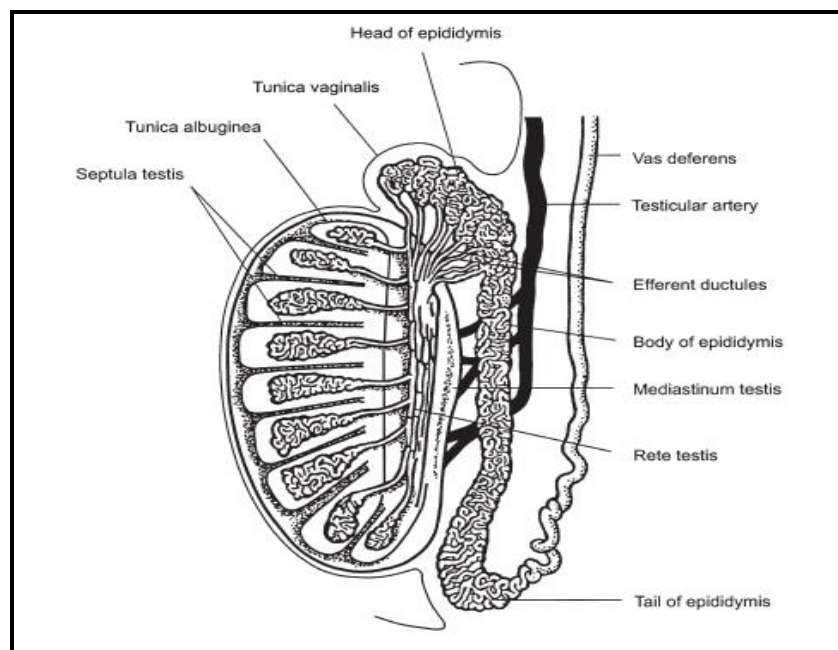


Fig. (2.3): Drawing showing the relationship of the tubules to the rete testis and the efferent ductules that lead out of the testis and form the epididymal duct. (Jequier, 2000).

Spermatozoids are produced in the seminiferous tubules at a daily rate of about 2×10^8 in the adult. Each testicle has 250—1000 seminiferous tubules that measure about 150-250 /μm in diameter and 30-70 cm in length. The tubules are convoluted and have the form of loops at whose ends the lumen narrows and continues in short segments, known as straight tubules, or tubuli recti **(Fig.2.2 and Fig.2.3).**These tubules connect the seminiferous tubules to the rete testis. About 10-20 ductuli efferentes connect the rete testis to the cephalic portion of the epididymis .The seminiferous tubules are lined with a complex stratified epithelium called germinal or seminiferous epithelium **(Fig.2.4)**.Interstitial

(Leydig) cells occupy much of the space between the seminiferous tubules .The seminiferous epithelium consists of two types of cells: Sertoli, or supporting, cells and cells that constitute the spermatogenic lineage **(Eroschenko ,2005;Junqueira & Carneiro ,2005)**.

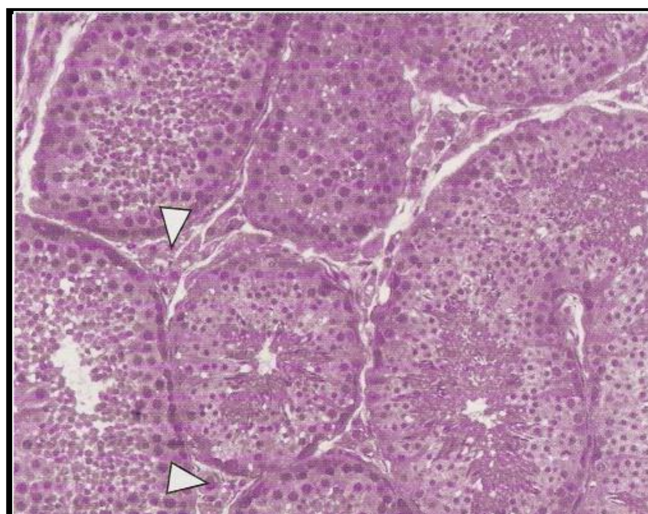


Fig.(2.4): Section of a testis showing seminiferous tubules and groups of pale-stained interstitial (Leydig) cells (arrowheads). Pararosaniline-toluidine blue (PT) stain. 40X magnification.(Junqueira & Carneiro ,2005)

2.1.2 Epididymis:

The epididymis consists of a markedly coiled duct that, at its lower pole (globus minor), is continuous with the vas deferens **(Fig.2.3)** . The epididymis lies posterolateral to the testis and is nearest to the testis at its upper pole **(Tanagho & McAninch , 2008).**

2.1.3 Spermatic cord:

The 2 spermatic cords extend from the internal inguinal rings through the inguinal canals to the testicles . Each cord contains the vas deferens, the internal and external spermatic arteries, the artery of the vas, the venous pampiniform plexus, lymph vessels, and nerves **(Baker , 1987;Wishahi ,1992 ; Tanagho and McAninch , 2008).**

2.2 Physiology of male reproductive organs :

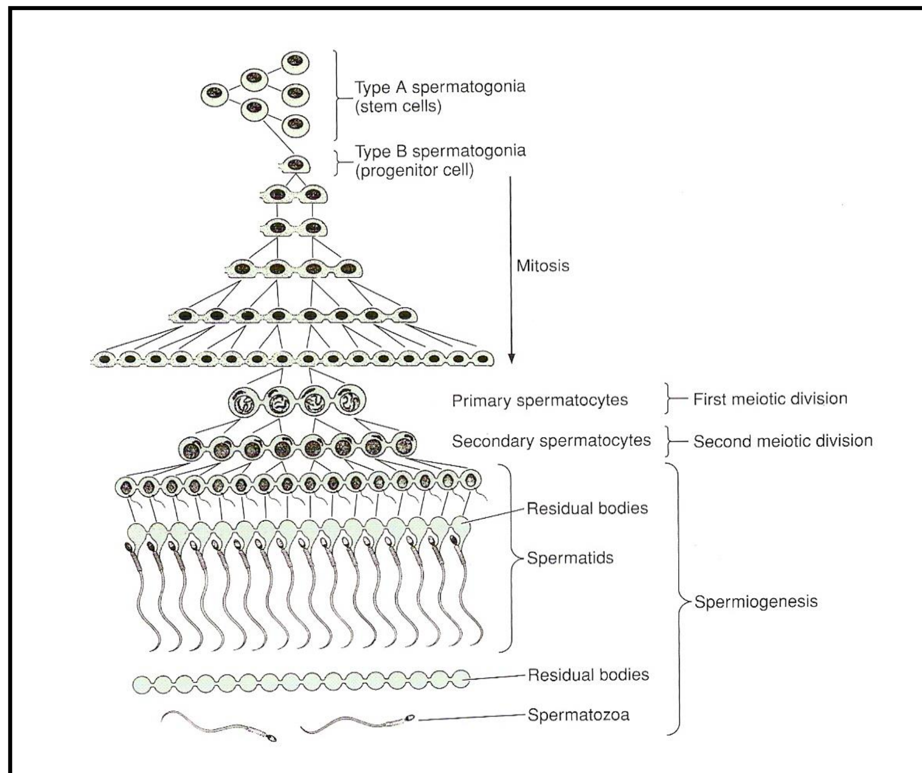
The dual function of the testes is to produce male sex androgens, mainly testosterone, and spermatozoa. The internal accessory organs produce the fluid constituents of semen, and the ductile system aids in the storage and transport of spermatozoa. The penis functions in urine elimination and sexual function **(Porth , *et al.*, 2007).**

2.2.1 Spermatogenesis:

2.2.1.1 Steps of spermatogenesis:

Spermatogenesis occurs in the seminiferous tubules. In the first stage of spermatogenesis, the spermatogonia migrate among *Sertoli cells* toward the central lumen of the seminiferous tubule . Spermatogonia that cross the barrier into the Sertoli cell layer become progressively modified and enlarged to form large *primary spermatocytes*.Each of these, in turn, undergoes meiotic division to form two *secondary spermatocytes*. After another few days, these too divide to form *spermatids* that are eventually modified to become *spermatozoa* (sperm)**(Fig.2.5**). During the change from the spermatocyte stage to the spermatid stage, the 46 chromosomes (23 pairs of chromosomes) of the spermatocyte are divided, so that 23 chromosomes go to one spermatid and the other 23 to the second spermatid **(Guyton & Hall,2006).**In humans, it takes an average of 74 days to form a mature sperm from a primitive germ cell by this orderly process of spermatogenesis (**Ganong, 2005).**

The diameter of the head of spermatozoon is 4–5 μm, the diameter of the flagellum is of 1–2 μm and the length of the spermatozoon measures 60 μm. The morphology of the human spermatozoon is depicted in **Fig.2.6** . Spermatozoa acquire their competence of motility during the transport throughout the epididymal ducts (**Ganong , 2005).**



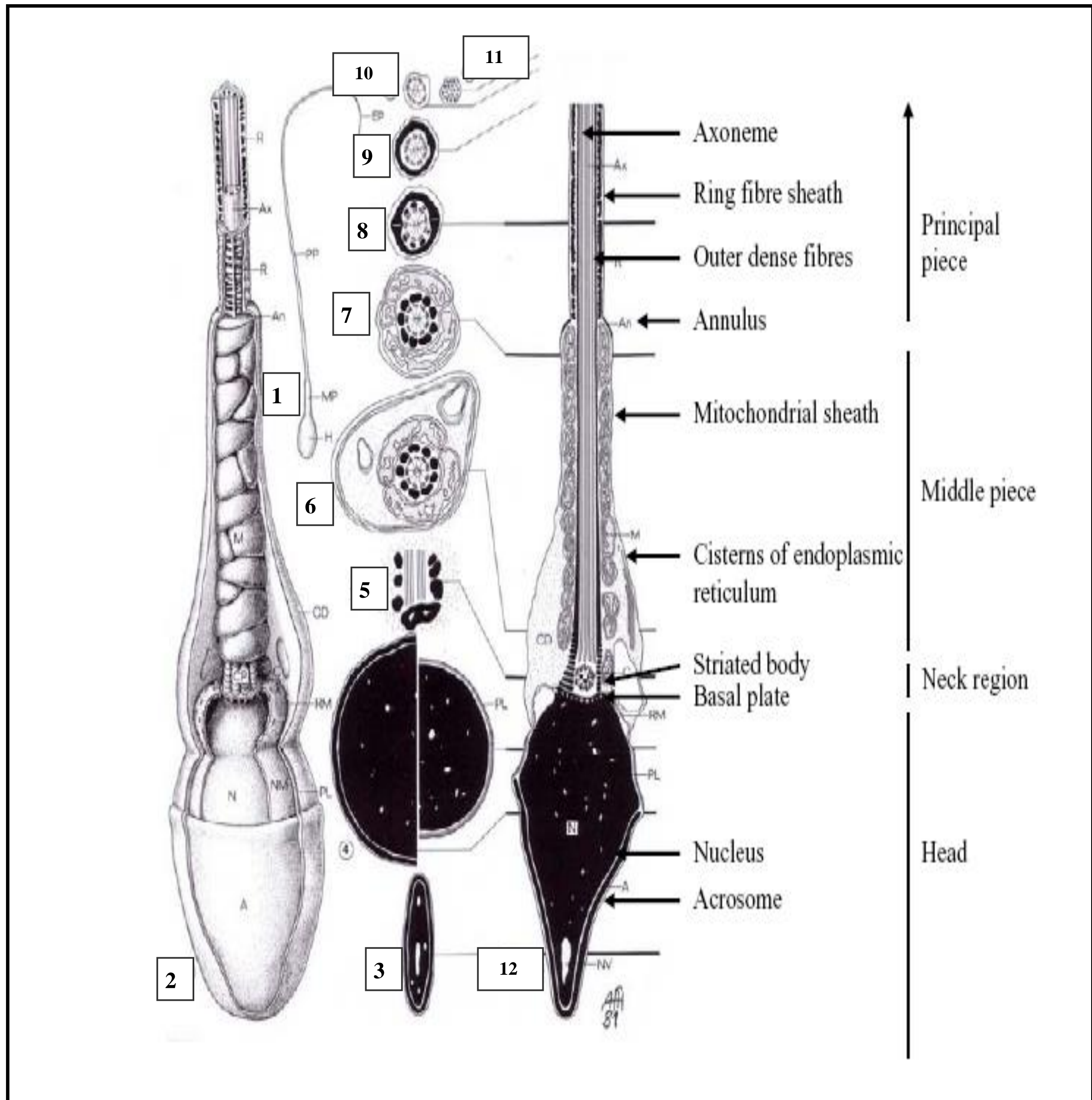
Fig(2.5):Stages of spermatogenesis (Junqueira & Carneiro ,2005).

2.2.1.2 Role of Sertoli cells in spermatogenesis:

Many functions are attributed to Sertoli cells :

1. Sustentacular and nutritive functions for the germ cells.
2. Organization of the delivery of mature spermatids into the tubular lumen (spermiation).
3. Production of endocrine and paracrine substances for the regulation of spermatogenesis.
4. Secretion of androgen binding protein (ABP) for the maintenance of epithelia of the excurrent duct system.
5. Interaction with the intertubular endocrine Leydig cells
6. Participation in Blood–Testis Barrier (**Holstein, *et al.*, 2003)** .

The Sertoli cells secrete androgen-binding protein (ABP) and inhibin. They do not synthesize androgens, but they contain aromatase (CYP19), the enzyme responsible for conversion of androgens to estrogens, and they can produce estrogens. ABP probably functions to maintain a high, stable supply of androgen in the tubular fluid. Inhibin inhibits Follicle Stimulating Hormone (FSH) secretion **(Ganong, 2005)**.



Fig(2.6): The human spermatozoon. (1) Light microscopical aspect, (2) Virtual preparation of the spermatozoon showing the acrosome, the nucleus and nuclear envelopes, the mitochondrial sheath of the main piece of the flagellum, (3–11) Cross sections of the human spermatozoon of different levels indicated in (12) longitudinal section of the human spermatozoon. Semi-schematic drawing on the basis of electron micrographs (Holstein ,*et al.*,2003)

2.2.1.3 Role of Leydig cell in spermatogenesis

Leydig cells produce and secrete among others androgens, the male sex hormone, the most well known of which is testosterone. Testosterone activates the hypophyseal-testicular axis, the masculinization of the brain and sexual behavior, the initiation, processing and maintenance of spermatogenesis, the differentiation of the male genital organs and secondary sex characteristics (**Holstein, *et al.*, 2003; Chen,*et al.*,2009).** Other studies pointed out elucidated that the Leydig cells possess neuroendocrine properties in addition to their endocrine functions. There is evidence that Leydig cells express serotonin, catecholamine synthesizing enzymes, different antigens characteristic for nerve cells as well as neurohormones and their receptors, neuropeptides, cell adhesion molecules, components of the NO/cGMP-system, components of the renin/angiotensin system, neurofilament proteins, synaptic and storage vesicle proteins, and numerous growth factors and their receptors (**Davidoff, *et al.* , 1993 ; Davidoff & Middendorff , 1996**). It was found that Transient hypothyroidism induced by propyl-2-thiouracyl blocks postpartum Leydig cell development**(Rijntjes , *et al.*, 2009).**

2.2.1.4 Regulation of spermatogenesis:

2.2.1.4.1 Intrinsic regulation of spermatogenesis:

The Leydig cells in the intertubular space secrete testosterone and additional neuroendocrine substances and growth factors.They are involved in maintenance of the trophic of Sertoli cells and the cells of peritubular tissue; they influence the contractility of myofibroblasts and in that way regulate the peristaltic movements of seminiferous tubules and the transport of spermatozoa. They also contribute to the regulation of blood flow in the intertubular microvasculature **(Middendorff , *et al.*, 1997).**

2.2.1.4.2 Extrinsic regulation of spermatogenesis:

The primary hormonal controls on spermatogenesis involve the action of FSH and testosterone on Sertoli cells **(Heckert & Griswold ,2002)** .The local regulation of spermatogenesis in the testis requires the well known extratesticular stimuli provided by the hypothalamus and pituitary gland.This is discussed in details in **section(2.2.6).**

2.2.2 Blood–Testis Barrier:

Tight junctions between adjacent Sertoli cells near the basal lamina form a blood–testis barrier that prevents many large molecules from passing from the interstitial tissue and the part of the tubule near the basal lamina (basal compartment) to the region near the tubular lumen (adluminal compartment) and the lumen(**Li,*et al.*, 2009; Lui,*et al.*,2009**). However, steroids penetrate this barrier with ease, and evidence suggests that some proteins pass from the Sertoli cells to the Leydig cells and vice versa in a paracrine fashion. In addition, maturing germ cells must pass through the barrier as they move to the lumen.

Maintenance of composition of intratubular composition presumably depends on the blood– testis barrier. The barrier also protects the germ cells from blood-borne noxious agents, prevents antigenic products of germ cell division and maturation from entering the circulation and generating an autoimmune response, and may help establish an osmotic gradient that facilitates movement of fluid into the tubular lumen. The mechanisms that regulate and coordinate the events of spermiation and blood–testis barrier (BTB) restructuring in the seminiferous epithelium are unknown **(Yan , *et al.*, 2008; Siu,*et al.*,2009).**

2.2.3 Functions of seminal vesicles:

In terms of volume, the seminal vesicles are the most important contributors to the seminal fluid as the secretions from these glands make up 60% of the total ejaculatory volume. Seminal vesical secretion is important for male fertility. It affects semen coagulation, sperm motility, stability of sperm chromatin and suppression of the immune activity in the female reproductive tract **(Wang & Zhang , 2007).**Each seminal vesicle is a tortuous, loculated tube lined with a secretory epithelium that secretes a mucoid material containing an abundance of fructose, citric acid, and other nutrient substances, as well as large quantities of prostaglandins and fibrinogen(**Said,*et al.*,2009**). Prostaglandins are believed to aid fertilization in two ways: (1) by reacting with the female cervical mucus to make it more receptive to sperm movement and (2) by possibly causing backward, reverse

peristaltic contractions in the uterus and fallopian tubes to move the ejaculated sperm toward the ovaries (a few sperm reach the upper ends of the fallopian tubes within 5 minutes)(**Guyton & Hall, 2006).**

2.2.4 functions of prostate:

The prostate gland is the second largest contributor to the ejaculate, for the prostatic contribution makes up around 10–30% of the total seminal plasma volume. Prostatic secretions contain large numbers of enzymes which are involved with semen clotting and liquefaction. The enzyme vesiculase induces clotting by its action on a substrate similar to fibrinogen and which is present in the seminal vesicular fluid **(Su,2009)**. Prostatic fluid also contains a number of proteases and peptidases and hyaluronidase, which are all enzymes that breakdown this clot. Thus, both clotting and liquefaction of the clot are induced by substances secreted by the prostate. The prostatic secretions also contain the bacteriostatic amine called spermine . The prostatic secretions also contain large amounts of the enzyme acid phosphatase ,citrates, zinc and magnesium**(Jequier ,2000)**.

2.2.5 semen:

The fluid that is ejaculated at the time of orgasm, the semen, contains sperms and the secretions of the seminal vesicles, prostate, Cowper's glands, and, probably, the urethral glands.The average pH of the combined semen is about 7.5 . The prostatic fluid gives the semen a milky appearance, and fluid from the seminal vesicles and mucous glands gives the semen a mucoid consistency(**Table (2.1)**)(**Guyton & Hall,2006).**

2.2.6 Endocrine regulation of male reproductive system

2.2.6.1 Hypothalamic-pituitary-gonadal axis:

The hypothalamus (the integrative center of the reproductive axis) receives neural input from many brain centers and is the *pulse generator* for the cyclical secretion of pituitary and gonadal hormones(**Veldhuis , 1997).** The hypothalamic-pituitary-gonadal axis consists of a closed-loop feedback control mechanism directed at maintaining normal reproductive function **(Fig.2.7).** The pulsatile secretion of Gonadotropins releasing hormone (GnRH) appears to be essential for the stimulatory effects on luteinizing hormone (LH) and follicle –stimulating hormone (FSH) release **(Patton & Battaglia ,2005).** The same GnRH peptide is

apparently responsible for the release of both gonadotropins, although evidence for a separate FSH releasing hypothalamic principle also exists (**Yu, *et al.*, 1997**). Luteinizing hormone stimulates in mature Leydig cells their steroidogenesis, hence being responsible for the supply of testosterone (T) for the maintenance of spermatogenesis and for extragonadal androgen effects. FSH in principle maintains the functional capacity of Sertoli cells in the support of spermatogenesis. (**Wang , 1999).**

FSH targets the epithelium of the seminiferous tubule and binds to membrane receptors on the Sertoli cells. This hormone acts by binding to specific receptors (FSH-Rs) confined to the gonads(**Foresta , *et al.*, 2004**) .The second messenger is the cAMP, and activation of adenylate cyclase stimulates cAMP-dependent protein kinase and RNA and protein synthesis, including synthesis of the androgen-binding protein and aromatase enzyme that converts testosterone to estradiol(**Fig.2.10).**

Table(2.1): Composition of Human Semen (Ganong, 2005).

Color: White, opalescent	
Specific gravity: 1.028	
pH: 7.35–7.50	
Sperm count: Average about 100 million/mL, with fewer than 20% abnormal forms	
Other components:	
Fructose (1.5–6.5 mg/mL)	From seminal vesicles (contributes 60% of total volume)
Phosphorylcholine	
Ergothioneine	
Ascorbic acid	
Flavins	
Prostaglandins	
Spermine	From prostate (contributes 20% of total volume)
Citric acid	
Cholesterol, phospholipids	
Fibrinolysin, fibrinogenase	
Zinc	
Acid phosphatase	
Phosphate	Buffers
Bicarbonate	
Hyaluronidase	

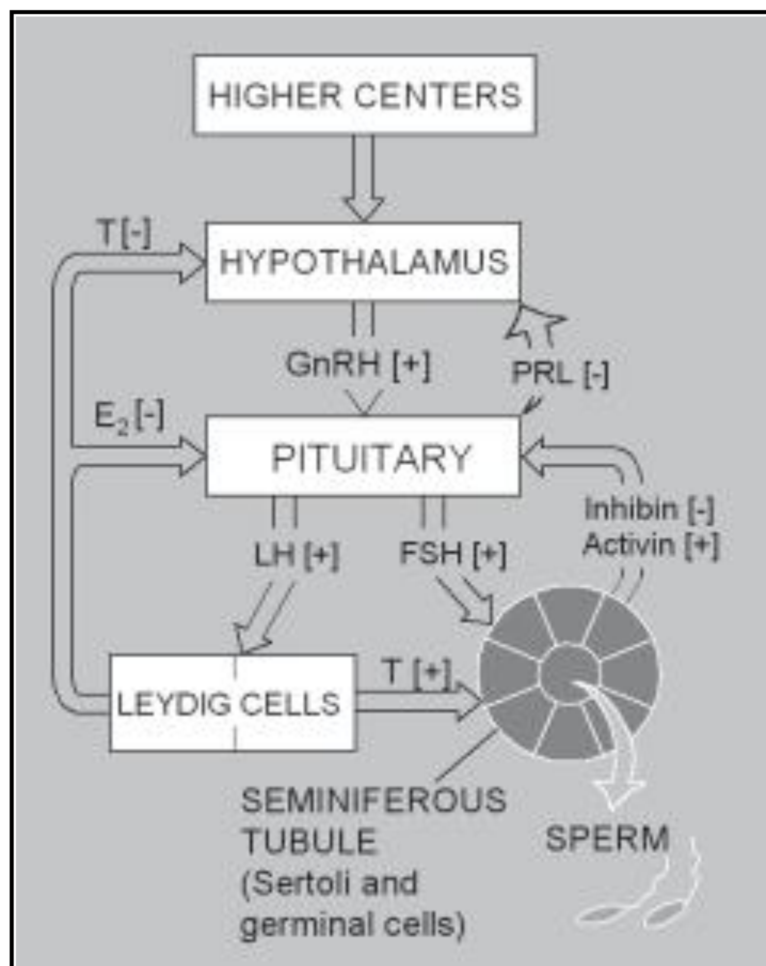
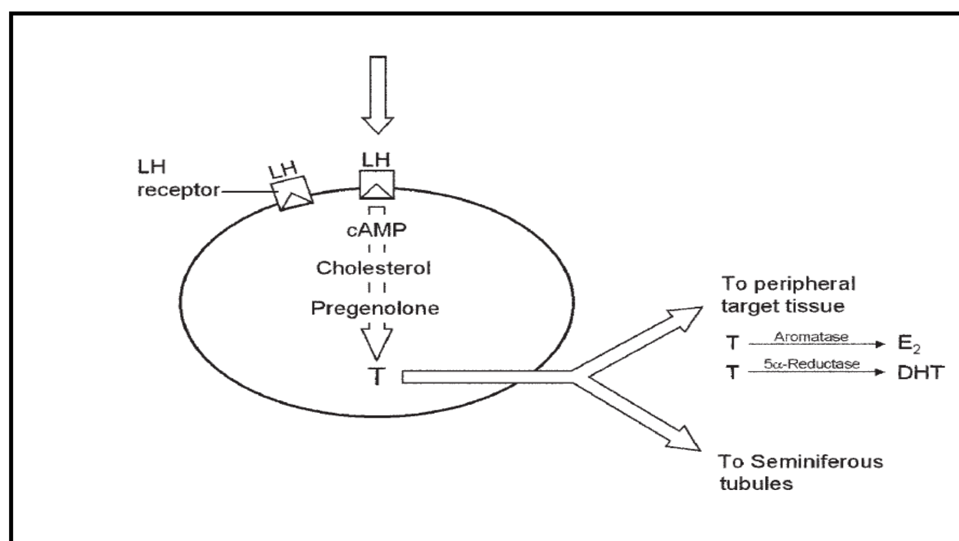


Fig.(2.7):Endocrine regulation of male reproductive system.GnRH:Gonadotropin Releasing Hormone;FSH:Follicle Stimulating Hormone ;LH:Luteinizing Hormone ;T:Testosterone; PRL:Prolactin; E:Estradiol; [-]:Negative feedback ; [+]:Positive feedback (Patton & Battaglia ,2005).

<u>Mechanisms of hormonal feedback controls:</u>

The major secretory product of the testes (testosterone hormone) is a primary inhibitor of LH secretion in men, other testes products, including estrogens and other androgens, also inhibit LH secretion(**Fig.2.7**).Testosterone is metabolized in peripheral tissues to the potent androgen dihydrotestosterone (DHT) or potent estrogen, estradiol **(Fig.2.8)** (**Swerdloff & Wang ,1998).** Here Testosterone, at least partly after conversion to estradiol, suppresses GnRH secretion at the hypothalamic level and gonadotropin synthesis in the pituitary gland(**Fig.2.9) (Wang,1999)** .The mechanism for the feedback control of FSH secretion is even more controversial than that of LH. After castration, FSH

increases the indication of a negative feedback from testicular products. Like LH, both testosterone and estradiol are capable of suppressing FSH serum levels, but the influence of these two gonadal steroids in physiological function is still undefined **(Veldhuis , 1997).**

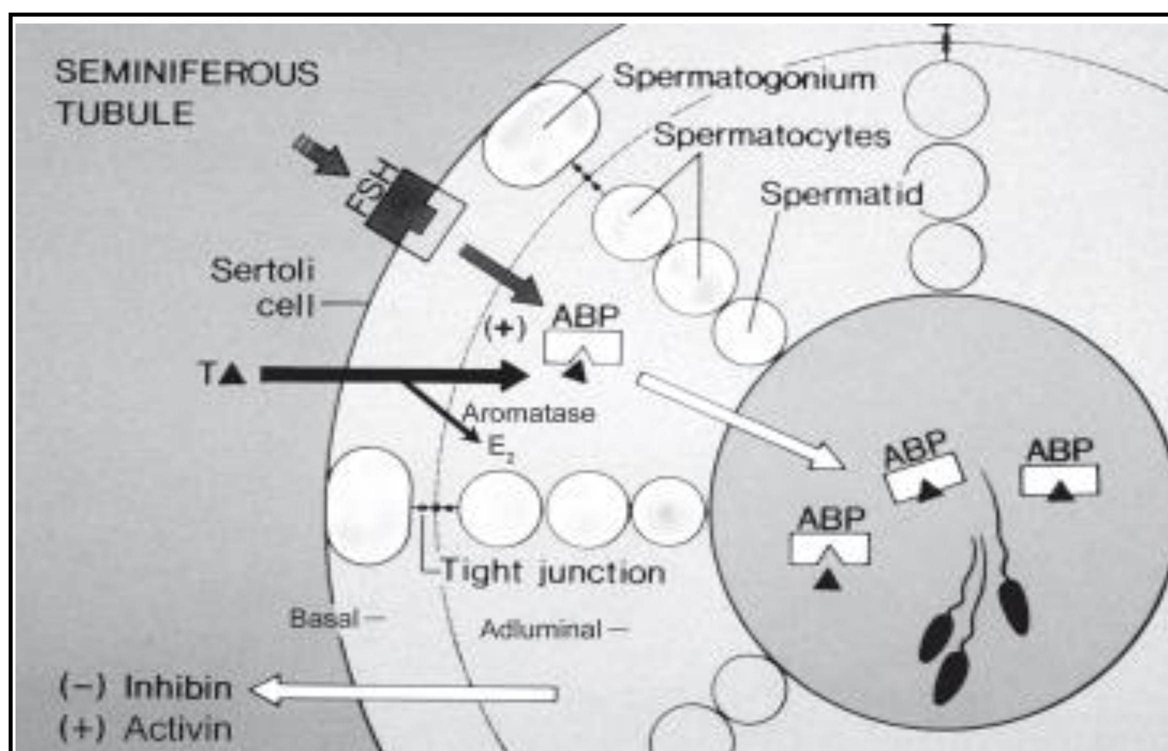


Fig(2.8): Testosterone production and metabolism. cAMP: cyclic adenosine monophosphate; LH:luteinizing hormone;T: testosterone; E2: estradiol; DHT: dihydrotestosterone (Patton & Battaglia ,2005).

A nonsteroidal tubular factor may also be significant in the feedback regulation of FSH. *Inhibin* has been isolated and is produced by the Sertoli cells of the testes. Two inhibin forms have been isolated—inhibin A (α and β A subunits) and inhibin B (α and β B subunits)—both of which have been shown to cause selective suppression of FSH release *in vitro*. The combination of the two β-subunits led to the formation of *activins*, which increase FSH secretion(**Okuma ,*et al.*,2005**). Reductions in spermatogenesis are accompanied by decreased production of inhibin, and this decline in negative feedback is associated with reciprocal elevation of FSH levels. Although testicular steroids also have effects on FSH, the specific regulation of this hormone takes place at the pituitary level through the two Sertoli cell proteins, activin and inhibin, the former a stimulator, the latter an inhibitor of FSH secretion **(Fig.2.7)**.

Considerable evidence indicates that testicular paracrine regulation involves the inflammatory cytokines, interleukin-1 (IL-1) and IL-6 , and activin, a member

of the transforming growth factor-β (TGFβ) family of cytokines with a broad range of immunoregulatory actions(**Okuma , *et al.*,2005; Guazzone,*et al.*,2009).**

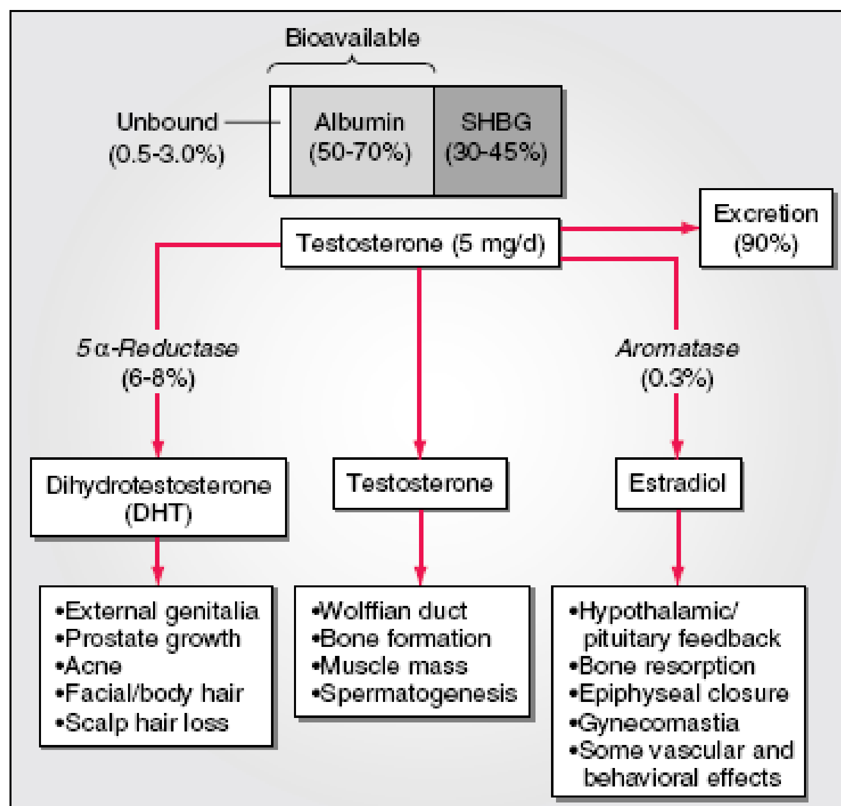


Fig(2.9): Schematic representation of the seminiferous tubule. Follicle-stimulating hormone (FSH) and testosterone (T) act on the Sertoli cells to produce androgen-binding protein (ABP), inhibin, activin, as well as estradiol (E2).(-): negative feedback;(+): positive feedback (Patton & Battaglia ,2005).

2.2.6.2 Androgens:

Androgens regulate gonadotropin secretion, initiation and maintenance of spermatogenesis, formation of male phenotype during sexual differentiation, promotion of sexual maturation at puberty, and controlling sexual drive and potency. There is a diurnal pattern: the peak level is in the early morning, and the nadir is in the evening **.** Testosterone circulates mainly bound to two plasma proteins: sex hormone–binding globulin (SHBG; also known as testosterone-binding globulin) and albumin. In young adult men, about 54% of testosterone is bound to albumin, 44% is bound to SHBG, and 2 to 3% is unbound, or free(**Goldman & Ausiello ,2006;Walker,2009**). The SHBG-testosterone fraction is tightly bound and serves a storage role. **(Fig.2.10)(Goldman & Ausiello ,2006).**Representative population-based cross-sectional studies of ethnically

comparable but geographically distinct cohorts have generally shown that total testosterone (TT) declines in adults with each year of age **(Ellison , *et al.*, 2002; Liu , *et al.*, 2006 ; Liu, *et al.*, 2007).**



Fig(2.10):Androgen metabolism and actions. SHBG: sex hormone–binding globulin (Kasper , *et al.*, 2005).

2.2.6.3 Prolactin and gonadotropins:

Prolactin is best known for the multiple effects it exerts on the mammary gland. However, it also exerts effects on other targets important to the reproduction of the mammalian species**(Freeman, *et al.*, 2000).**Hyperprolactinemia is linked with disturbed reproductive function reflected by a variety of symptoms and signs of hypogonadism. Luteinizing hormone levels are inappropriately low relative to low-serum testosterone levels, indicating that the hypothalamic- pituitary axis fails to respond to reduced testicular testosterone production. Prolactin may inhibit GnRH secretion either directly or through modulation of the dopaminergic pathways. Excessive prolactin may affect sexual functions by having a direct effect on the central nervous system and also from inhibition of androgen

secretion. In individuals with elevated prolactin, libido does not return to normal as long as the prolactin levels remain elevated despite use of androgen therapy**(Patton & Battaglia ,2005).**

2.3 Infertility:

Infertility is a problem of global proportions. The WHO estimates that 8–12% of couples around the world experience difficulty conceiving a child . The infertility rates vary between countries and regions**(Wiersema,*et al* .,2006)**. The consequences of infertility in these countries range from economic hardship, to social isolation, violence and denial of proper death rites. Many families depend on children for economic survival, especially in old age. In oriental cultures reproduction is one of the highest valued factors.A psychological crisis may occur when reproduction appears impossible. **(Wiersema,*et al* .,2006; Gurkan,*et al.*,2009)**. Approximately 20% of cases of infertility are caused entirely by a male factor, with an additional 30% to 40% of cases involving both male and female factors**(Table 2.2)**. Therefore, a male factor is present in one half of infertile couples(**Walsh , *et al.*, 2003**). About 15% of couples will have more than one cause for their subfertility **(Cahill & Wardle ,2002)**.

infertility is defined as the failure of a couple to conceive after 1 year of regular, unprotected intercourse **(Wallace, 1995; Buckett & Bentick, 1997;Snick , *et al.*, 1997; Philippov, *et al.*, 1998 ;Ebisch , *et al.*, 2007**). The chance of a normal couple conceiving is estimated to be 20% to 25% per month, 75% by 6 months, and 90% by 1 year **(Walsh , *et al.*, 2003).**Infertility is classified as primary infertility if no previous pregnancies have occurred, and secondary infertility if it occurred after one or more pregnancies.Approximately 10–17% of all couples experience primary or secondary subfertility at some time during their reproductive life **(Wallace, 1995; Buckett & Bentick, 1997;Snick , *et al.*, 1997; Philippov, *et al.*, 1998 ;Ebisch , *et al.*, 2007).**

2.4 Male infertility:

In the past, women were commonly considered to be the major cause of couples' infertility, because the influence of male fertility disturbances on a couple's fecundity was not adequately addressed. In the past several decades it became progressively more apparent that the male partner is an important

component of a couple's fertility potential.However,considerable controversy exists concerning the details of the male contribution to a couple's infertility and the mechanism by which it affects the couple's fertility and fecundity(**Ayala,*et al.*, 1996**). Evidence suggests that human semen quality may have been deteriorating in recent years. Measures of male infertility are needed if we want to monitor the biological capacity for males to reproduce over time or between different populations. We also need these measures in analytical epidemiology if we want to identify risk indicators, risk factors, or even causes of an impaired male fecundity—that is, the male component in the biological ability to reproduce (**Pasqualotto, 2003**).Male-related factors play a role in approximately half of cases of infertility (**Athayde & Cocuzza, 2007**).

2.4.1 Causes of male infertility:

2.4.1.1 Categories of the causes of male infertility

Similar to many other diseases, the aetiology of male infertility is commonly multifactorial .The causes underlying male infertility are numerous but are conveniently grouped by effects at one or more of the following levels: pretesticular, testicular, and posttesticular levels (**Tanagho & McAninch , 2008).**They also can be classified according to seminal fluid analysis (SFA) findings **(Table 2.3) (Walsh, *et al.*, 2006).**

Table (2.2) : The causes of subfertility and their approximate frequencies (Ebisch , *et al.*, 2007)

Type of subfertility	Cause	Frequency (%) *
Male factor subfertility	Sperm defects or dysfunction	30
Female factor subfertility	Ovulation failure (amenorrhoea or oligomenorrhoea)	25
	Tubal damage	20
	Endometriosis	5
	Cervical mucus defects or dysfunction	3
	Uterine abnormalities (such as fibroids or abnormalities of shape)	(<1)
Male & Female factors	Unexplained subfertility	25
	Coital failure or infrequency	5

*Total exceeds 100% as 15% of couples have more than one cause of subfertility

Table (2.3) :Classification of male infertility status by criteria of semen analysis (Walsh , *et al.* , 2003).

I. Low Ejaculate Volume
A. Medications.
B. Retroperitoneal or bladder neck surgery .
C. Ejaculatory duct obstruction.
D. Diabetes mellitus.
E. Spinal cord injury .
F. Psychologic disturbances.
G. Idiopathic .
H. Incomplete collection .
II. Azoospermia
A. Hypogonadotropic hypogonadism.
1. Kallmann's syndrome.
2. Pituitary tumor.
B. Spermatogenic abnormalities .
1. Chromosomal abnormalities.
2. Y chromosome microdeletions .
3. Gonadotoxins
4. Varicocele.
5.Viral orchitis.
6. Torsion .
7. Idiopathic .
C. Ductal obstruction .
1. Congenital bilateral absence of the vas deferens .
2. Vasal obstruction .
3. Epididymal obstruction .
4. Ejaculatory duct obstruction .
III. Oligasthenoteratospermia (OTA)
A. Varicocele .
B. Cryptorchidism
C. Idiopathic .
D. Drugs, heat, toxins.
E. Systemic infection .
F. Endocrinopathy .
IV. Normal but infertile
A. Gynecologic abnormality.
B. Abnormal coital habits .
C. Acrosomal defects .
D. Antisperm antibodies.
E. Unexplained .
V. Asthenospermia
A. Spermatozoal structural defects .
B. Prolonged abstinence .
C. Idiopathic .
D. Genital tract infection .
E. Antisperm antibodies.
F. Varicocele.
G. Partial obstruction .

2.4.1.2 Treatable and modifiable causes of male infertility:

2.4.1.2.1 Hypothalamic Disease:

1.Gonadotropin deficiency (Kallmann syndrome)
2.Isolated LH deficiency “fertile eunuch”
3.Isolated FSH deficiency
4.Congenital hypogonadotropic syndromes**(Tanagho & McAninch , 2008).**

2.4.1.2.2 Pituitary insufficiency:

Pituitary insufficiency may result from tumors, infarcts, surgery, radiation, or infiltrative and granulomatous processes. In sickle cell anemia, pituitary and testicular microinfarcts from sickling of red blood cells are suspected of causing infertility. Men with sickle cell anemia have decreased testosterone and variable LH and FSH levels **(Tanagho & McAninch , 2008).**

2.4.1.2.3 Hyperprolactinemia:

When the diagnosis of hyperprolactinemia is made, secondary causes such as stress , systemic diseases, and medications should be ruled out. When these causes excluded, the most common and important cause of hyperprolactinemia is a prolactin secreting pituitary adenoma. Elevated prolactin usually results in decreased FSH, LH, and testosterone levels and causes infertility. Associated symptoms include loss of libido, impotence, galactorrhea, and gynecomastia **(Tanagho & McAninch , 2008)**.**Table (2.4)** shows the causes of hyperprolactinemia.

Table (2.4):Causes of hyperprolactinaemia(Williams, *et al.*, 2003)

Causes
Physiological (pregnancy , stress , nipple stimulation , coitus).
Pituitary prolactinoma .
Any pituitary /hypothalamic tumour that compresses the pituitary stalk
Idiopathic hypersecretion .
Primary hypothyroidism .
Pharmacological causes (phenothiazines , butyrophenones, pimozide , cimetidine, methyldopa).
Renal/liver failure
Traumatic or neoplastic lesions of the thorax or spine
Ectopic production of prolactin by extrapituitary tumour .

2.4.1.2.4 Exogenous or endogenous hormones:

A. Estrogens and Androgens:

An excess of sex steroids, either estrogens or androgens, can cause male infertility due to an imbalance in the testosterone-estrogen ratio. Excess estrogens mediate infertility by decreasing pituitary gonadotropin secretion and inducing secondary testis failure**(Tanagho & McAninch , 2008; Sinkevicius,*et al.*,2009)**.An excess of androgens can suppress pituitary gonadotropin secretion and lead to secondary testis failure. The use of exogenous androgenic steroids (anabolic steroids) may result in temporary sterility due to this effect**(Tanagho & McAninch , 2008)**.

B. Glucocorticoids:

Exposure to excess glucocorticoids either endogenously or exogenously can result in decreased spermatogenesis. Elevated plasma cortisone levels depress LH secretion and induce secondary testis failure**(Tanagho & McAninch , 2008)**.

C. Hyper- and hypothyroidism:

Abnormally high or low levels of serum thyroid hormones (Thyroxine (T_4) and Triiodothyronine (T_3)) affect spermatogenesis at the level of both the pituitary and testis. Thyroid balance is important for normal hypothalamic hormone secretion and for normal sex hormone-binding protein levels that govern the testosterone-estrogen ratio**(Tanagho & McAninch , 2008)**. Hyperthyroidism has been found to cause oligozoospermia, asthenozoospermia, abnormal sperm morphology, or occasionally infertility in males**(Krassas, *et al.*, 2002)**. The effects of hypothyroidism on male reproduction appear to be more subtle than those of hyperthyroidism and reversible. Severe, prolonged hypothyroidism in childhood may be associated with permanent abnormalities in gonadal function**(Krassas,2003)**.The routine assessment of thyroid hormones and antibodies in infertile men is not recommended**(Trummer& Schwarzer, 2003)**.

2.4.1.2.5 Genetic factors:

Many recent studies had identified genetic defects in men with infertility **(Welaa, 2005;Burton , *et al.*, 2006; Sun, *et al.*, 2006; Welaa ,*et al.*, 2007;Martin , *et al,* 2008.; Okada , *et al.*, 2008; Smith , 2008 ; Kumtepe,*et al.*,2009)**.Genetic defects include chromosomal abnormalities, Y chromosome microdeletions, gene

mutations, etc. Klinefelter's syndrome and microdeletions in the long arm of Y chromosome (Yq) represent the most frequent molecular genetic cause of severe infertility**(Patton and Battaglia, 2006)**. Gene mutations involved in male infertility include the cystic fibrosis transmembrane conductance regulator (CFTR) gene, androgen receptor (AR) gene, insulin-like factor 3 (INSL3) gene and leucine-rich repeat-containing G-protein coupled receptor 8 (LGR8) gene.Chromosomal abnormalities presented with infertility include Klinefelter syndrome (47,XXY) and 46,XX male**(Patton and Battaglia, 2006)**.The X-linked genetic defects include Kallmann's syndrome and Androgen Insensitivity and others**(Schill, *et al.*, 2006)**. In addition to an increase in sex chromosome abnormalities there is also an increase in autosomal abnormalities in populations of men with non-obstructive azoospermia or severe oligozoospermia**(Schill, *et al.*, 2006)**.**Tempest , (2004)** suggested that the association between sex chromosome disomy and oligozoospermia may be due to reduced recombination in the XY pairing region and discussed the relevance of the correlations between sperm disomy and sperm motility and morphology.In one study ,it was stated that genome-wide gene expression analyses were used to identify genes involved in the pathogenesis of non-obstructive azoospermia (NOA), and ART3 was subsequently identified as a susceptibility gene for NOA. These findings clarify the molecular pathophysiology of NOA and suggest a novel therapeutic target in the treatment of NOA **(Okada , *et al.*, 2008)**. Physiological, cellular and molecular studies can be complemented by an evolutionary perspective to produce increased insights into the genes and mechanisms underlying the complex phenotype of fertility **(Smith , 2008)**.

2.4.1.2.6 Gonadotoxins(Drugs and environmental toxins):

Gonadotoxins can result in infertility by various mechanisms: Ketoconazole, spironolactone, and alcohol inhibit testosterone synthesis, whereas cimetidine is an androgen antagonist. Recreational drugs such as marijuana, heroin, and methadone are associated with lower testosterone levels. Certain pesticides, like dibromochloropropane, are likely to have estrogen-like activity.**(Tanagho & McAninch , 2008).Table 2.5** shows the mechanisms by which certain drug groups affect spermatogenesis**.**Many studies had shown increased risk of male infertility in those exposed to environmental toxins like cigarette smoking, lead, cadmium, pesticides, , air pollutant,organic solvents,flame retardants, Gulf-War toxicants and even recently cell phones **(Swan , *et al.*, 2003 ;Maconochie, *et al.*, 2004; Shiau ,**

***et al.*, 2004;Sokol , *et al.*, 2006 ; Meeker , *et al.*, 2008; Makker,*et al.*,2009; Paradisi,*et al.*,2009).**

Table (2.5) :Drugs with negative effects on male fertility (Schill, *et al.*, 2006)

I- Suppression of spermatogenesis
1. Cytostatic agents
2. Hormones and hormonally active drugs: androgens , antiandrogens , oestrogens , progestagens, glucocorticaids, anabolice, cimetidine, spironolactone, digoxin, ketoconazole, .
3. Psychotropic drugs : antiepileptics , antiemetics, analgesics, certain antibiotics and chemotherapeutics, antihelmintics such as niridazole, salazosulphapyridine.
II- Impairment of sperm function
1. Calcium channel blockers (sperm motility and sperm – egg binding).
2. Antiepileptics (sperm motility).
3. Sulphasalazine (sperm count and motility).
4. Antibiotics (sperm motility).
5. Amantadine and colchicines (Sperm- egg interaction).
6. Psychotropic drugs, alpha – and beta – blockers (sperm motility).
7. Inhibition of sperm transport .
8. Antihypertensive drugs.
9. Psychotropic drugs

2.4.1.2.7 <u>Systemic Disease:</u>

A. <u>Renal Failure:</u>

Reduced sexual activity and libido as well as diminished fertility have consistently been reported in the dialysis population. Azoospermia, severe oligozoospermia, and decreased sperm viability are common in patients with chronic renal failure. Especially when FSH is elevated, sperm deficiency is usually severe **(Schill, *et al.*, 2006).**

B. <u>Liver cirrhosis:</u>

Hepatic cirrhosis is associated with hypogonadism and signs of feminization irrespective of the direct toxic effect of ethanol upon the testes. Testicular atrophy, low testosterone levels, decreased libido, infertility, reduced secondary sex hair and gynecomastia are found in men with cirrhosis. Fifty percent of patients with cirrhosis present reduced spermatogenesis and peritubular fibrosis**(Karagiannis,2005; Tanagho & McAninch,2008).**

C. Orchitis and epididydimo-orchitis:

Infection and inflammation are relevant entities of male factor infertility. Bacterial infections are mostly the consequence of an ascending infection of the genito-urinary tract which can ultimately lead to epididymo-orchitis. Bacterial toxins and the innate immune responses directed against them may have a significant impact on male reproductive function **(Bhushan,*et al.*, 2009; Pelletier,*et al.*,2009)**. There is clinical and pathological evidence that chronic inflammatory conditions of the testes can disrupt spermatogenesis and irreversibly alter both sperm number and quality **(Schuppe & Meinhardt ,2008).**

2.4.1.2.8 Torsion:

Testicular ischaemia-reperfusion injury is commonly seen in childhood. Infertility occurs in 25% of patients after unilateral testicular ischaemia**(Inan, 2008)**. The primary pathophysiologic event in testicular torsion is ischemia followed by reperfusion.Testicular torsion and detorsion causes morphological and biochemical changes by both ischemia and reperfusion of the tissues**(Dokmeci,2006)**.It is unclear whether these abnormalities are due to an autoimmune process that occurs after the rupture of the hematotesticular barrier leading to formation of antisperm antibodies or as a result of reperfusion-induced injury to the testis**(Arap , *et al.*, 2007).**

2.4.1.2.9 Trauma:

Most of testicular traumas are blunt traumas caused by a direct blow on the scrotum. The testicle is projected against the pubic arch. Early surgical investigation has considerably improved the prognosis of testicular trauma, and reduced orchidectomy rate**(Culty,2006)**. Because of the peculiar immunologic status of the testis in the body (i.e, it is an immunologically privileged site), trauma to the testis can invoke an abnormal immune response in addition to atrophy resulting from injury. Both may contribute to infertility**(Tanagho & McAninch , 2008)**.

2.4.1.2.10 Cryptorchidism:

Undescended testis or cryptorchidism is the most common genitourinary disorder in boys. The true undescended testis has stopped along the pathway of

normal descent. The ectopic testis is in an aberrant location, such as the base of the penis, the superficial inguinal pouch, the medial aspect of the upper thigh, or the contralateral scrotal sac .Cryptorchidism must be differentiated from the retractile testis, which is not in the scrotum but can be manipulated without tension into the scrotum. Retractile testes normally descend by puberty**(Callaghan , 2000).** Major regulators of testicular descent are the hormones insulin-like factor 3 (INSL3) and testosterone, and disruption of these pathways might cause cryptorchidism(**Ferlin ,*et al.*,2008).** Maldescended testes is commonly mentioned as an important cause for defective spermatogenesis **(Ferlin ,*et al.*,2008;Prasad , 2008;Bonney,*et al.*, 2009).** There has been a long-standing debate over whether the increased incidence of malignancies in retained testes can be alleviated by bringing it into the scrotum. This has recently been shown to be the case**(Ritzén , 2008).**

2.4.1.2.11 <u>Varicocele:</u>

varicocele is a condition of varicosity and tortuosity of the pampiniform plexus that is often associated with a reduction in the volume of the affected testicle. The rate of clinical varicocele ranges from 9% to 23%, but this increases to 40% in infertile patients **(Zucchi & Mearini , 2006).** There is indisputable evidence that the varicocele affects semen quality(**Acar,et al., 2009; Zorba,*et al.*,2009**). In fact, a classic semen analysis pattern has been attributed to varicoceles in which low sperm count and motility is found in conjunction with abnormal sperm morphology. It has not yet clearly been established if treatment is truly useful to restore testicular function. After treatment of varicocele, a sperm count must be performed approximately every three months for one year or until pregnancy is achieved(**Wagner & Tostain ,2008).**

2.4.1.2.12 <u>Free radicals and male infertility:</u>

Oxidative stress and oxidative damage were increased significantly in spermatozoa with declined motility, and the antioxidant capacities in the spermatozoa and seminal plasma were lower in males who had infertility or subfertility**(Oborna,*et al.*,2009)** .Human spermatozoa are redox active cells that are capable of generating superoxide and hydrogen peroxide. This activity is of fundamental biological importance in regulating the signal transduction pathways that control sperm capacitation. However, excess exposure to reactive oxygen species can lead to pathological damage to human spermatozoa curtailing their

competence for fertilization and disrupting their genetic integrity**(Sanocka & Kurpisz ,2004 ; Baker & Aitken,2005; Basar, *et al.*, 2006).**

2.4.1.2.13 Genital Tract Obstruction:

Obstructive azoospermia occurs in 15%–20% of men with azoospermia. Common causes of obstructive azoospermia are summarized in **table (2.6) (Schill, *et al.*, 2006**).Because the majority of seminal fluid is contributed by the seminal vesicles, in the absence of retrograde ejaculation, a low-volume ejaculate suggests the lack of seminal vesicle contribution. Partial or complete ejaculatory duct obstruction and vasal agenesis will also cause a low-volume ejaculate.Genital tract obstruction also can be acquired and both congenital and acquired can lead to infertility **(Walsh , *et al.*, 2003).**

Table (2.6) : Classification of obstructive azoospermia (Schill, *et al.*, 2006).

1.Epididymal obstruction	
Congenital forms	Idiopathic epididymal obstruction
Acquired forms	Post-infective (epididymitis)
	Post-surgical (epididymal cyst)
2.Vas deferens obstruction	
Congenital forms	Congenital absence of the vas deferens
Aquired forms	Post-vasectomy
	Post-surgical (hernia, scrotal surgery)
3.Ejaculatory duct obstruction	
Congenital forms	Prostatic cysts (Müllerian cysts)
Acquired forms	Post-surgical (bladder neck surgery)
	Post-infective

2.4.1.2.14 Disorders of sperm function or motility:

A. Immunologic infertility:

Protection against autoimmunity is provided by the blood-testis barrier. Several other immunoregulatory mechanisms also play a significant role in the prevention of anti-sperm immunity, such as immunosuppressive factors of seminal plasma, as well as both systemic nonspecific and specific factors (immunoregulatory cells, cytokines, absence of co-stimulatory molecules

expression etc.) . In some cases, auto-immunization with sequestered sperm molecules happened after disruption of blood-testis barrier by disease and/or injuries. Generally, humoral immune response such as anti-sperm antibodies (ASA) formation can be induced primarily during infectious and noninfectious inflammations, or by obstruction of testicular efferent duct. The ASA was also induced after accidental and/or surgical injury of testicles, exposure to very low temperature or cryptorchidism **(Bubanovic & Najman ,2004)** . Several reports have indicated that immunological causes may be present in 3%–36% of infertile couples . Although ASA were considered to cause a relative reduction in fertility, but not to absolutely prevent conception, it is now clear that the presence of ASA on the surface of spermatozoa can impair the sperm fertilizing capacity at different levels, including diminished sperm vitality, motility and mucous penetration, and impaired sperm-egg interaction **(Schill, *et al.*, 2006; Ulcova-Gallova,*et al.*,2009).** Sperm antibodies in semen belong almost exclusively to two immunoglobulin classes: IgA and IgG. Immunoglobulin M (IgM) antibodies, because of their large molecular size, are rarely found in semen. The screening test for antibodies is performed on the fresh semen sample and makes use of either the immunobead test (IBT) or the mixed antiglobulin reaction (MAR) test (**WHO, 1999 ; NAFA & Eshre-Siga , 2002**).

B. Infection

Bacterial and viral infection of male genital tract may be an important etiological factor for male infertility.Infectious process may lead to deterioration of spermatogenesis , impairment of sperm function and/or obstruction of the male genital tract(**Keck , 1998).** The agents most commonly responsible for male genital tract infections are listed in **Table 2.7.**In a study in Sfax, Tunisia (2007), genital mycoplasmas and ureaplasmas seem to be widespread among the male partners of infertile couples(**Gdoura , *et al.*, 2007**). A correlation exists between leukocytes in semen and the generation of superoxide anions, hydrogen peroxide, and hydroxyl radicals (reactive oxygen species), all of which can damage sperm membrane(**Dejucq & Je´Gou ,2001**).

Table (2.7) :Most Common Organisms in Male Genital Infection. (Tanagho & McAninch ,2008).

Neisseria gonorrhoeae	Cytomegalovirus
Chlamydia trachomatis	Herpes simplex II
Trichomonas vaginalis	Human papilloma virus.
Ureaplasma coli (other gram-negative bacilli).	Hepatits B virus
Mycoplasma hominis	Human immunodeficiency virus.

2.4.1.2.15 Disorders of Coitus:

Sexual dysfunction stemming from low libido or impotence is a frequent cause of infertility**(Tanagho& McAninch ,2008)**.

2.4.1.2.16 Hypospadias:

Hypospadias is a relatively common genital anomaly with a prevalence of 0.5% . It may be classified into glandular, penile, and scrotal/perineal types on the basis of the anatomical location of the urethral meatus **(Muroya , *et al.*, 2001).**

2.4.1.2.17 Timing And Frequency:

An appropriate frequency of intercourse is every 2 days, performed within the periovulatory period, the window of time surrounding ovulation when egg fertilization is possible.Inappropriate timing and frequency may lead to infertility problem**(Tanagho& McAninch ,2008)**.

2.4.1.2.18 Retrograde ejaculation:

Retrograde ejaculation is an uncommon cause of infertility and can be defined as the escape of seminal fluid from the posterior urethra into the bladder**(Malossini , *et al.*, 1999).**

2.4.2 Evaluation of male infertility:

2.4.2.1 General considerations:

The goals of the evaluation of the infertile male are multiple. First, to identify reversible conditions.Second, to search for irreversible causes that may be managed by assisted reproductive techniques (ARTs) using the male partner's sperm.Third, to explore irreversible conditions that may not be managed by these techniques and in which the couple should be advised to pursue adoption.Fouth, to

investigate significant underlying medical pathology.Finally, to find out any genetic and/or chromosomal abnormalities that may affect either the patient or his offspring **(Walsh , *et al.*, 2003; Sokol,*et al.*,2009).**

2.4.2.2 History:

Infertility is a disorder of a couple, not of an individual. For this reason, both partners must be involved in the investigation of infertility as factors that may contribute to their problem of childlessness can interact in many often fairly subtle ways**(Jequier , 2000).** The cornerstone of the male partner evaluation is the history. A comprehensive list of information relevant to the infertility history is given in **table 2.8 (Tanagho & McAninch ,2008**)

2.4.2.3 Examination:

Scarce facial, pubic, and auxiliary hair are common signs of hypogonadism and may be seen in Klinefelter syndrome. Visual field changes or new onset of blurred vision may indicate intracranial and pituitary tumors. Gynecomastia is common in adolescents and young obese males, but gynecomastia suddenly developing may indicate hormonally active testicular tumor (**Patton & Battaglia ,2005).** During an examination, testicular size and consistency are noted, and volume is assessed . Any suspicious mass in the testis should be further evaluated with a scrotal ultrasound because testicular cancer can present in men with infertility**(Simon , *et al.*,2001).** The presence, location, and size of the epididymis, as well as any tenderness, are also recorded. Dilated veins of pampiniform plexus (varicocele) are seen much more commonly in patients evaluated for infertility than in the general population, and are considered to be the leading cause of male infertility. The vas deferens should be palpable on both sides. Pubic hair distribution, penile length, and the localization and configuration of the meatus should be assessed also**(Patton & Battaglia ,2005 ;Quaas & Dokras , 2008).**

Table (2.8): Components of the male Infertility History (Tanagho & McAninch ,2008).

Medical history
Fevers
Systemic illness(diabetes, cancer, infection,etc.)
Genetic diseases (cystic fibrosis , Klinefelter syndrome, etc.)
Surgical history
Orchidopexy , cryptorchidism .
Herniorraphy
Trauma, torsion
Pelvic , bladder , or retroperitoneal surgery .
Transurethral resection for prostatism
Pubertal onset .
Fertility history
Previous pregnancies (present and with other partners).
Duration infertility treatments
Female evaluation
Sexual history
Erections
Timing and frequency
Lubricants
Family history
Cryptorchidism
Midline defects (Kartagener syndrome).
Hypospadias
Exposure to diethylstilbestrol and other chemicals.
Other rare syndromes (prune belly , etc.)
Medication history
Nitrofurantion
Cimetidine
Sulfasalazine
Spironolactone
Alpha blockers & others
Social history
Ethanol
Smoking/tobacco
Cocaine
Anabolic steroids
Occupational history
Exposure to ionizing radiation
Chronic heat exposure (saunas)
Aniline days
Pesticides
Heavy metals (lead)

2.4.2.4 <u>Laboratory investigations:</u>

2.4.2.4.1 <u>Semen analysis:</u>

I- <u>Manual semen analysis:</u>

The semen analysis remains the cornerstone of the laboratory evaluation of the infertile man. Despite this, it is important to realize that the measurement of semen parameters does not necessarily constitute a measure of fertility**(Walsh , *et al.*, 2003)** . Except in cases of azoospermia, the semen analysis does not allow for the definitive separation of patients into sterile and fertile groups(**Walsh , *et al.*, 2003 , Björndahl & Haugen , 2008**). As semen parameters decrease in quality, the statistical chance of conception decreases but does not reach zero.Minimum requirements for semen analysis and semen parameter standards were established in 1951 by the American Fertility Association, in 1966 by Freund, and in 1971 by Eliasson.This was followed by publications from the World Health Organization (WHO) of the normal range for SFA in 1980,1987,1992 and 1999**(WHO, 1980; WHO, 1987; WHO, 1992; WHO, 1999 ; Menkveld, 2001**). The latest recommended reference values of SFA by WHO are those that were provided in 1999 manual **(Table 2.9)**. The method for SFA describedby 1999 WHO manual (*WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction*) is the most reliable and accepted method by large numbers of researchers and scientific centers **(Williams, *et al.*, 2003 ; Quaas & Dokras ,2008).**

Many studies tried to establish reference range for SFA parameters for fertile populations in their countries**(Van Der Merwe , *et al.*, 2005 ; Haugen , *et al.*, 2006;Pasqualotto, *et al.*, 2006; Athayde & Cocuzza ,2007).**

II- <u>Computer assisted semen analysis (CASA):</u>

Computer-assisted semen analysis (CASA) refers to a semiautomated technique used to individualize and digitalize static and dynamic sperm images using computer-assisted image analysis.Some studies have suggested that computer-assisted semen analyzer (CASA) estimates of concentration and movement characteristics of progressively motile spermatozoa are significantly related to the fertilization rate in vitro and the time to conception .Sperm motion characteristics, specifically amplitude of lateral head displacement (ALH) and

linearity (LIN), have been related to sperm fertilizing capacity **(Bedaiwy , *et al.*, 2003).** Although the technology is promising, when manual semen analyses are

Table (2.9): Reference values of seminal fluid analysis (WHO , 1999)

Volume	2.0 ml or more
PH	7.2 or more
Sperm concentration	20×10^6 spermatozoa / ml or more
Total sperm number	40×10^6 spermatozoa per ejaculate or more
Motility	50% or more motile (grade a+b) or 25 % or more with progressive motility (grade a) within 60 minutes of ejaculation
Morphology*	
Vitality	50% or more live (i.e., excluding dye)
White blood cells	Fewer than 1×10^6 /ml
Immunobead test	Fewer than 50% motile spermatozoa with bead bound
Mixed antiglobulin reaction (MAR) test	Fewer than 50% motile spermatozoa with adherent particles

***Data from assisted reproductive technology programs suggest 15% or more should be in normal forms(WHO,1999).**

compared to CASA on identical specimens, CASA can overestimate sperm counts by 30% with high levels of contaminating cells such as immature sperm or leukocytes**(Tanagho & McAninch ,2008).**

III- Patterns of seminal fluid analysis (SFA) results:

After the history, physical examination, and initial laboratory studies, a differential diagnosis should be developed. In addition, more specific testing results in a narrowing of the differential diagnosis and allows the physician to place the patient in a causative category**(Jarow, 2007):**

1. **Absent or low-volume ejaculate:**when there is no ejaculation at all or ejaculated semen volume below 2 ml**(WHO, 1999).**
2. **Azoospermia :** when sperm concentration equal zero million/ml after centrifugation**(WHO, 1999).**

3. **Oligozoospermia :** when sperm concentration above zero and below 20 million/ml **(WHO, 1999).**
4. **Asthenozoospermia :** when sperms motility (grade a+b) < 50 % or (grade a) < 25% after 1 hour of ejaculation**(WHO, 1999).**
5. **Teratozoospermia :** when the percentage of sperms with normal morphology below 15 %.
6. **Multiple seminal defects (WHO, 1999).**
7. **Normal semen parameters (WHO, 1999).**

2.4.2.4.2 Hormonal evaluation:

There is no agreement as to what should constitute the initial endocrine evaluation of infertile men. Some clinicians recommend that all men with an indication in the history or physical examination or a sperm density less than 10 million/ml should have measurement of serum FSH and testosterone levels , because endocrine abnormalities are rarely present when the sperm concentration is greater than 10 million/ml . However, some experts believe that all infertile men should undergo endocrine testing and others recommend a more comprehensive panel of tests, including serum LH, prolactin, and thyroid function tests on preliminary screening**.(Walsh , *et al.*, 2003 ; Geidam , *et al.*, 2008**).An evaluation of the pituitary-gonadal axis can provide valuable information on the state of sperm production. In turn, it can reveal problems with the pituitary axis that can cause infertility (hyperprolactinemia, gonadotropin deficiency, congenital adrenal hyperplasia)**.**The patterns of endocrine profiles of infertile men are listed in **Table 2.10**.

Table (2.10): Characteristic Endocrine Profiles in Infertile Men.(Tanagho & McAninch ,2008)

Condition	T	FSH	LH	PRL
Normal	NL	NL	NL	NL
Primary testis failure	Low	High	NL/High	NL
Hypogonadotropic hypogonadism	Low	Low	Low	NL
Hyperprolactinemia	Low	Low/NL	Low	High
Androgen resistance	High	High	High	NL

T,testosterone; FSH, follicle stimulating hormone; LH, luteinizing hormone ; PRL,prolaction;NL, normal

2.4.2.4.3 Other tests:

The purpose of these additional tests is to rule in or out specific causes for male infertility. It is important to keep in mind that there are specific indications for each of these tests and that they do not need to be performed on a routine basis(**Walsh , *et al.*, 2003 ;Patton & Battaglia ,2005).**These includes:

1. **Antisperm Antibody Test:**

   Antisperm antibodies can be found in three locations: serum, seminal plasma, and sperm-bound. Among these, sperm-bound antibodies are the most relevant. The antibody classes that appear to be clinically relevant include immunoglobulin G (IgG) and IgA. Immunoglobulin G antibody is derived from local production and from transudation from the blood stream (1%). IgA is thought to be purely locally derived **(Tanagho & McAninch ,2008)**.An assay for ASA should be obtained when:

   A. The semen analysis shows sperm agglutination or clumping.
   B. Low sperm motility exists with history of testis injury or surgery.
   C. There is confirmation that increased round cells are leukocytes.
   D. There is unexplained infertility.

2. **Post-coital test**
3. **Leukocyte Staining**
4. **Semen Cultures**
5. **Ultrastructural Evaluation**
6. **Radiologic Evaluation:**
   a. Vasography
   b. Transrectal ultrasound
   c. Venography
   d. Scrotal ultrsonography
   e. Abdominal ultrasonography
7. **Sperm function testings:**
8. **Sperm viability assays**
9. **Reactive oxygen species tests**
10. **Genetic tests**
11. **Testicular biopsy**

2.4.3 Treatment of male infertility:

2.4.3.1 General considerations:

There are many medical and surgical options that can help most couples overcome male factor infertility **(Table 2.11)** . When applicable, medical therapies are used as an initial strategy to improve sperm production or as a preliminary therapy to boost sperms production transiently in anticipation of a surgical sperm retrieval attempt. Hormonal therapies bear good promise, but only in cases with hormonal deficiency. Because the etiology differs a lot in infertility cases, it is difficult to estimate the success rate of hormone therapy(**Madhukar & Rajender ,2009)** A range of surgical options is available to correct varicoceles, reconstruct the obstructed system, or retrieve sperm for assisted reproduction **(Schiff , *et al.*, 2007).** The alternative strategy is to optimize the fecundity of the male partner through the use of assisted reproductive technologies (ARTs) **(Jarow , 2007).**

2.4.3.2 Assisted reproduction techniques:

2.4.3.2.1 Intrauterine insemination (IUI):

The three main types of assisted conception are intrauterine insemination(IUI), *in vitro* fertilization (IVF), and intracytoplasmic sperm Injection (ICSI). For intrauterine insemination, the sample of washed, prepared, motile sperm is deposited in the uterus just before the release of an ovum or ova in a natural or a stimulated cycle. The technique is most effective when it is combined with mild superovulation using gonadotropins. The indications for (IUI) are shown in **table 2.12**.

Semen samples are prepared for assisted conception (sperm activation) by selecting for a population of highly motile, morphologically normal sperm and removing the seminal plasma, leucocytes and bacteria **(Rowell and Braude 2003)**.The most simple and cheapest is the conventional swim-up procedure**.** A more sophisticated and most gentle migration method is migration-sedimentation. However, its yield is relatively small and the technique is therefore normally only limited to ejaculates with a high number of motile spermatozoa. Recently, however, the method was also successfully used to isolate spermatozoa for intracytoplasmic sperm injection (ICSI). Sperm separation methods that yield a higher number of motile spermatozoa are glass wool filtration or density gradient centrifugation with different media**(Henkel, 2003)**.

Table(2.11):Etiology and relevant treatment options of male infertility(Madhukar & Rajender ,2009)

Cause of Infertility	Treatment Options
Extratesticular causes of male infertility	
I- Gonadotropin deficiency	
Kallmann syndrome	Not available
Hemochromatosis	Venesection [a]
Laurence – Moon- Biedl syndrome	Not available
Prader – Willi syndrome	Gonadotropins
Familial cerebral ataxia	Not available
Pituitary tumor	Surgery [a]
Prolactionoma	Dopamine agonist [a]
Hypothalamic tumor	Surgery [a]
Tuberculosis	Antibacterials [a]
Fungal infections	Antifungals [a]
Sarcoidosis	Corticosteroids [a]
Histiocytosis	Not available
Opioids , psychotropic drugs	Change drugs [a]
Postpituitary surgery	Gonadotropins [a]
Postpituitary irradiation	Gonadotropins [a]
Malnutrition	Nutrition [a]
Obesity	Weight loss [a]
Chronic illness	Specific treatment according to disease .
II - Ejaculatory dysfunction	
Psychosexual	Psychotherapy [a]
Iatrogenic Postgenitourinary surgery Retrograde ejaculation	Ephedrine/amitriptyline [a]
III- Epididymal obstruction	
Congenital absence of vas deferens	Sperm retrieval [b]
Infection (gonorrhea, Chlamydia)	Sperm retrieval [b]
IV- Vasal obstruction	
congenital	Sperm retrival [b]
Infection	Antibacterial [b]
Vasectomy	Vasectomy reversal
V- Others	
Erectile dysfunction	Specific to the patient's condition
Sperm autoantibodies	ICSI [b]
Epididymal dysfunction	ICSI/IVF[b]
Accessory gland infection	ICSI/IVF[b]
Testicular causes of male infertility	
Acquired	
Viral orchitis	ICSI/IVF[b]
Bacterial orchitis	ICSI/IVF[b]
Vascular (torsion)	ICSI/IVF[b]
Irradiation	ICSI/IVF[b]
Chemotherapy	ICSI/IVF[b]
Castration	ICSI/IVF[b]
Drugs (marijuana, etc)	Rehabilitation [a]
Heat	ICSI/IVF[b]
Trauma	ICSI/IVF[b]
Idiopathic	ICSI/IVF[b]
Genetic causes of male infertility	Not available

a Specific treatment.
b Empirical treatment
ICSI: intracytoplasmic sperm injection;IVF: ***in vitro*** **fertilization**

Table (2.12):Indications for interuterine insemination(IUI) (Rowell and Braude ,2003)

• Unexplained infertility .
• Male infertility – mild oligozoospermia , asthenozoospermia , or teratozoospermia .
• Failure to conceive after ovulation induction treatment .
• Immunological (antisperm antibodies).
• Ejaculatory failure .
• Retrograde ejaculation .

2.4.3.2.2 ***In vitro* fertilization (IVF):**

It involves controlled ovarian stimulation and ultrasound-guided transvaginal egg retrieval from the ovaries before normal ovulation. *In vitro* fertilization has become increasingly used to treat infertile couples. In vitro insemination is performed by mixing processed sperm with recovered oocytes. Eggs are then fertilized in petri dishes with anywhere from 500,000 to 5 million motile sperm.In standard IVF, when fertilization occurs, the developing embryos are incubated for 2 to 3 days in culture media and then placed transcervically into the uterus. Only 20% to 30% of transferred embryos will implant and produce clinical pregnancies.The indications for IVF are listed in **table 2.13**. The clinical pregnancy rates by standard IVF average 20% to 30% per initiated cycle. There is a significant effect of the female age on pregnancy rates with IVF(**Walsh , *et al.* , 2003).** The cumulative chance of a successful pregnancy is about 13% after 1 transfer, 22% after 2 transfers, 31% after 3 transfers and 50% after 6 transfers **(Wang , 1999).**

Table (2.13) :Indications for *in vitro* fertilization (IVF) (Braude , 2003)

• Severe tubal damage.
• Bilateral salpingectomy .
• Endometriosis.
• Mild male infertility.
• Idiopathic infertility.
• Immunologic infertility .

2.4.3.2.3 intracytoplasmic sperm injection (ICSI):

The application of intracytoplasmic sperm injection (ICSI) is becoming more and more popular in assisted reproduction technology (ART) units throughout the world **(Yoeli , *et al.*, 2008).** Because ICSI involves the injection of a single spermatozoon directly into the oocyte , this procedure bypasses all the preliminary steps of fertilization. Matured eggs are obtained from the woman by hormonal ovarian hyperstimulation **(Wen , 2004).** Only mature eggs are suitable for injection with prepared sperm. As ICSI is usually used when sperm quality is extremely poor, each sperm can be examined and selected for normality of its morphology before being picked up individually with a fine glass needle and inserted directly into the cytoplasm of the egg **(Fig.2.11)**. Sperm do not have to be motile but should show evidence of viability. The "best" sperm are selected on the basis of morphology and mobility then injected inside the ovum **(Rowell & Braude ,2003).** The indications for ICSI can be divided into two main categories; established and possible indications **(Table 2.14)(Wen , 2004).**

Table(2.14): Indications for intracytoplasmic sperm injection ICSI (Wen , 2004).

I- Established indications :
1. Sever deficit in semen quality.
2. Obstructive azoospermia.
3. Nonobstructive azoospermia
4. Failed IVF or very poor fertilization
II- Possible indications :
1. Sperm count < 1 million
2. Sperm morphology < 4% normal forms.

IVF,*in vitro* fertilization.

2.4.3.2.4 Sperm Retrieval:

For patients with obstructive azoospermia, sperm may be retrieved from the ductal system or from the testicular parenchyma. In contrast, only testicular sperm retrieval is applicable for patients with nonobstructive azoospermia. Microsurgical epididymal sperm aspiration (MESA) is commonly employed to retrieve sperm out of the ductal system in cases with obstructive azoospermia such as congenital vassal obstruction (CBAVD). Some have advocated percutaneous epididymal

sperm aspiration (PESA) as a less invasive technique that does not require microsurgical skills **(Walsh , *et al.*, 2003)**

Fig(2.11) : Micromanipulation equipment required to undertake ICSI procedures. The embryologist guides the needle into the egg using joystick directed servomotors (Braude ,2003).

Chapter Three
Materials and methods

The study conducted during the period from November 2008 – May 2009 at Babylon Hospital for Maternity and Childhood in Hilla city, Babylon Province, Iraq .

3.1 Materials:

3.1.1 Patients:

The patients were male patients with infertility attending Infertility Center at Babylon Hospital for Maternity and Childhood. They were already diagnosed by a specialist doctor and referred to the center or they were attending the center for the first time . All patients enrolled in the study fulfilled the following criteria:

Inclusion criteria:

1. The patient was unable to achieve pregnancy in the period of last 12 months or more despite regular unprotected intercourse **(Gracia, *et al.*, 2005)**.
2. He had abnormal sperms concentration with or without other SFA abnormalities, according to WHO guidelines **(WHO, 1999)**
3. The wife's infertility evaluation by a specialist gynecologist revealed no abnormalities in the female side.
4. The patient agreed to participate in the study.

Exclusion criteria:

1. Patient's refusal.
2. History of recent administration of hormonal therapy.
3. Patient who can not provide semen sample (e.g. impotence or inability to ejaculate at the hospital and can not bring the sample from home within 1 hour to the laboratory).
4. Patient with normal semen analysis or with asthenozoospermia plus normal sperms concentration ($>$ 20 million/ml).This is because these patients with normal sperm concentration usually have normal sex hormones levels **(Walsh, *et al.* , 2003; Tanagho & McAninch ,2008)**

A total number of 110 male patients with infertility fulfilled our inclusion and exclusion criteria were selected; of whom 9 patients with normal SFA and 20 patients with asthenozoospermia plus sperms concentration > 20 million/ml were excluded from the study.The remaining 81 patients had become the patients group in our study.They were divided by their SFA results into the following groups **(Table 3.1)**:

1. Patients with azoospermia (n = 38).
2. Patients with oligoasthenozoospermia (n = 31).
3. Patients with oligozoospermia only (n = 12).

3.1.2 Control:

This group consisted of fertile men who succeeded to meet the following criteria:

Inclusion criteria:

1. Achieved pregnancy in the last 24 months .
2. Have normal SFA according to WHO criteria (**WHO, 1999**).

Exclusion criteria:

1. Subject's refusal
2. History of recurrent abortions in the wife.
3. History of varicocelectomy.

30 fertile men who met the above criteria were selected as control group for the study. Some of them were the husbands of pregnant women attending Babylon Hospital for Maternity and Childhood for various reasons.

Table(3.1): Frequencies of infertile men groups and control.

Subjects	group	No.	Total
Patients	Azoospermia	38	81
	Oligoasthenozoospermia	31	
	Oligozoospermia	12	
Control		30	30
Total		111	111

3.1.3 Apparatus :

The apparatus that were utilized in this study and their manufacturing companies are shown in **table 3.2**

3.1.4 Chemicals:

The chemical materials that were used together with their producing companies are listed in **table 3.3.**

3.2 Methods:

3.2.1 Medical History (Questionnaire):

Every patient or control (who fulfilled the selection criteria) was interviewed in a private room in the Infertility Center on individual basis. All the patients and control were interviewed and examined by the same researcher for the sake of the consistency of the data. A well-structured questionnaire **(Appendix – I)** was developed for the study and was filled for every patient and control. The questionnaire covered the most important aspects of medical history relevant to male infertility including: demographic data, type and duration of infertility, history of abortions, family history of infertility, use of lubricants and contraceptives, sexual history, medical history, surgical history and finally drugs and social history.

3.2.2 Physical examination:

The patients and control then were physically examined by the same researcher.The physical examination that was performed was focused on points that were essential and specific for male infertility. It was divided into two divisions. First, general non-scrotal examination which included the examination for the presence of secondary sexual characteristics, gynecomastia, features of Klinefelter syndrome and features of endocrine disorders. The other division was directed toward scrotal examination. This included searching for undescended or ectopic testis, varicocele, testicular mass or tenderness, absent vas, and finally small penis or congenital anomalies in penis like Hypospadias.

Table (3.2) : The apparatus used in the study.

No.	Apparatus	Manufacturers
1.	Hematocrit centrifuge	Hettich ,Germeny
2.	Light microscope	Olympus , Japan
3.	Centrifuge	Rotofix 32A,Germany.
4.	Graded pH Paper	Shangahai SSS Reagents Co.Ltd.,China
5.	Plain test tube	Afco – Dispo, Jordan.
6.	EDTA tube	Afco – Dispo, Jordan.
7.	Micropipette 100 -1000 µl	Slamed ,USA.
8.	Micropipette 10-100 µl	Slamed ,USA.
9.	Microscope slide	Sail Brand ,China
10.	Refrigerator	Liebherr, Austria
11.	Disposable syringes (10ml) G21	Mediplast ,U.A.E
12.	Coverslip 22×22 mm	Marienfeld, Germany
13.	Incubator	Fisher Scientific, U.S.A
14.	Disposable semen collection containers	RSD,China
15.	Improved Neubauer hemocytometer (0.1 mm)	Marienfeld , Germany
16.	Disposable graded plastic test tubes	RSD,China
17.	Woody sticks	Locally produced in Iraq
18.	Hematocrit capillaries	Hirschmann Laborgerate , Germany
19.	EDTA tube shaker	Karlkolb , Germany
20.	Westergren tube	Alfa,Germany
21.	Eliza Microtiter Reader	Beckman,Inc.U.S.A

Table (3.3) : The chemical materials utilized in the study.

No.	Chemical material	Producing company
1	Saturated NH_4Cl solution(250g/l)	Fluka , Switezerland
2	Na_2EDTA (50 g/l) in phosphate buffer (PH 6.0)	Fluka , Switezerland
3	Ortho-toluidine stain (0.25 mg/ml)	Alfa,Germany
4	H_2O_2 (30 %) in distilled water	Crescent ,Saudi Arabia
5	Eosin Y stain (0.5 %) in distilled water	Alfa,Germany
6	Ethanol (95 %)	Charlton, Ltd.England
7	Testosterone (ELIZA) kit	Biocheck , U.S.A.
8	FSH (ELIZA) kit	Monobind , U.S.A.
9	LH (ELIZA) kit	Monobind , U.S.A.
10	Prolactin (ELIZA) kit	Monobind , U.S.A.
11	$NaHCO_3$	Crescent ,Saudi Arabia
12	Formalin solution (35 %) (v/v)	Jaljyoti International,India
13	Turk's solution (Acetic acid (2%) + Gention violet (1%))	Crescent ,Saudi Arabia
15	Distilled water	Crescent ,Saudi Arabia
16	Trisodiun citrate (3.8 %)	Merck , U.S.A.

3.2.3 <u>Laboratory investigations:</u>

3.2.3.1 <u>Seminal fluid analysis (SFA) :</u>

For each patient and control , SFA was performed according to latest published WHO guidelines in (*WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction*) **(WHO , 1999)** and NAFA (Nordic Association of Andrology) and Eshre-Siga (European Society of Human reproduction and Embryology, Special Interest Group on Andrology (*Manual on basic semen analysis*) **(NAFA & Eshre-Siga , 2002) .**The WHO protocol is highly recommended and adopted by large numbers of research centers and andrologists (**Quaas & Dokras ,2008).**All the components of the test was completed by the same researcher to avoid interpersonal variations.The WHO form for SFA was adopted to report the results **(Appendix - II**).The SFA parameters

that were measured and their methods are listed below (N.B.All what was applied to the patients was applied to the control).

3.2.3.1.1 Semen collection:

All the men were given clear instructions regarding the accurate semen collection to minimize error. They were asked to avoid sexual intercourse for 3 days.All men who came to the Infertility Center with sexual abstinence between 2-7 days were enrolled in the study because this duration is accepted according to the WHO manual. However we tried as much as possible for the standardization. The study subjects were given a disposable ,sterile, wide-mouth, non sperm-toxic semen container for semen collection .They were asked to collect the sample by masturbation without using any lubricants. Ideally , the sample should be collected in a private room near the lab. Collection of the whole sample was stressed on. The samples were collected during the period 9.00-11.00 a.m.They were brought to the andrology laboratory immediately for examination.Persons who were unable or embarrassed to provide semen sample at hospital and can bring the sample from home to the laboratory within 1 hour were accepted(only 2 patients were so). However, they were given special instructions regarding keeping the sample away from the extreme temperatures by covering the container with tissue papers and keeping the container near the body. *Coitus interruptus* was not acceptable as a means of collection (because the first portion of the ejaculate, which usually contains the highest concentration of spermatozoa, may be lost. Moreover, there will be cellular and bacteriological contamination of the sample and the acid pH of the vaginal fluid adversely affects sperm motility).Immediately after the delivery of the sample to the lab , a label containing the patient’s name and the time of sample delivery is applied to sample container.The sample then was introduced into the incubator (37°C)for liquefaction **(WHO , 1999 ; NAFA & Eshre-Siga , 2002)**

3.2.3.1.2 Patients’ informations:

At the beginning, the patient’s name and study unique number was recorded.Then the date and time of collection of the sample as well as the duration of sexual abstinence was provided.Other SFA parameters were recorded in the form as soon as they were measured.

3.2.3.1.3 Macroscopic SFA findings:

3.2.3.1.3.1 Liquefaction:

Every semen sample was mixed gently immediately after delivery to the lab then introduced into the incubator at 37°C. After that, the sample was examined every 5 minutes for evidence of liquefaction. When the sample was completely liquefied (semen should not be as a clot and contained no mucous streaks) , the time of liquefaction was recorded. A normal semen sample liquefies within 60 minutes at room temperature (25°C) , although usually this occurs within 15 minutes. In some cases, complete liquefaction does not occur within 60 minutes, and this was recorded.Then presence of mucous streaks may interfere with semen analysis. Occasionally, samples may not liquefy, in which case additional treatment, mechanical mixing or enzyme digestion may be necessary**(WHO , 1999)** .This was not performed in our study due to unavailability of the specific equipments and materials for this purpose.

3.2.3.1.3.2 Appearance:

The semen sample was examined immediately after liquefaction by simple inspection at room temperature and the appearance was recorded in the SFA form.A normal sample has a homogenous, grey-opalescent appearance. It may appear less opaque if the sperm concentration is very low, red-brown when red blood cells are present or yellow in a patient with jaundice or taking some vitamins(**WHO , 1999 ; NAFA & Eshre-Siga , 2002).**

3.2.3.1.3.3 Volume:

The volume of the ejaculate was measured using a disposable graduated cylinder with a conical base . Plastic syringes should not be used because they may affect sperm motility(**WHO , 1999 ; NAFA & Eshre-Siga , 2002).**

3.2.3.1.3.4 Viscosity:

In our study, the viscosity(sometimes referred to as 'consistency') was evaluated by introducing a woody rod into the sample and observing the length of the thread that formed on withdrawal of the rod. If the threads length exceeded 2 cm , then the result was reported as abnormal viscosity. The viscosity of the liquefied sample should be recognized as being different from coagulation. High

viscosity can interfere with determinations of sperm motility, concentration and antibody coating of spermatozoa. The methods to reduce viscosity are the same as those for delayed liquefaction **(WHO , 1999 ; NAFA & Eshre-Siga , 2002).**

3.2.3.1.3.5 <u>Semen pH:</u>

A drop of semen was spread evenly onto graded pH paper (range: pH 1.0 to 14.0). After 30 seconds, the color of the impregnated zone was uniform and was compared with the calibration strip to read the pH. Measurement of pH should not exceed 60 minutes after ejaculation. If the pH is less than 7.0 in a sample with azoospermia, there may be obstruction of the ejaculatory ducts or bilateral congenital absence of the vasa **(WHO,1999; NAFA & Eshre-Siga , 2002**).

3.2.3.1.4 <u>Microscopic SFA findings:</u>

3.2.3.1.4.1 <u>Initial microscopic investigation:</u>

During the initial microscopic investigation of the sample, estimates were made of the concentration, motility, agglutination of spermatozoa, and presence of cellular elements other than spermatozoa.

3.2.3.1.4.2 <u>Preparation for routine semen analysis</u>

The volume of semen and the dimensions of the coverslip must be standardized so that the analyses are always carried out in a preparation of fixed depth of about 20 μm. This allows a rough estimate of sperm concentration to be made in order to determine how to prepare the semen for the accurate determination of sperm concentration. Depths less than 20 μm may constrain the rotational movement of spermatozoa**(WHO,1999; NAFA & Eshre-Siga , 2002**).

A fixed volume of 10 μl semen was delivered onto a clean glass slide with a positive displacement pipette and covered with a 22 mm × 22 mm coverslip. The weight of the coverslip spreads the sample for optimum viewing and care was taken to avoid forming and trapping bubbles between the coverslip and the slide. The freshly made wet preparation was left to stabilize for approximately one minute. The examination was carried out at room temperature for all the samples. Initial evaluation at 100 X total magnification (i.e., 10 X objective and 10 X ocular) provided an overview for determining mucus strand formation, sperm

aggregation, and the evenness of spread of spermatozoa on the slide. The preparation was then examined at a magnification of 400 X total magnification (**WHO, 1999 ; NAFA &Eshre-Siga , 2002**).

3.2.3.1.4.3 Preliminary estimation of sperm concentration

Scanning the slide and estimating the number of spermatozoa per field or part of a field equivalent to 1 µl gives an approximate sperm concentration in 10^6/ml. This estimate was used to decide the dilution for determining the sperm concentration by haemocytometry: < 15 spermatozoa / field, dilution 1:5; 15-40 spermatozoa / field , dilution 1:10; 40-200 spermatozoa / field , dilution 1:20; >200 spermatozoa / field, dilution 1: 50 **(Table 3.3) (WHO, 1999)**.If the number of spermatozoa per visual field varies considerably, it indicates that the sample is not homogeneous. In such cases, the semen sample was mixed again thoroughly. Lack of homogeneity may also result from abnormal consistency, abnormal liquefaction, aggregation of spermatozoa in mucous threads, or from sperm agglutination **(WHO, 1999)**.

Table (3.4) :Dilutions and conversion factors for sperm concentration measurement using the improved Neubauer hemocytometer (WHO, 1999).

Spermatozoa per (400 X) field	Dilution (semen + diluent)	Conversion factors		
		Numbers of large squares counted		
		(5)	(10)	(25)
<15	1:5 (1+ 4)	20	8	4
15-40	1:10 (1+ 9)	10	4	2
40-200	1:20 (1+ 19)	5	2	1
>200	1:50 (1+49)	2	0.8	0.4

All samples in which no spermatozoa were detected by microscopy were centrifuged to detect the presence of spermatozoa in the sediment. Centrifugation at 2000 rpm for 5 minutes was applied. Only when no spermatozoa were found after a complete and systematic search of the resuspended precipitate , samples were classified as azoospermic (**WHO, 1999 ; NAFA & Eshre-Siga , 2002**).

3.2.3.1.4.4 Assessment of sperm motility:

A simple grading system was recommended by WHO manual and was used in this study which provides an assessment of sperm motility without the need for complex equipment. At least five microscopic fields were assessed in a systematic way to classify 200 spermatozoa. The motility of each spermatozoon is graded **'a'**, **'b'**, **'c'**, or **'d'**, according to whether it shows:

a. Rapid progressive motility (i.e., >25µm/s at 37°C; note that 25µm is approximately equal to five head lengths or half a tail length);
b. Slow or sluggish progressive motility (5- 25µm/s);
c. Nonprogressive motility (<5 µm/s);
d. Immotilily

Within a microscopic field , all spermatozoa with grade **a** and **b** motility were counted first. Subsequently spermatozoa with nonprogressive motility **(grade c)** and immotile spermatozoa **(grade d)** were counted in the same area. The count should be in the area 5 mm away from the edge of the coverslip and in systematic way(**Fig.3.1**). The count of 200 spermatozoa was sufficient to get then the percentage of each category(**WHO, 1999 ; NAFA & Eshre-Siga , 2002**).

3.2.3.1.4.5 Agglutination:

Agglutination of spermatozoa means that motile spermatozoa stick to each other head to head, tail to tail or in a mixed way, e.g., head to tail. The adherence either of immotile spermatozoa to each other or of motile spermatozoa to mucous threads, cells other than spermatozoa, or debris is not sufficient evidence for, an immunological cause of infertility. Agglutination was assessed at the time of determining sperm motility. A semiquantitative grading was used : **(-)** (no agglutination),(+)(mild agglutination), (++)(moderate agglutination) and **(+ + +)** (severe clumping in which all the motile spermatozoa are agglutinated **(WHO, 1999**).

3.2.3.1.4.6 Sperm vitality by dye exclusion

Sperm vitality is reflected in the proportion of spermatozoa that are 'alive' as determined by either dye exclusion or hypo-osmotic swelling .The first test was

used in this study **(Appendix-III)**. The vitality of sperms should be determined if the percentage of immotile spermatozoa exceeds 50%**(WHO,1999)**. The proportion of live spermatozoa can be determined by using staining techniques that

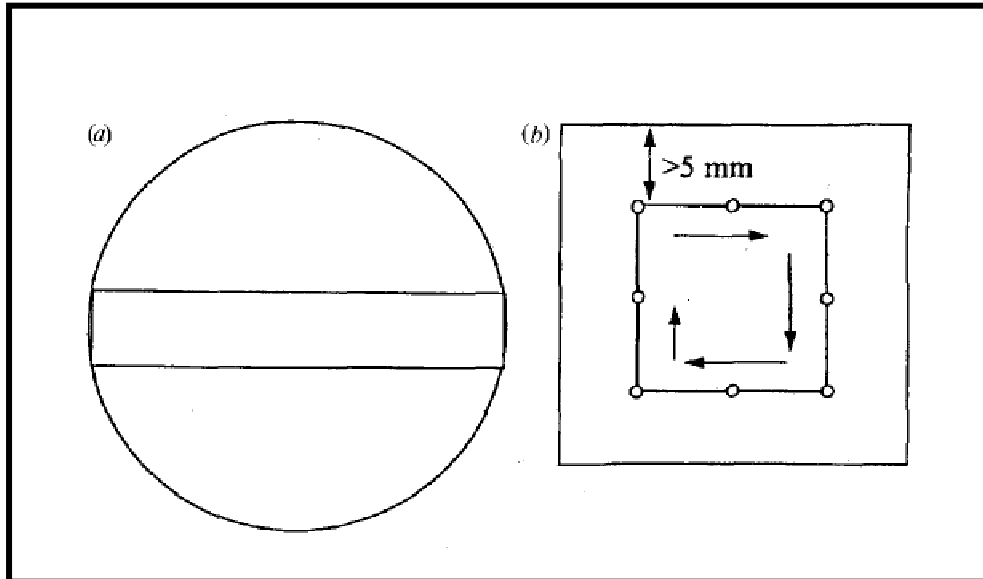


Fig. (3.1) Sperm motility assessment in light microscope, (a)Tramlines in the field of view of the microscope to aid assessment of sperm motility. (b) Systematic selection of eight fields for assessment of sperm motility at least 5 mm from the edges of the cover slip (WHO, 1999).

are based on the principle that dead cells with a damaged plasma membrane take up certain stains. Two hundred spermatozoa were counted with the light microscope, differentiating the live (unstained) spermatozoa from the dead (stained) cells(**WHO, 1999 ; NAFA & Eshre-Siga , 2002**).The presence of a large proportion of vital but immotile cells may be indicative of structural defects in the flagellum (**WHO, 1999 ; NAFA & Eschre-Siga , 2002**).

3.2.3.1.4.7 <u>Assessment of sperm concentration</u>

The concentration of spermatozoa was determined using the haemocytometer method **(Appendix-IV)** .

3.2.3.1.4.8 <u>Cellular elements other than spermatozoa</u>

The ejaculate invariably contains cells other than spermatozoa collectively referred to as 'round cells'. These include epithelial cells from the genitourinary

tract, prostate cells, spermatogenic cells, and leukocytes . As a general guide, a normal ejaculate should not contain more than 5×10^6 round cells/ml**(WHO,1999)**.

Leukocytes predominantly neutrophils, are present in most human ejaculates. Excessive numbers of these cells (leukocytospermia) may be associated with infection and poor sperm quality . The number of leukocytes should not exceed 1×10^6 / ml(**WHO, 1999 ; NAFA & Eshre-Siga , 2002**). Several techniques have been devised for quantifying the leukocyte population in semen. Two cytochemical techniques based on the presence of intracellular peroxidase and on leukocyte-specific antigens(**WHO, 1999 ; NAFA & Eshre-Siga , 2002**).The peroxidase technique was used in this study **(Appendix-V)**. The immature germ cells (the round cells other than leukocytes) include round spermatids, spermatocytes, spermatogonia, and exfoliated epithelia1 cells. These are often degenerating and difficult to identify.The different types of immature germ cells appearing in semen are usually indicative of disorders of spermatogenesis; their identification can be aided by the use of the special stain Bryan-Leishman stain(**WHO, 1999 ; NAFA & Eshre-Siga , 2002**), however, unfortunately this stain was not available for the researcher.

Counting cells other than spermatozoa

The concentration of such cells can be estimated in wet preparations using a suitable haemocytometer in the same way as spermatozoa **(WHO,1999).**

3.2.3.1.4.9 Assessment of sperm morphology

Although the morphological variability of the human spermatozoon makes sperm morphology assessment difficult, observations on spermatozoa recovered from the female reproductive tract (especially in postcoital cervical mucus) or from the surface of the zona pellucida have helped to define the appearance of a normal spermatozoon **(WHO, 1999)** .

Preparation of smears:

Smears were made from the fresh semen sample for assessment .A small drop of semen (5 to 20 µl) was applied to the slide. If the sperm concentration is over 20×10^6/ml , then 5 µl of semen was used; if the sperm concentration is less than 20×10^6/ml, then 20 µl of semen was adopted**(WHO,1999)**. The 'feathering'

technique (whereby the edge of a second slide is used to drag a drop of semen along the surface of the cleaned slide was used to make smears of spermatozoa, but care was taken not to make the smears too thick. These slides were allowed to dry in air and then fixed by (95 %) ethanol . The fixation procedure depends on the staining method. Modified Papanicolaou stain is the method most widely used in andrology laboratories and is the method recommended by WHO manual. It gives good staining of the spermatozoa and other cells. It permits staining of the acrosomal and post-acrosomal regions of the head, the cytoplasmic droplet, the midpiece, and the tail. Unfortunately , the modified stain was unavailable for the researcher at the time of the study so we examined the slides after fixation with (95 %) ethanol without staining that was able to highlight the details of the sperms. Other methods for assessing sperm morphology include Shorr method and rapid staining method **(WHO, 1999)**.

Criteria of normal sperm morphology:

For a spermatozoon to be considered normal, the sperm head, neck, midpiece, and tail must be normal. The head should be oval in shape. Allowing for the slight shrinkage that fixation and staining induce, the length of the head should be 4.0-5.0 µm and the width 2.5-3.5 µm .The length-to-width ratio should be 1.50 to 1.75. Estimation of the length and width of the spermatozoon can be made with an ocular micrometer. There should be a well-defined acrosomal region comprising 40-70% of the head area. The midpiece should be slender, less than 1 µm in width, about one and a half times the length of the head, and attached axially to the head. Cytoplasmic droplets should be less than half the size of the normal head. The tail should be straight, uniform, thinner than the midpiece, uncoiled and approximately 45 µm long. This classification scheme requires that all 'borderline' forms be considered abnormal **(WHO, 1999)** .

The following categories of defects should be noted **(Fig.3.2):**

1. **Head defects:** namely large, small, tapered, pyriform, round, and amorphous heads, vacuolated heads (>20% of the head area occupied by unstained vacuolar areas), heads with small acrosomal area (<40% of head area) and double heads, or any combination of these.

2. **Neck and midpiece defects:** namely 'bent' neck (the neck and tail form an angle of greater than 90% to the long axis of the head), asymmetrical insertion of the midpiece into the head, thick or irregular midpiece, abnormally thin midpiece (i.e., no mitochondrial sheath), or any combination of these.
3. **Tail defects:** namely short, multiple, hairpin, broken tails, bent tails (>90°) ,tails of irregular width, coiled tails, or any combination of these Cytoplasmic droplets greater than one-half of the area of a normal sperm head. The droplets are usually located in the midpiece **(WHO, 1999)** .
4. **Combination of the above defects**.

Only recognizable spermatozoa with a tail were considered in a differential morphology count; immature cells up to and including the round spermatid stage were not counted as spermatozoa **(WHO, 1999)**.

Performing a sperm morphology count:

With fixed semen smears , a 100 X oil-immersion lens were used. Morphological evaluation was performed in several systematically selected areas of the slide **(Fig. 3.1(b))**.As the slide was examined systematically from one microscopic field to another, all normal spermatozoa were assessed and scored. It is essential to use an ocular micrometer to estimate the size of the spermatozoa, but this was not at hand for the researcher.We counted 200 spermatozoa in systematic way and were classified into normal and abnormal categories then the percentage of each category was obtained **(WHO, 1999)**.

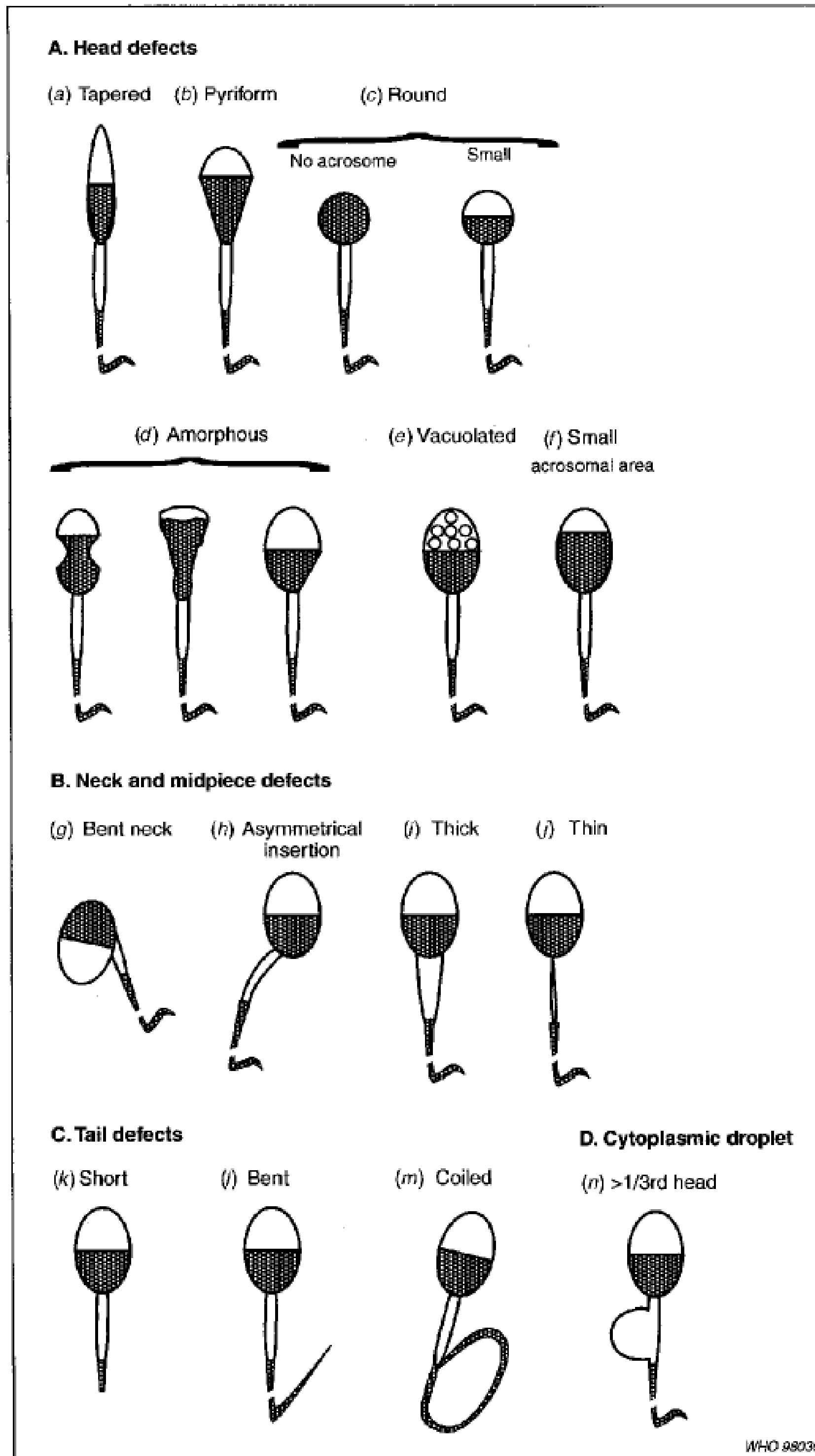


Fig.(3.2) Sperm morphological abnormalities classification (WHO, 1999).

3.3.3.2 Hormonal assays:

All the hormonal assays were performed at the Public Health Laboratory in Hilla City which is one of the specialized laboratories of the Iraqi Ministry of Health.

3.2.3.2.1 Blood samples collection:

Blood samples were obtained from the patients and control. All men were invited to a quiet room. Sterile disposable syringes (G21 needle) and plain plastic tubes and ethylenediaminetetraacetic acid (EDTA) tubes were prepared and labeled. Blood samples of 6 ml were taken by an antecubital vein venepuncture in the morning between 9:00am-12.00am.The blood sample obtained from each man was divided into 2 parts. The 1st part was (4 ml) of blood transferred into plain tube for separation of serum while the 2nd part consisted of 2 ml of blood which was put in an EDTA tube to prevent coagulation and this fraction of blood was used for hematological investigations. Then blood in the plain tubes was allowed to clot at room temperature (25 °C) for 1 hour . After that centrifugation was done at (3000) rpm for 3 minutes to separate the serum. The serum was transferred by micropipette and divided into 4 equal fractions in 4 test tubes , one fraction for each hormonal assay. The sera were stored at -20 °C until the assay was done. The blood samples in the EDTA tubes was transferred immediately to an auto shaker for mixing of blood with the anticoagulant then the hematological investigations were performed **(Lewis , et al., 2006**).

3.2.3.2.2 Serum Testosterone measurement:

The level of serum testosterone for male patients with infertility and control was measured using enzyme-linked immunosorbent assay (ELIZA) method **(Appendix-VI)** .The ELIZA kit that was used was manufactured by BioCheck , Inc. company (U.S.A.).We adopted test procedure and protocol recommended by the kit manufacturer which was given in details in the kit's insert.

3.2.3.2.3 Serum Follicle Stimulating Hormone (FSH) measurement:

The level of serum FSH for male patients with infertility and control was measured using enzyme-linked immunosorbent assay (ELIZA) method **(Appendix-VII)**. The ELIZA kit that was used was manufactured by Monobind ,

Inc. company (U.S.A.). We adopted test procedure and protocol recommended by the kit manufacturer which was given in details in the kit's insert.

3.2.3.2.4 Serum Luteinizing Hormone (LH)measurement:

The level of serum LH for male patients with infertility and control was measured using enzyme-linked immunosorbent assay (ELIZA) method **(Appendix-VIII)**. The ELIZA kit that was used was produced by Monobind , Inc. company (U.S.A.). We applied test procedure and protocol recommended by the kit manufacturer which was given in details in the kit's insert.

3.2.3.2.5 Serum Prolactin Hormone measurement:

The level of serum Prolactin for male patients with infertility and control was measured using enzyme-linked immunosorbent assay (ELIZA) method **(Appendix-IX)** . The ELIZA kit that was used was produced by Monobind , Inc. company (U.S.A.). We used test procedure and protocol recommended by the kit manufacturer which was given in details in the kit's insert.

3.2.3.3 Hematological tests:

3.2.3.3.1 Determination of Packed Cells Volume (PCV)(Appendix-X)

3.2.3.3.2 Total White Blood Cells count (WBCs count)(Appendix-XI)

3.2.3.3.3 Erythrocyte Sedimentation Rate (ESR) measurement (Appendix-XII)

3.2.4 Statistical analysis:

Data analysis was performed using Statistical Package for the Social Sciences (SPSS) software v.12.0 (by SPSS,Inc. ,Chicago, U.S.A.) and Openstat software (by Miller W.G.).Z-test was applied to determine the statistical significance of the difference between proportions. Whenever Z-test was not applicable for this purpose (because of low frequencies),Fisher Exact Test was used instead. Chi-Square test was utilized to obtain the significance for 2×2 tables.

Analysis of Variance (ANOVA) test was used to compare the means for multiple groups. Finally, Pearson's Correlation Coefficient was applied to get the strength of association between different variables(**Daniel, 1999**).

Chapter Four
The Results

4.1 Study groups characteristics:

In this study the mean age was 31.33±6.28 & 33.15±5.25 years for patients & control respectively. Duration of infertility in patients group was 3.89±2.4 (range 1-10) years. Primary infertility constituted 81.48% while secondary type formed 18.52 % of the total infertile men. This difference was statistically significant ($P<0.001$) **(Table 4.1).**

Table (4.1): Infertile men and control characteristics.

Character	Patients	Control
number	81	30
Age (years) (mean ± SD)	31.33 ± 6.28	33.15 ± 5.25
Duration of infertility (years) (mean ± SD)	3.89 ± 2.4 Range (1-10) years	
Type of infertility: Primary [No.(%)] secondary	 66 (81.48 %)* 15 (18.52 %)	

*** P < 0.001**

Table (4.2) shows the distribution male patients with infertility and control by age group. In both patients and control , the age groups and their percentages were: [(20-24): (9.8%, 13.3%)], [(25-29): (29.6% , 26.6%)], [(30-34) :(33.3%, 33.33%)], [(35-39): (19.7%, 20%)] and [(≥40): (7.6% , 6.8%)] years respectively.

Table (4.2): Frequency distribution of male patients with infertility and control by age group.

Age group	Patients		Control	
	No.	(%)	No.	(%)
20-24	8	9.8	4	13.3
25-29	24	29.6	8	26.6
30-34	27	33.3	10	33.33
35-39	16	19.7	6	20
≥40	6	7.6	2	6.8
Total	**81**	**100**	**30**	**100**

4.2 Residence:

Fig.(4.1) demonstrates the distribution of infertile men and fertile control according to their residence. The percentages of patients and control with urban residence were 76.54% & 86.66% respectively while the percentages of those with rural residence were 23.46 % & 13.34% respectively. The percentage of urban residence was significantly higher (P < 0.01) than that of rural residence in both patients and control.

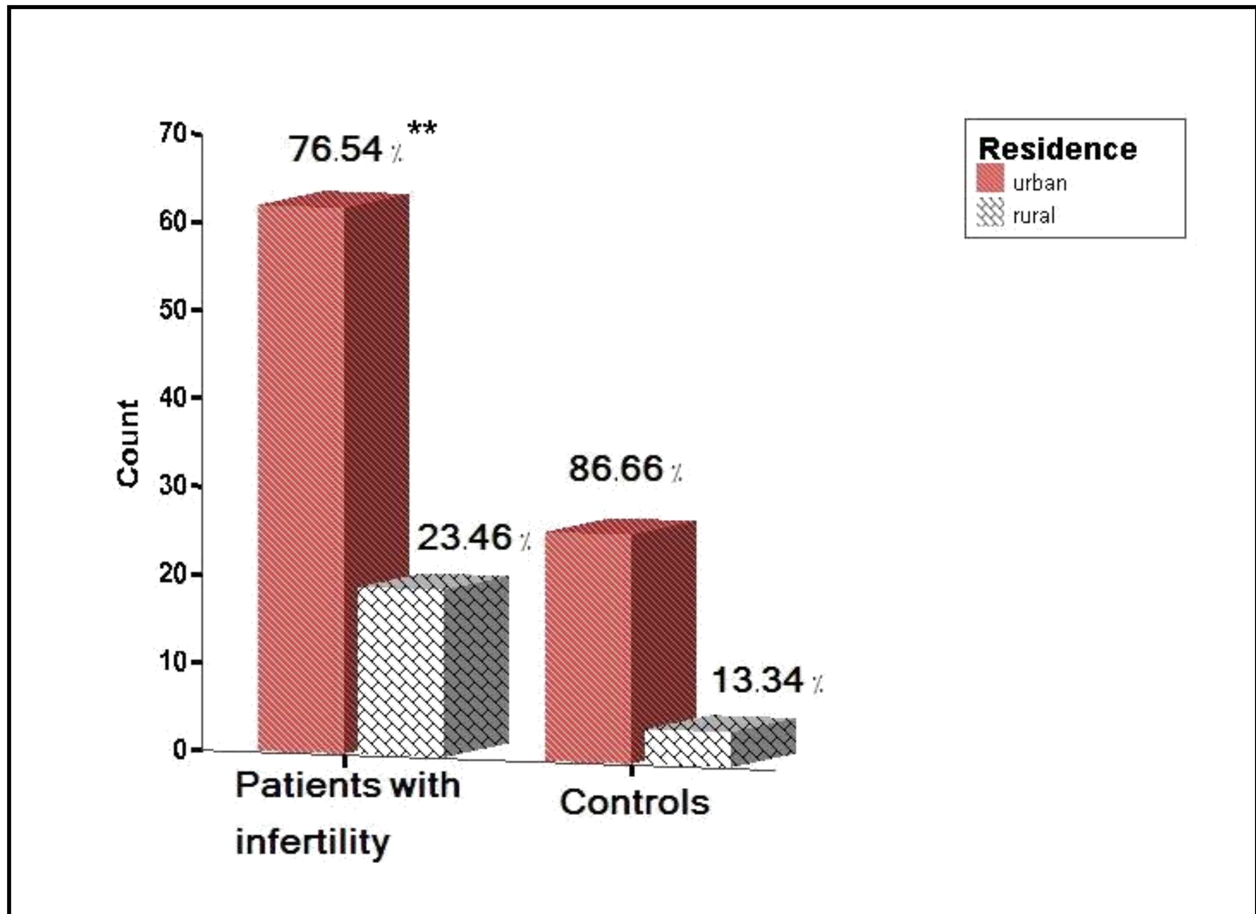


Fig.(4.1): Distribution of male patients with infertility and control by residence
**** P<0.01**

4.3 Sexual history:

History of abortion in the wife was positive in (46.66%) of patients with secondary infertility as compared to (6.66%) of fertile control (P<0.01) **(Fig.4.2)**.

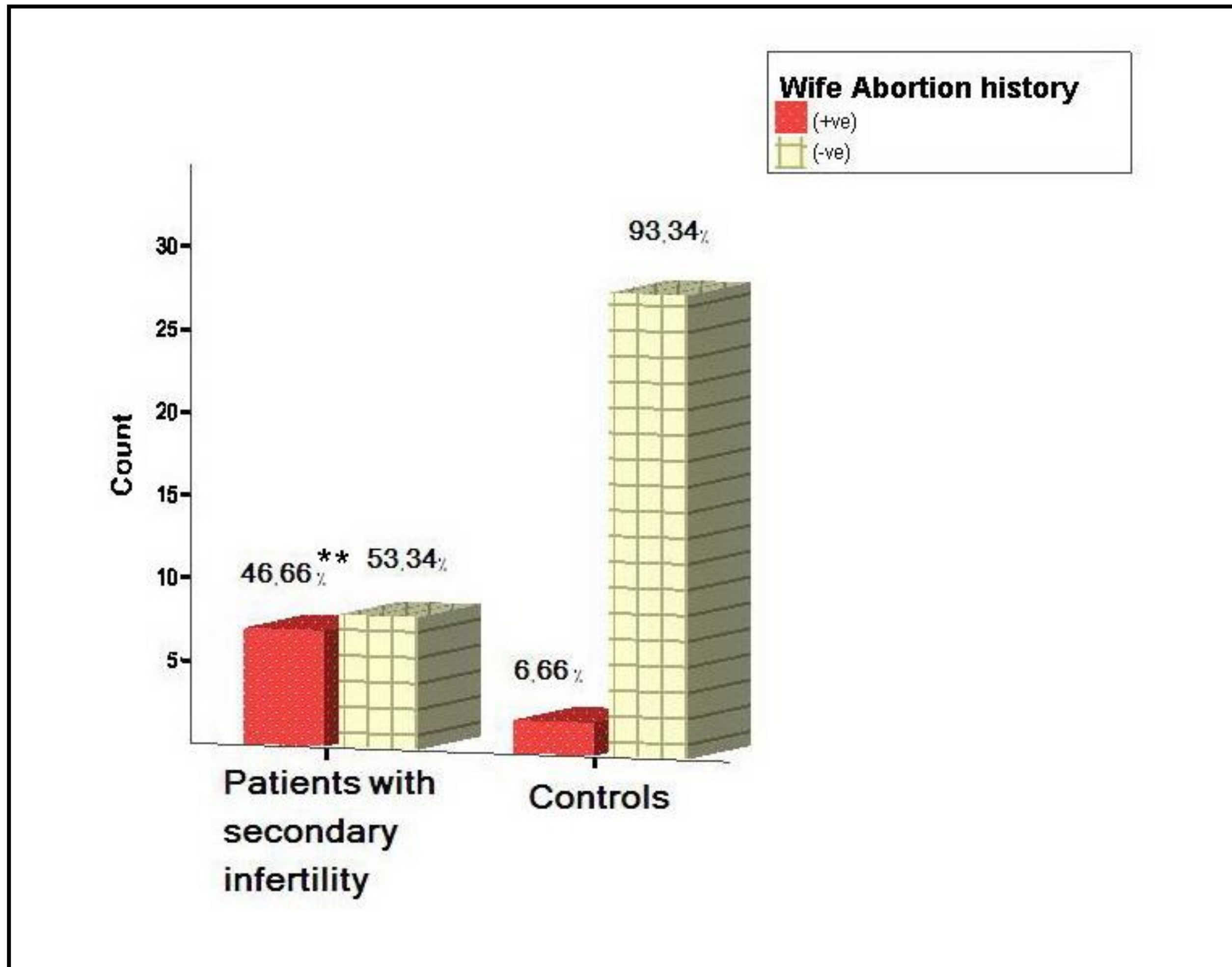


Fig.(4.2):Distribution of men with secondary infertility and fertile control according to history of wife's abortion
**** P<0.001**

Frequency distribution of infertile and fertile men by sexual history is shown in **table (4.3)**.The percentages for patients and control for each parameter were as follows: infrequent sexual intercourse (once every 4 days or more) constituted 6.18% & 3.34% ; those who do not know the proper timing for sexual intercourse formed 75.31% & 83.34% ; those who declared decreased libido were 8.64% & 0% , those who complained of delayed ejaculation formed 1.23%,0% ; users of lubricant formed 1.23% & 0 % ; low socio-economic status persons formed 60.5% & 36.6% ; medium socio-economic status constituted

25.9% & 33.3% ; high socio-economic status constituted 13.6% & 30.1% and users of contraceptive methods formed 1.23% & 26.66% respectively.

Table (4.3): Distribution of the men with infertility and control according to sexual history.

Sexual history	Patients		Control	
	No.	(%)	No.	(%)
Intercourse frequency				
Frequent (≥ 1 per 3 days)	76	93.82	29	96.66
Infrequent (once every 4 days or more)	5	6.18	1	3.34
Proper timing				
know	20	24.69	5	16.66
don't know	61	75.31	25	83.34
Decreased libido				
(+ve)	7	8.64 *	0	0
(-ve)	74	91.36	30	100
Delayed ejaculation				
(+ve)	1	1.23	0	0
(-ve)	80	98.77	30	100
Lubricant use				
(+ve)	1	1.23	0	0
(-ve)	80	98.77	30	100
Socioeconomic status				
low	49	60.5 *	11	36.6
medium	21	25.9 *	10	33.3
high	11	13.6 *	9	30.1
Contraception				
(+ve)	1	1.23 **	8	26.66
(-ve)	80	98.77	22	73.34
Total	**81**		**30**	

*** P < 0.05**

**** P < 0.01**

respectively. Decreased libido history was significantly higher in patients than in control (P<0.05).Also socioeconomic status was significantly lower in infertile men than fertile group (P< 0.05).Furthermore, the use of contraceptives was ,of course ,lower in patients than in control (P<0.01).However, for other parameters no statistically significant differences were observed(P>0.05).

4.4 Family history of male infertility:

Fig.(4.3) shows family history of male infertility in the sample. The proportion of patients with positive family history (20.98%) were significantly higher (P<0.05) than control (6.66%).

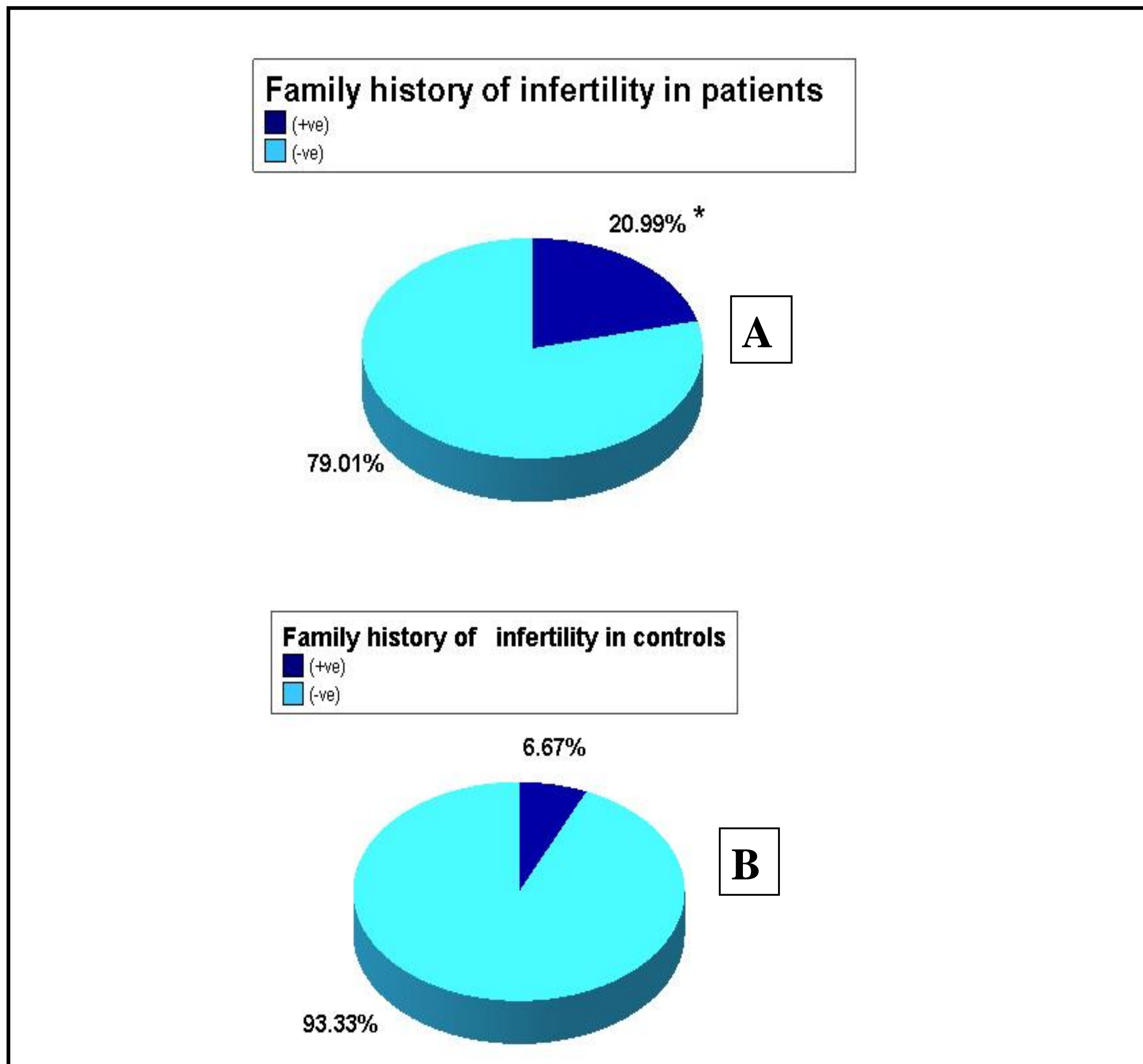


Fig.(4.3) : Family history of male infertility in infertile men (A) and Control (B)
*** P<0.05**

4.5 Medical history;

Regarding medical history and as shown in **table (4.4)**, The percentages of each parameter studied in patients and control were as follows: genital tract infection formed 7.4% &3.33% ; urinary tract infection constituted 14.81% & 13.33% ; febrile illness before 3 months formed 2.46% & 0% , chronic diseases constituted 9.87% & 0% ; endocrine disease feature by history constituted (1.23% & 0%) and congenital disease proportion were 2.46% & 0% respectively. History of chronic disease was significantly higher among infertile men ($P<0.05$) than fertile group while we could not obtain a statistical significance with respect to other parameters($P>0.05$).The chronic diseases that were mentioned by patients and their frequencies were: diabetes (n=3); hypertension (n=2); epilepsy (n=2)and tuberculosis (n=1).Genital tract infections that were stated by the patients and the number of patients were: orchitis (n=3), prostatitis (n=2) and gonorrhea (n=1).The endocrine disease feature by history was mentioned by one patient only and it was related to hypothyroidism whereas the congenital diseases that were declared by 2 patients were undescended testis and single testis.

Table (4.4): Frequency distribution of men with infertility and control by medical history.

Medical history	Patients		Control	
	No.	(%)	No.	(%)
Genital tract infection				
(+ve) Orchitis (n=3) Prostatitis (n=2) Gonorrhea (n=1)	6	7.4	1	3.33
(-ve)	75	92.6	29	96.67
Urinary tract infection				
(+ve)	12	14.81	4	13.33
(-ve)	69	85.19	26	86.67
Febrile illness before 3 months				
(+ve)	2	2.46	0	0
(-ve)	79	97.54	30	100
Chronic diseases				
(+ve) Diabetes (n=3) Hypertension (n=2) Epilepsy (n=2) Tuberculosis(n=1)	8	9.87 *	0	0
(-ve)	73	90.12	30	100
Endocrine disease feature				
(+ve) (hypothyroidism features)	1	1.23	0	0
(-ve)	80	98.77	30	100
Congenital disease				
(+ve) (undescended testis, single testis)	2	2.46	0	0
(-ve)	79	97.54	30	100
Total	**81**		**30**	

*** $P < 0.05$**

4.6 History of injuries:

Distribution of patients and control according to history of injuries is shown in **table (4.5)**.The proportions of each parameter in infertile and fertile men were: testis injury formed 7.4% &3.33% ; pelvic injury constituted 1.23% & 0% ; head injury proportions were 3.7% & 6.66% ; spinal cord injury proportions were 0% & 3.33% and radiotherapy or chemotherapy formed 0% & 0% respectively (P>0.05).

Table (4.5): History of injuries in men with infertility and control.

Injuries history	Patients		Control	
	No.	(%)	No.	(%)
Testis injury				
(+ve)	6	7.4	1	3.33
(-ve)	75	92.6	29	96.67
Pelvic injury				
(+ve)	1	1.23	0	0
(-ve)	80	98.77	30	100
Head injury				
(+ve)	3	3.7	2	6.66
(-ve)	78	96.3	28	93.34
Spinal cord injury				
(+ve)	0	0	1	3.33
(-ve)	81	100	29	96.67
Radiotherapy or chemotherapy				
(+ve)	0	0	0	0
(-ve)	81	100	30	100
Total	**81**		**30**	

4.7 History of varicocele:

The history of varicocele in men with infertility as well as in fertile control is clarified in **table 4.6** .History of varicocele was positive in 23.45% of infertile men as compared to %3.33 in control. It was significantly higher in patients than control (P<0.01).The proportion of left-sided varicocele in patients and control were 84.21% & 100% respectively and the remaining were right –sided 15.79% & 0% respectively and none was bilateral. Nine patients of those who had got varicocele had performed varicocelectomy which constituted 47.3 % .History of varicocelectomy was significantly higher in patients (P<0.01) than in control

Table (4.6): History of varicocele in infertile men and control.

Varicocele history	Patients		Control	
	No.	(%)	No.	(%)
(+ve)	19	23.45*	1	3.33
(-ve)	62	76.55	29	96.67
Varicocele side				
Left	16	84.21*	1	100*
Right	3	15.79	0	0
Bilateral	0	0	0	0
Varicocelectomy				
(+ve)	9	47.3*	0	0
(-ve)	10	52.7	1	100

***P < 0.01**

4.8 Surgical history:

The distribution of male patients with infertility and control by surgical history is demonstrated in **table 4.7** .The proportions of the surgical histories that were investigated in patients and control were : inguinal surgery formed 3.7% & 0% ;scrotal surgery constituted 30.86% & 0% ; testis biopsy formed 8.64% & 0% ; prostate or bladder surgery proportions were 0% & 0% and abdominal surgery percentages were 3.7% & 3.33% respectively. History of scrotal surgery was significantly higher (P<0.001) in patients than in control .The surgical conditions that were operated and mentioned by the patients together with their frequencies were :varicocele(n=9); testicular biopsy (n=7); hernia (n=6); hydrocele (n=4) and undescended testis(n=1).Testicular biopsy history was also remarkable in infertile men (P<0.01) than fertile group. Nevertheless, we observed no significant differences with regard to other parameters(P>0.05).

Table (4.7): Distribution of the male patients with infertility and control by surgical history.

Surgical history	Patients		Control	
	No.	(%)	No.	(%)
Inguinal surgery				
(+ve)	3	3.7	0	0
(-ve)	78	96.3	30	100
Scrotal surgery				
(+ve) varicocelectomy (9)† Testicular biopsy (7) Hernia (6) Hydrocele (4) Undescended testis (1)	25	30.86 *	0	0
(-ve)	56	69.14	30	100
Testis biopsy				
(+ve)	7	8.64 **	0	0
(-ve)	74	91.36	30	100
Prostate or bladder surgery				
(+ve)	0	0	0	0
(-ve)	81	100	30	100
Abdominal surgery				
(+ve)	3	3.7	1	3.33
(-ve)	78	96.3	29	96.67
Total	**81**		**30**	

*** P < 0.05**
**** P < 0.01**
† total is (25) and not (27) because 2 patients performed both varicocelectomy and testicular biopsy in the same operation

4.9 Social history:

Concerning social history and as shown in **table 4.8**, the percentages of the following parameters in men with infertility as well as in fertile control were as follows: smoking 48.14% & 30% ; alcohol intake 8.64% & 10% ; pesticides exposure 1.23% & 0% ; intense exercises 3.7% & 0% and excessive heat exposure 8.64% & 0% respectively. Both history of smoking and excessive heat exposure were higher ($P<0.05$) in men with infertility than fertile group.

Table (4.8): Frequency distribution of infertile men and control according to social history.

Social history	Patients		Control	
	No.	(%)	No.	(%)
Smokers				
(+ve)	39	48.14 *	9	30
(-ve)	42	51.86	21	70
Alcoholism				
(+ve)	7	8.64	3	10
(-ve)	74	91.36	27	90
Pesticides exposure				
(+ve)	1	1.23	0	0
(-ve)	80	98.77	30	100
Intense exercises				
(+ve)	3	3.7	0	0
(-ve)	78	96.3	30	100
Excessive heat exposure				
(+ve)	7	8.64 *	0	0
(-ve)	74	91.36	30	100
Total	**81**		**30**	

*** P < 0.05**

4.10 Drug history:

Distribution of patients and control by drug history of medications that affect sexual function is shown in **table (4.9)** .The percentages of those who administer drugs that affect sperm function in patients and control were 4.93% & 3.33% respectively while those of drugs that affect ejaculation were 1.23% & 0% respectively. The differences between infertile as compared to fertile men were not significant(P>0.05).The drugs that were mentioned included synthetic androgen, cimetidine,amlodipine,valproic acid and imipramine.

Table (4.9): History of drugs affecting sexual function in men with infertility and control

Drugs history	Patients		Control	
	No.	(%)	No.	(%)
Drugs affect sperm function				
(+ve) synthetic androgen (1) cimetidine(1) amlodipine(1) valproic acid (1)	4	4.93	cimetidine(1)	3.33
(-ve)	77	95.07	29	96.67
Drugs affect ejaculation				
(+ve) imipramine (1)	1	1.23	0	0
(-ve)	80	98.77	30	100
Total	**81**		**30**	

4.11 Physical examination:

The distribution of patients and control by general non-scrotal examination findings is shown **table 4.10**.The percentages of the following parameters in infertile men versus fertile control were as follows: diminished secondary sexual characters 2.46% & 0% ; Klinefelter syndrome features 1.23% & 0% , gynecomastia 0% & 0% ; goiter 0% & 0% ; endocrine disease features 1.23% & 0% respectively. All the differences were insignificant ($P>0.05$).

Table (4.10): Distribution of male patients with infertility and control by general non-scrotal examination findings

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Non-scrotal examination	Patients		Control	
	No.	(%)	No.	(%)
Diminished secondary sexual characters				
(+ve)	2	2.46	0	0
(-ve)	79	97.54	30	100
Klinefelter syndrome features				
(+ve)	1	1.23	0	0
(-ve)	80	98.77	30	100
Gynecomastia				
(+ve)	0	0	0	0
(-ve)	81	100	30	100
Goiter				
(+ve)	0	0	0	0
(-ve)	81	100	30	100
Endocrine disease features				
(+ve) (Hypothyroidism)	1	1.23	0	0
(-ve)	80	98.77	30	100
Total	**81**		**30**	

Regarding local scrotal examination, The parameters proportion in males with infertility and fertile males were **table 4.11** : undescended ,or ectopic testis formed 1.23% & 0% , single testis constituted 1.23% & 0% ; retractile testis constituted 0% & 0% ; atrophied testes formed 17.28% & 0% ; varicocele (by examination) proportions were 3.7% & 3.33% ; testes tenderness percentages were 4.93% & 0% ; absent vas formed 0% & 0% ; testis mass constituted 0% & 0% ; hernia formed 1.23% & 0% ; small penis formed 2.46% & 0% respectively. The proportion of those with atrophied testes was significantly higher ($P<0.01$) in infertile than fertile men.

Table (4.11): Frequency distribution of male patients infertility and control according to scrotal examination findings.

	Patients		Control	
	No.	**(%)**	**No.**	**(%)**
Undescended ,or ectopic testis				
(+ve)	1	1.23	0	0
(-ve)	80	98.77	30	100
Single Testis				
(+ve)	1	1.23	0	0
(-ve)	80	98.77	30	100
Retractile testis				
(+ve)	0	0	0	0
(-ve)	81	100	30	100
Atrophied testes				
(+ve)	14	17.28 *	0	0
(-ve)	67	82.72	30	100
Varicocele				
(+ve)	3	3.7	1	3.33
(-ve)	78	96.3	29	96.67
Testes tenderness				
(+ve)	4	4.93	0	0
(-ve)	77	95.07	30	100
Absent vas				
(+ve)	0	0	0	0
(-ve)	81	100	30	100
Testis mass				
(+ve)	0	0	0	0
(-ve)	81	100	30	100
Hernia				
(+ve)	1	1.23	0	0
(-ve)	80	98.77	30	100
Small Penis				
(+ve)	2	2.46	0	0
(-ve)	79	97.54	30	100
Total	**81**		**30**	

*** P < 0.01**

4.12 Seminal fluid analysis:

Regarding type of SFA abnormalities, the percentages of patients with azoospermia, oligoasthenozoospermia and oligozoospermia were 46.9% , 38.2% and 14.9% respectively. The difference in the frequencies of the 3 groups was significant (P < 0.01) **(Table 4.12)**.

Table (4.12) : Frequency distribution of patients with male infertility by seminal fluid analysis abnormalities.

SFA abnormalities	No.	(%)
Azoospermia	38*	46.9
Oligoasthenozoospermia	31*	38.2
Oligozoospermia	12*	14.9
Total	**81**	**100**

*** P value for the difference in the frequencies of the 3 groups is < 0.01**

The means for abstinence time for the 4 groups (patients with azoospermia, oligoasthenozoospermia, oligozoospermia and control) were 3.4±0.84 , 3.2±0.78 , 2.8±0.78 and 2.3±0.48 days respectively **(Table 4.13)**.The mean for control was significantly lower (P<0.05) than other groups. The two groups (azoospermia patients and oligozoospermia patients) are significantly different (P<0.05) in means of abstinence time from each other (azoospermia patients > oligozoospermia patients).The means time of liquefaction for the patients and control were 32.75±12.1, 34.16±10.35, 33.24±9.72 and 28±11.63 minutes respectively. The means of oligoasthenozoospermia patients and control were different (oligoasthenozoospermia patients > control) at (P<0.05).The means of pH values were 8.1±0.55, 7.92±0.42, 8.42±0.61, 8.35±0.52 respectively. The mean values for sample volumes were 1.95±0.74, 2.05±0.68, 2.1±0.52, 2.15±0.63 ml respectively. The proportions of abnormal semen viscosity for patients and control groups were 26.3%,34.4 %,25 % and 6.8% respectively **(Table 4.13)**.

Table(4.13): Macroscopic seminal fluid analysis parameters of groups of male patients with infertility and control

Parameter	Azoospermia	Oligoastheno-zoospermia	Oligozoospermia	Control
Abstinence (days)	3.4±0.84 a (2-4)	3.2±0.78 (2-4)	2.8±0.78 b (2-4)	2.3±0.48 * (2-3)
Liquefaction time (min)	32.75±12.1 (15-60)	34.16±10.35 a (15-60)	33.24±9.72 (10-55)	28±11.63 b (10-45)
PH	8.1±0.55 (7-9)	7.92±0.42 (7-9)	8.42±0.61 (7-9)	8.35±0.52 (7-9)
Volume (ml)	1.95±0.74 (0.5-2.5)	2.05±0.68 (0.5-3.5)	2.1±0.52 (1-3.5)	2.15±0.63 (1-4)
Viscosity				
Normal (No.) (%)	28(73.7%)	20 (64.6%)	9 (75 %)	28(93.34%)
Abnormal (No.) (%)	10 (26.3%)	11 (34.4 %)	3 (25 %)	2 (6.8%)

Results were expressed as means ± SD and range unless otherwise is mentioned.
Pairs of means with different small letters horizontally have (P <0.05)
*** mean for this group is different from other means at (P < 0.05)**

The means for sperm concentration were 0±0 , 9.03±5.06, 12.41±5.14, 89±26.43 ($\times 10^6$ sperm/ml) for the 4 groups respectively **(Table 4.14)**.The means of oligoasthenozoospermia patients, oligozoospermia patients vs. control have statistically significant difference (oligoasthenozoospermia patients and oligozoospermia groups <control) (P<0.01).Patients with azoospermia mean for sperm concentration was lower than other groups means at (P<0.01).Regarding sperm motility, the means for percentages of (grade a + b) motility in the samples were 0±0, 29.19±11.76, 54.58±3.96, 60.33±6.55 respectively. Oligoasthenozoospermia patients, oligozoospermia patients and control have different means (oligoasthenozoospermia patients < oligozoospermia patients < control) at (P<0.01).The mean of azoospermia patients is different from other means at (P<0.001).

The means for the percentages of normal sperm morphology were 0± 0, 32.5± 6.77, 34.5± 6.85, 36.5± 5.79 respectively. The means of oligoasthenozoospermia patients ,oligozoospermia patients and control were different (oligoasthenozoospermia < oligozoospermia < control)(P<0.05). The mean of azoospermia was lower than other means (P<0.001).Regarding the means of sperms vitality, these for the 4 groups were 0± 0, 41.53±10.87, 65.31± 7.18, 73.33± 5.68 respectively. Oligoasthenozoospermia patients , oligozoospermia patients and control means were different (oligoasthenozoospermia patients < oligozoospermia patients < control) at (P<0.01). The mean of azoospermia patients was lower than other means at (P<0.001).

The proportions for samples with grade (+) agglutination were 0 %,6.4%,8.3%,3.3% respectively, grade (++) were 0 %,6.4, 8.3%,0% while those of grade (+++) were 0 %,3.2%,0%,0% respectively. WBCs in semen means count were (0.87±0.59, 0.88±0.65, 0.64±0.51, 0.41±0.4) ($\times 10^6$ cell/ml) respectively for the 4 groups. The means of azoospermia patients and oligoasthenozoospermia patients vs. control have statistically significant difference(azoospermia patients and oligoasthenozoospermia patients < control) at (P<0.01) **(Table 4.14)**.

4.13 Hormonal analysis and correlations:

With regard to hormonal ELIZA assays of serum FSH.The means of the 4 groups were 22.63±21.52, 10.41±8.42, 11.22±7.41, 2.81±2.22 (mIU/ml) respectively **(table 4.15)**.The mean for azoospermia patients was significantly higher than other groups (P<0.01).The means of oligozoospermia patients and control were statistically different from each other (oligozoospermia > control) (P<0.01).Serum LH means were 14.84±14.33, 12.04±11.12, 13.55±12.66, 3.80±2.67 (mIU/ml) respectively. The mean for control was lower than other groups means (P<0.05).The means for serum testosterone were 2.39±2.34, 4±0.71, 3.8±1.64, 5.1±1.07 (ng/ml) respectively. The mean for azoospermia patients was significantly lower (P<0.05) than the mean of other groups. The means for serum prolactin formed 7.12±4.34 , 7.56±6.15, 9.28±8.97, 4.01±1.83 (ng/ml). Respectively. The means of patients were significantly higher (P<0.05) than those of control.

Table(4.14): Microscopic seminal fluid analysis parameters of groups of infertile men and control

Parameter	Azoospermia	Oligoastheno-zoospermia	Oligozoospermia	Control
Sperms concentration ($\times 10^6$ sperm / ml)	0±0 †	9.03±5.06 A (1-18)	12.41±5.14 A (3-18)	89±26.43B (40-140)
Sperms motility				
(grade a + b) (%)	0±0 **	29.19±11.76 A (10-45 %)	54.58±3.96B (50-60%)	60.33±6.55C (50-80%)
(grade c +d) (%)	0±0 **	67.58±16.32 B (55-90%)	49.16±5.81 C (40-50%)	42.06±8.08 D (20-50%)
Normal sperm morphology (%)	0± 0 **	32.5± 6.77 a (22-40)	34.5± 6.85 (19-43)	36.5± 5.79 b (24-46)
Sperms vitality (%)	0± 0 **	41.53±10.87 A (31-62)	65.31± 7.18 B (53-76)	73.33± 5.68 C (66-84)
Sperms agglutination (No.) (%)				
—	0 (0 %)	26(84%)	10(83.4%)	29(96.7%)
+	0 (0 %)	2(6.4%)	1(8.3%)	1(3.3%)
++	0 (0 %)	2(6.4%)	1(8.3%)	0(%)
+++	0 (0 %)	1(3.2%)	0(0%)	0(%)
WBCs count ($\times 10^6$ cell/ml)	0.87±0.59 A (0-1.8)	0.88±0.65 A (0-2)	0.64±0.51 (0-1.4)	0.41±0.4B (0-1.2)

Results were expressed as means ± SD and (range) unless otherwise is mentioned.
Pairs of means with different capital letters horizontally have (P <0.01)
Pairs of means with different small letters horizontally have (P <0.05)
† mean for this group is different from other means at (P < 0.01)
**** mean for this group is different from other means at (P < 0.001)**

Table (4.15): Serum hormonal levels distribution in the groups of male patients with infertility and control

Serum hormones	Azoospermia	Oligoastheno-zoospermia	Oligozoospermia	Control
FSH (mIU/ml)	22.63±21.52†	10.41±8.42	11.22±7.41a	2.81±2.22b
LH (mIU/ml)	14.84±14.33	12.04±11.12	13.55±12.66	3.80±2.67*
Testosterone (ng/ml)	2.39±2.34*	4±0.71	3.8±1.64	5.1±1.07
Prolactin (ng/ml)	7.12±4.34	7.56±6.15	9.28±8.97	4.01±1.83*

Results were expressed as means ± SD
† mean for this group is different from other means at (P < 0.01)
*** mean for this group is different from other means at (P < 0.05)**

Table (4.16) demonstrates distribution of all patients with oligospermia by their sperm concentration and serum hormones levels. Serum FSH levels for the 3 groups (<5, 5-10 and 10-20 million/ml sperm concentration) were 13.1±5.2, 12±6.4, 10.3±8.2 (mIU/ml) respectively. Serum LH levels for the 3 groups were 14.05±9.1 , 13.09±10.3, 12.4±11.1 (mIU/ml) respectively. Both the group of <5 and 5-10 million/ml had serum FSH and serum LH higher (P<0.05) than the group 10-20 million/ml sperm concentration(P<0.05).However, no differences between the <5 and 5-10 million/ml groups was found (P>0.05).For the 3 groups, S.Testosterone levels were 2.7±2.6, 3.8±1.6, 4.5±1 (ng/ml).Those with sperm concentration <5 million/ml demonstrated lower values (P<0.05) for S.Testosterone than those with sperm concentration of 5-10 and 10-20 million/ml P<0.05 .Nevertheless, no significant difference was found between the 5-10 & 10-20 million/ml groups regarding S.Testosterone level. Finally, no significant differences were found among the 3 groups of oligozoospermic patients regarding their S.Prolactin levels(P>0.05).

Table (4.16):Distribution of all patients with oligozoospermia by their sperm concentration and serum hormones levels

Serum Hormones	Sperm concentration (million /ml)		
	< 5	5-10	10-20
FSH (mIU/ml)	13.1±5.2 a	12±6.4 a	10.3±8.2 b
LH (mIU/ml)	14.05±9.1 a	13.09±10.3a	12.4±11.1 b
Testosterone (ng/ml)	2.7±2.6 a	3.8±1.6 b	4.5±1 b
Prolactin (ng/ml)	9.3±8.2	8± 6.2	8.2±6.6

Results were expressed as means ± SD and (range) unless otherwise is mentioned.
Pairs of means with different small letters horizontally have (P <0.05)

Correlation between age and serum hormones was examined in this study. Pearson's correlation coefficient (r) in patients and control for: age versus S.Testosterone it was − 0.39, − 0.45 ,for age versus S.FSH it was 0.43 , 0.62 ,for age versus S.LH it equaled 0.38, 0.56 and age versus S.Prolactin it equaled 0.35, 0.27 respectively **(Table 4.17)**.All these correlations did not reached the statistical significance.

Table (4.17) : Correlation between age and serum hormones of the infertile men and control

Correlation variables	Pearson's correlation coefficient (r)	
	Patients	Control
Age * serum Testosterone	− 0.39	− 0.45
Age * serum FSH	0.43	0.62
Age * serum LH	0.38	0.56
Age * serum Prolactin	0.35	0.27

Regarding correlation between serum hormones and sperms concentration of the study groups , the Pearson's correlation coefficient (r) was as follow each pair of the following parameters: for serum Testosterone and sperms concentration it was 0.91, 0.93 ;for S.FSH and sperms concentration it was − 0.89, − 0.72 ; for serum LH and sperms concentration it equaled − 0.65, − 0.42 and finally for serum Prolactin and sperms concentration it equaled − 0.43 , − 0.39 respectively

(Table 4.18). Correlation between serum Testosterone and sperms concentration was statistically significant ($P<0.05$).This also the case for the correlation between serum FSH and sperms concentration ($P<0.05$).

Table (4.18) : Correlation between serum hormones and sperms concentration of the male patients with infertility control.

Correlation variables	**Pearson's correlation coefficient (r)**	
	Patients	**Control**
Serum Testosterone * sperms concentration	0.91 **	0.93 **
Serum FSH * sperms concentration	– 0.89 **	– 0.72
Serum LH * sperms concentration	– 0.65	– 0.42
Serum Prolactin * sperms concentration	– 0.43	– 0.39

**** $P < 0.05$**

With respect to correlation between serum hormones and sperms motility , the Pearson's correlation coefficient was as follow each pair of the following parameters: for S.Testosterone and sperms motility it equaled 0.46, 0.27 ; for S.FSH and sperms motility it was – 0.54, – 0.12 ; for S.LH and sperms motility it equaled – 0.67, – 0.09 and for S.Prolactin and sperms motility it was 0.11, 0.24 respectively **(Table 4.19)**. Correlation between S.Testosterone and sperms concentration was statistically significant ($P<0.05$).This also the case for the correlation between S.FSH and sperms concentration ($P<0.05$). All the other correlations were statistically insignificant.

Table (4.19) : Correlation between serum hormones and sperms motility of patients with infertility and control .

Correlation variables	**Pearson's correlation coefficient (r)**	
	Patients	**Control**
Serum Testosterone * sperms motility	0.46	0.27
Serum FSH * sperms motility	– 0.54	– 0.12
Serum LH * sperms motility	– 0.67	– 0.09
Serum Prolactin * sperms motility	0.11	0.24

4.14 Hematological investigations:

The frequency distribution of the packed cell volume (PCV),total WBCs and erythrocytes sedimentation rate(ESR) among the patients and control groups (azoospermia, oligoasthenozoospermia,oligozoospermia and control) is clarified in **table (4.20)** .The means of PCV among the patients and control groups were 0.45±.031, 0.41±.02, 0.41±.03 and 0.46±.02 respectively.Patients had got lower (P<0.01) PCV means than control.Azoospermic patients had higher (P<0.01) PCV than other patients groups. The means of total WBCs count for the patients and control groups were 9.4±7.2, 10.3±7.6, 7.2±2.57, 5.1±.87 respectively.Patients demonstrated higher (P<0.001) total WBCs count means than control.The azoospermia, oligoasthenozoospermia and oligozoospermia patients groups were different from each other at (P<0.01).Means for ESR among the patients and control groups were 6±1.33, 7.9±2.89, 8.4±2.27, 5.9±0.87 respectively.The groups were different from each others (P<0.05).It is important to mention that although many hematological significant differences identified, all of them fell within normal reference ranges.

Table (4.20): Distribution of the groups of male patients with infertility and control by some hematological parameters levels .

Parameter	Azoospermia	Oligoastheno-zoospermia	Oligozoospermia	Control
PCV	0.45±.031A	0.41±.02 B	0.41±.03 B	0.46±.02†
Total WBC count (*10^9 cell/l)	9.4±7.2A	10.3±7.6 B	7.2±2.57 C	5.1±.87**
ESR (mm / hour)	6±1.33 a	7.9±2.8 9b	8.4±2.27 c	5.9±0.87 a

Results were expressed as means ± SD
Pairs of means with different small letters horizontally have (P <0.05)
† mean for this group is different from the means of patients with oligoasthenozoospermia and oligozoospermia at (P < 0.01)
**** mean for this group is different from other means at (P < 0.001)**

Chapter Five
Discussion

Infertility affects about 15% of couples during their reproductive years, and male-related factors play a role in approximately half of these cases. The studies of trends in semen parameters in developing countries were sparse. There are great differences in environment, culture, lifestyle and risk factors for infertility between different countries **(Fekri ,*et al.* ,2009).**Participation rates in epidemiologic studies on semen quality are generally very low, raising concerns as to the potential for selection bias (**Meeker & Bailey , 2007).**

5.1 Patients and control characteristics:

In this study and among 81 patients with male infertility and 30 fertile control , the most common age group among infertile patients and was the age group 30-34 years followed by the age group 25-29 years **(Table 4.1 and Table 4.2)**.The control most common age group was also 30-34 years. The mean of age in patients and control was 31.33 ± 6.28 & 33.15 ± 5.25 years respectively. Our results were in good agreement with those of **Geidam & Yawe, (2008)** who observed that among infertile men the most common age group was 25-40 years which constituted 76 % .Also we were in line with the findings of **De Castro & Mastrorocco , (1984);Trummer & Habermann , (2002); Taskiran *et al.*, (2006)** ;**Gao *et al.*,(2007) and Fekri *et al.*, (2009)** who reported similar results to the results of this sudy.

However, the findings of this study were in contrast with the findings of **Okonofua *et al.*, (2005)** who found that the most common age group for patients and control in his sample was 40-44 years. This difference may be due to selection criteria or selection method. Besides, this study disagreed with **Rehan *et al.*,(1975) and Eskenazi *et al.*,(2003)** who mentioned higher mean for the age in their samples than we did. The majority of our patients were young and this is probably due to their great concern regarding getting a child which made them consult the fertility specialist at earlier age .

Infertility duration ranged from 1 to 10 years. The majority of patients had got primary infertility (81.48 %) and this percentage was significantly

higher($P<0.001$) than those with secondary type of infertility **(Table 4.1)**.Our results were in accordance with those of **Abramsson & Ducheck , (1989); Abramsson & Ducheck, (1989) and Geidam & Yawe, (2008)** who found that the primary infertility percentage was higher than secondary type. Primary infertility in these three studies constituted 86.5 % , 86 % & 70.8 % respectively. The wide variation in the duration of infertility may be due to that some patients consult a specialist less frequently than others or their infertility problem is long lasting either because of non-compliance to the treatment or the exact diagnosis for their cause of infertility had not reached. The primary infertility was higher ($P<0.001$) than those with secondary type and this is probably due to that many causes of the secondary type are correctable or probably that patients with secondary type are in less urge for seeking medical help since they already got children.

Regarding the residence, in both infertile and fertile men we observed that urban proportion was significantly higher($P<0.001$) than rural one **(Fig.4.1)**. We were in good agreement with **Gao *et al.*,(2007)** who reported that among fertile men in his study in China, 53.2% were urban while 46.8% were rural. The high prevalence of infertility in urban community is possibly due to wide-spread environmental pollutants exposure as well as the type of lifestyles and habits. Another reasonable explanation is that men with infertility in our rural areas tend to blame the wife frequently as the one who is responsible for the infertility of the couple because in rural areas the usual tradition is that men infertility explained as a kind of male weakness and bring some sort of shame to the man.

5.2 Sexual history:

History of abortion was remarkably higher ($P<0.001$) in infertile men than fertile group **(Fig.4.2)**.With respect to sexual history, we reported significant differences between infertile and fertile men in decreased libido, socio-economic status and the use of contraception techniques **(Table 4.3)**.

Decreased libido was higher ($P<0.05$) in infertile patients than fertile control with the socio-economic status and the use of contraceptive methods were lower($P<0.05$ & $P<0.01$ respectively) in patients than in control. Nevertheless , we detected no significant variations between the infertile and fertile group in

intercourse frequency , proper sexual timing knowledge , painful intercourse , delayed ejaculation and lubricant use **(Table 4.3)**.Our results agreed with **Okonofua *et al.*,(2005)** who detected no difference in sexual intercourse frequency between infertile and fertile men in Nigeria.

Family history of infertility was significantly higher (P<0.05) in infertile than fertile men **(Fig.4.3)**.Decreased libido may be due to the influence of decreased level of testosterone. This is supported by our observation of lower(P<0.05) serum Testosterone in azoospermic patients than control in our study .Low socio-economic status among infertile men was expected because patients with high socio-economic status usually prefer to attend private clinics may be because they think that they will obtain better privacy or better care. High proportion of those with positive family history of male infertility is supported and explained by large numbers of studies who identified or investigated the genetic defects in men with infertility(**Burton , *et al.* , 2006;Christensen, *et al.*, 2006; Sun, *et al.*, 2006 ;Okada , *et al.*, 2008; Martin , *et al.*, 2008.;Smith , 2008; ; Tanagho & McAninch , 2008).**

5.3 Medical history:

Following obtaining medical history relevant to male infertility, we detected that history of chronic disease was significantly higher (P<0.05) in infertile patients than control **(Table 4.4)**.In our sample, the most common disease was diabetes, followed by hypertension and epilepsy while the last one was tuberculosis.

On the other hand, we found no significant differences between patients and control with regard to history of genital tract infection , urinary tract infection , febrile illness before 3 months , endocrine disease feature or congenital disease **(Table 4.4)** .The genital tract infections that were mentioned were orchitis , prostatitis and gonorrhea in descending order. Only one patient reported the features of hypothyroidism. Besides, one patient reported history of undescended testis and another one mentioned that he had got single testis. Our observations were in accordance with those of **Okonofua *et al.*,(2005)** who stated that there was no significant difference between patients and control regarding history of frequent painful discharge, frequent painful urination or genital ulcer. **Gdoura *et al.*,(**

2007) detected that genital mycoplasmas and ureaplasmas were widespread among the male partners of infertile couples in Tunisia. Furthermore, they mentioned that genital mycoplasmas infections of the male genital tract could negatively influence semen quality. **Gubin *et al.*,(1998)** mentioned that prior genital infection is a significant risk factor for the development of anti-sperm antibodies. **Eskenazi *et al.*,(2003)** reported in a study on fertile men in U.S.A. that UTI history was positive in 12% of the sample.

5.4 History of injuries:

In this study no significant differences between infertile and fertile men were observed regarding history of testis injury , pelvic injury , head injury , spinal cord injury and radiotherapy or chemotherapy exposure **(Table 4.5)**.**Patki *et al.*, (2008)** reported that after spinal cord injury (SCI) ,there is ample evidence of disturbance of sperm production, maturation and storage and transport due to abnormal neuroendocrine milieu.

According to **Naderi & Safarineiad ,(2003)** , hypogonadotropism in SCI subjects is likely to be secondary to altered neural or hormonal pathway between hypothalamus and pituitary gland and that these endocrine abnormalities may be the mechanism causing impairment of semen. Our data demonstrated that various kinds of testicular or other injuries are infrequent in our sample of infertile men and may be of less importance as risk factor contributing for male infertility in our community

5.5 History of Varicocele:

The proportion of infertile men who reported history of varicocele was remarkably higher($P<0.01$) than in fertile men **(Table 4.6)**.The prevalence in patients was 23.45% while in control it was 3.33 % .The majority of varicoceles were left-sided. Nine patients of those who had got varicocele had performed varicocelectomy which constituted 47.3 % . **Nielsen *et al.* (2006)** observed that varicocele prevalence among infertile men was 18.9 % which was lower than our prevalence. In a study conducted by **Handel *et al.*, (2006)** ,it was concluded that varicocele was present in 34% of men with infertility. This result was higher than the result we obtained. Using ultrasonography to diagnose varicocele, **Qublan *et al.*,(2007)** found that varicocele found in 35.5% & 16% among infertile and fertile men respectively. Four studies**(Ghazzl,2006;Prabakara,2006;Kadyrov,2007;**

Kumarov,2008) investigated the prevalence of varicocele among adolescents and schoolchildren and mentioned that the prevalence was 2.7%,22.8%,10.5% & 7.9% respectively . In a study performed by **De Castro & Mastrorocco , (1984),** they reported that the prevalence of varicocele among prevasectomy fertile men was 16.2 % which was higher than our value. Our results showed that varicocele is widely-prevalent risk factor for male infertility in our sample and most likely in our Iraqi community. Although the other studies mentioned above had detected higher or lower proportion of varicocele in infertile men than we did, our prevalence of varicocele (i.e., 23.45%) is still high prevalence. This indicates that varicocele is an important contributor to infertility in Iraqi men and attention should be directed toward it. Grade I varicocele is impalpable while grade II is palpable only in the hand of the specialist doctor so the majority of patients are unaware of it and it is searched for only when the issue of infertility arises. Hence, both health education as well as screening programs for varicocele are needed. The high proportion of varicocelectomy operations in our infertile men is probably due to the great concern of these men regarding having children together with the low risk and complications expected from these operations. This makes the patients prefers to undergo surgery whenever varicocele is diagnosed.

The relation of varicocele to male infertility was postulated many decades ago by Tulloch in 1951. Seminal abnormalities observed in infertile men with varicocele were described extensively by MacLeod in 1965 who introduced the concept of “seminal stress pattern”: decreased sperm count, low motility, and abnormal sperm morphology**(De Castro & Mastrorocco,1984)**. **Tang *et al.*, (2007)** detected that varicocele causes damage to sperm DNA and changes sperm motility which may result in male infertility. **Safarinejad *et al.*, (2008)** , concluded that infertility were observed as significantly higher in the presence of varicocele (Odds Ratio OR 2.85). Varicocele have been clearly identified as an important cause of male infertility. However, the influence of varicocele on the men’s reproductive capacity has been subjected to debate due to its markedly diverse effects on the testicles. Varicocele’s elementary pathologies include variations in size, intratesticular temperature , hydrostatic pressure in the internal spermatic vein , different degrees of venous stasis and alteration in the hypothalamic-pituitary-hypogonadal axis which often cause deleterious effects on spermatogenesis. Varicocele can adversely affect sperm concentration, motility , morphology and

membrane , acrosome and chromatin integrity. Likewise, it increase the level of reactive oxygen species in the semen **(Andrade-Rocha , 2007; Benoff,*et al.*,2009)**. **Paduch & Skoog , (2001)** conducted a study for varicocele in adolescents and stated that varicocele is associated with time-dependent testicular growth arrest and is the most common correctible cause of male infertility. **Agarwal *et al.*, (2007)** observed that surgical varicocelectomy significantly improved semen parameters in infertile men with palpable varicocele and abnormal semen parameters. **Okeke *et al.*, (2007)** showed that no significant improvement in semen parameters may be obtained in patients with clinical varicocele and preoperative normospermia. It is possible that only patients with preoperative oligozoospermia may benefit from varicocelectomy.

5.6 <u>Surgical history:</u>

With respect to surgical history, history of scrotal surgery as well as testicular biopsy performance was significantly higher in infertile men than fertile group **(Table 4.7)**.This is expected because varicocele as well as azoospermia are common in our infertile men as we obtained in this study. Infertile men prefer to pass through the surgical options (i.e., varicocelectomy and testicular biopsy) in the hope of improving their fertility and then having a child. One facilitating factor for this high rate of performing of these operations is low risk and complications expected from those patients. Testicular biopsy is widely practiced in patients with azoospermia to differentiate between obstructive and nonobstructive causes of azoospermia as well as to assess spermatogenesis status. The most common surgical operations that were performed were surgery for varicocele , testicular biopsy ,scrotal hernia , hydrocele and undescended testis in decreasing frequencies. **Safarinejad *et al.*, (2008)** found that infertility was observed as significantly higher in the presence of history of cryptorchidism (Odds Ratio OR 3.81).Since varicocele may be widely prevalent in our community so it is expected to obtain high frequency of positive varicocele history and this is also the case for varicocelectomy operation. Besides, testicular biopsy procedure is also frequently performed in patients with azoospermia which constituted good percentage from the total infertile men in our study group.

5.7 Social and drug history:

In our study , infertile patients who were smokers were significantly higher (P<0.05) in proportion than fertile control **(Table 4.8)**.Our findings were in contrast with the observations of **Okonofua *et al.* , (2005)**who detected that the prevalence of smoking among infertile and fertile men was 46 % & 34.7 % respectively but here there was no statistical significance for the difference. Besides, **Goverde *et al.* , (1995)** stated that there was high but statistically insignificant proportion of heavy smokers in poor semen quality group compared to fertile control. Among fertile men , **Gao *et al.*, (2007)** found the prevalence of smoking to be 47.1 % whereas **Eskenazi *et al.*,(2003)** reported it to be 28% .

Furthermore, history of excessive heat exposure e.g. in job was also higher in patients than in control **(Table 4.8**). Two studies reported lower semen quality in those exposed to high temperature **(Bonde ,1992 ;Dada ,*et al.*,2002).** However , we reported no difference between infertile and fertile men regarding history of alcoholism , pesticides exposure , lead exposure and intense exercises. We were in line with **Goverde et al., (1995) and Okonofua *et al.,* (2005)** who found that there was no significant difference between infertile and fertile men with respect to history of alcohol intake. However, we disagreed with **Florack *et al.*, (1994)** observed that in males there was high fecundability in those with lower alcohol intake. Among fertile men , **Eskenazi *et al.*,(2003)** found the prevalence of alcohol intake as 65% while **Gao *et al.*,(2007)** reported the value of 42.2% .

Due to the large number and multiple systemic effects of smoking on human body, it is not surprising that cigarette smoking has negative effects on male reproductive system same as other tissues. But the relationship between cigarette smoking and male fertility remains controversial. Studies had shown that cigarette smoking affects sperm concentration , motility , and morphology and related with poor semen quality (**Florack ,*et al.*, 1994;Goverde, *et al.*,1995; Sepaniak, *et al.*, 2004)** .Recently ,studies show that cigarette smoking cause low semen quality with several mechanisms. One of these mechanisms is seminal oxidative stress induced reactive oxygen species (ROS) which has destructive effects on sperm structure and function.**(Colagar ,2007**).

Occupational exposure to high temperatures adversely affect testicular function causing partial or complete spermatogenic arrest. Dyers, cooks, blast furnace workers and men with varicocele are known to develop testicular hyperthermia, which leads to oligoasthenoteratozoospermia (OAT) and azoospermia (**Dada , *et al.*,2002)** . Spermatogenesis is sensitive to a variety of chemical and physical stressors. Testicular hyperthermia has been known to have a deleterious effect on male fertility since the time of Hippocrates and is a well-recognized cause of impaired sperm production . Its detrimental effect has been demonstrated in both animal models and in humans . Whether due to endogenous (such as high fevers) or exogenous stimuli, heat decreases sperm concentration, impairs motility, and reduces the number of morphologically normal sperm . This effect is striking enough that the effect of laptop computers on scrotal hyperthermia has recently been reported and the use of heat exposure as a male contraceptive has been studied **(Shefi, *et al.,*2007)**.

No significant difference was found between infertile and fertile men neither in the history of drugs that affect sperm function nor in the history of drugs that alter normal ejaculatory function **(Table 4.9)**.The drugs that were mentioned were synthetic androgen, cimetidine, amlodipine, valproic acid and imipramine. Our findings demonstrated that offending drugs administration may be of low importance as a contributing factor that can explain the cause for male infertility in our study group and possibly in our community .

5.8 Physical Examination:

Regarding general examination findings that were relevant to male infertility, no significant variations were demonstrated between patients and control in some important physical findings including diminished secondary sexual characters , klinefelter syndrome features , gynecomastia , goiter or endocrine disease features by examination **(Table 4.10)**.

On the other hand and concerning the local scrotal examination , we detected higher (P<0.01) proportion of infertile men with atrophied testis than in fertile men **(Table 4.11)**. Nevertheless, we obtained no differences with regard to relevant physical examination features like undescended ,or ectopic testis , single

testis , retractile testis , varicocele , testes tenderness , absent vas , testis mass , scrotal hernia or small penis.

Our findings were in accordance with **Okonofua *et al.*, (2005)** , who found that there was no significant difference between infertile men and fertile control with regard to testicular swelling or tenderness. **Arai *et al.*, (1998)** stated that testicular volume had the strongest positive correlation with sperm density followed in decreasing order by total sperm count per ejaculate. It also had the strongest negative correlation with serum FSH concentration followed by serum LH. However, they observed no significant correlation between testicular volume and serum Testosterone. Finally, they concluded that the measurement of testicular volume can be helpful for rapidly assessing fertility in initial physical examination. **Abramsson & Ducheck , (1989)** reported that men with low testicular volume had high concentration of serum FSH and serum LH and low concentration of serum testosterone due to negative feedback mechanism. The high prevalence of atrophied testes in our sample are expected finding in patients with primary or secondary hypogonadism .This because in both primary and secondary hypogonadism there is loss of the seminiferous tubules which form the bulk of testis substance and this lead to testicular atrophy.

5.9 Seminal Investigations:

Semen analysis is the backbone of male infertility evaluation **(Athayde & Cocuzza,2007) .**Several different approaches have been used to identify standards for normal semen measurements. Some focus on infertile couples, comparing those who conceive with those who do not. Others have compared fertile men with infertile men or have followed couples after they discontinued the use of contraception. Studies focusing on infertile couples undergoing treatment ,i.e., those comparing couples who conceive with those who do not conceive are limited by the inclusion of infertile couples only; in order to define the fertile ranges of semen measurements, fertile men must also be evaluated **(Guzick ,*et al.*,2001)**.

Semen quality has been considered as one of the most important indicators of environmental pollution . Previous retrospective studies have indicated that semen quality has been declining in the past several decades . However, semen quality is affected by various factors other than physical environments, such as age

occupation , cigarette smoking and other lifestyle factors(**Gao ,*et al.*,2007**). The WHO manual provides guidelines for assessing the various semen variables; however, it is still difficult to compare the values between different laboratories. Furthermore, several studies have indicated geographical differences in semen quality, probably related to environmental factors; however, ethnic or genetic differences cannot be excluded. A common set of reference values may therefore not be appropriate to use worldwide. **(Haugen ,2006) .**

For all the above, we included a control group to compare with the SFA findings of patients rather than comparing our patients' SFA results with the reference values of the WHO manual for semen analysis mentioned above. Furthermore, due to lack of the Iraqi studies that investigated the cut-off thresholds for the normal reference SFA values ,we were obligated to use the reference values of the manual to select fertile control.

5.9.1 Types of SFA abnormalities:

In our study, the percentage of patients with azoospermia were significantly higher ($P<0.01$) than those with oligoasthenozoospermia which was in turn higher than that of patients with oligozoospermia **(Table 4.12)** .Our findings were in contrast with those of **Trummer & Habermann , (2002)** who found that among infertile men in Austria, the highest percentage was in oligoasthenozoospermia group followed by asthenozoospermia, oligozoospermia then azoospermia groups in descending pattern. **Acacio *et al.*, (2000)** pointed out in a study conducted in U.S.A. that the proportions of semen samples of infertile men with abnormal sperm motility were significantly higher than samples with abnormal sperm concentration. A probable cause that explain the high proportion of azoospermia in our samples is that many cases of azoospermia are caused by primary testicular failure and the treatments options other than Assisted Reproductive techniques (ART) are less helpful, making their infertility problem remain unresolved and making them frequent visitors to the Infertility Center.

5.9.2 Abstinence :

Abstinence time was higher ($P<0.05$) in patients group than in control group but no significance difference was found among patients groups **(Table 4.13)**.In general, the range was 2 to 4 days. Our observations were in good agreement with

the findings of **Eskenazi *et al.*, (2003)** who showed that among fertile men group studied in U.S.A., the majority had abstinence time of 2 to 5 days range. Our results were in contrast with the results of **Menkveld *et al.* ,2001** who detected no difference in abstinence time between infertile patients and control in Netherlands. **Fekri *et al.*, (2009)** studied a group of 2940 infertile men in Tunisia and stated that even if a time of abstinence from 3 to 5 days was recommended before ejaculation, only 71% of men respected this period and the duration of abstinence varied between 1 and 8 days . This mean that our patients were more compliant to the instructions given for proper semen collection. **Pellestor *et al.*, (1994)** studied the effect of long abstinence periods on human sperm quality in French population. They found that lengthy sexual abstinence affects all semen characteristics. Besides, they mentioned that semen volume and concentration and total sperm count showed significant increases, whereas motility and normal morphology decreased significantly with duration of abstinence. The abstinence of 2 to 3 days is ideal and standardization for all patients is important although the range of 2 to 7 days is acceptable according to WHO manual **(WHO,1999)**.

5.9.3 Liquefaction time:

We detected that liquefaction time was longer($P<0.05$) in patients with oligoasthenozoospermia than control whereas no significant difference was found among patients groups **(Table 4.13)**.Among our infertile patients groups, the means for liquefaction time were 32.75±12.1, 34.16±10.35 and 33.24±9.72 minutes respectively. Our results were in agreement with **Trummer & Habermann, (2002)** who observed that the mean of liquefaction time among infertile men was 36 ±1.8 minutes. Liquefaction of semen depends on the factors and enzymes produced by the seminal vesicles and prostate as we mentioned above. So different liquefaction times in our oligoasthenozoospermic patients may indicates different status of function for these glands.

5.9.4 Semen pH:

No difference in the semen pH mean values was found neither between patients and control nor within patients groups **(Table 4.13)** .Furthermore, the vast majority of men studied showed normal semen pH according to WHO reference values **(WHO,1999)**.Our findings were in good agreement with those of **Menkveld *et al.*,(2001)** who pointed out no difference in semen pH between fertile

versus infertile men in Netherlands. Also our results were in consistence with those of **Haugen ,(2006)** who found similar results among fertile men in Norway. This indicates that pH abnormality was not an important contributing factor for men infertility in our sample because the majority of our infertile patients had got normal semen pH values. Normal semen pH is essential for sperm vitality.

5.9.5 Volume:

Although ejaculate volume varied from man to man, no significant difference was obtained in patients or control **(Table 4.13)**.These results agreed with the findings of many researchers who compared semen parameters in fertile versus infertile men (**Portuondo & Colabozo,1983;Ombelet &Bosmans,1997 ;Zinaman & Brown , 2000 and Menkveld, *et al.*, 2001)**.Our findings also were in line with many studies who investigated the infertile men alone (**Chia, *et al.*, 1998 ; Trummer & Habermann ,2002; Eskenazi, *et al.*, 2003 and Fekri , *et al.*,2009)** or fertile men alone **(Rehan *et al.* , 1975 ;De Castro & Mastrorocco,1984 and Li , *et al.*,2009)**.However our results were in contrast with the observations of **Haugen, (2006)** and **Small *et al.* , (1987)** who showed mean values for semen volumes in fertile men higher than our values. While in fertile men in our study the mean of semen volume was 2.15±0.63 ml, **Bang *et al.* (2005)** observed that the mean of ejaculate volume for fertile men studied in Denmark was 3.2±1.4 ml.We were in contrast also with the findings of **Menkveld *et al.*, (2001)** who mentioned that there was no significant difference in the ejaculate volumes between fertile and infertile males. Semen volume depends mainly on the secretions of seminal vesicles and prostate. Normal semen volumes in the majority of our patients may point out to a normal contribution of these glands to semen composition.

5.9.6 Viscosity:

This study demonstrated no significant variation in semen abnormal viscosity proportions neither between patients and control nor among patients groups themselves **(Table 4.13)** .Again, this may highlight the normal functions of seminal vesicles and prostate in these persons. Unfortunately, the studies that investigated semen parameters which was mentioned above did not mention any details regarding semen viscosity.

5.9.7 Sperm concentration:

Patients presented significantly lower (P<0.01) sperm concentrations than control **(Table 4.14)**.This study was in accordance with large number of studies who observed that infertile men had lower sperm concentration than fertile group **(Portuondo & Calabozo , 1983; Ayala , 1996; Ombelet & Bosmans , 1997 ; Menkveld , *et al.*, 2001 ; Athayde & Cocuzza, 2007 and Guzick, *et al.,*2007).** In our study the sperm concentrations in patients and control group were 0±0 , 9.03±5.06 , 12.41±5.14 and 89±26.43 ($\times 10^6$ million/ml) respectively (Table 4.14) .The means of sperm concentrations in fertile and infertile men in these studies were [47.7±33.43 , 15.76±10.66] ,[61.45 , 41.23] , [53.1 , 32.9],[81.07±49.7 , 18.97±26.5] ,[89.1±52.14,29 ±17.78] and [<48 ,<13.5] ($\times 10^6$ million/ml) respectively. With regard to our findings among infertile men, we were in disagreement with **Small *et al.*, (1987) ; Chia & Tay , (1998); Trummer & Habermann , (2002) ; Eskenazi *et al.*, (2003); Taskiran *et al.* (2006); Meeker & Bailey, (2007) and Fekri *et al.*, (2009)** who reported higher values for sperm concentrations than ours. This study were in line with the reports of **Rehan *et al.*, (1975) ;Sobrero & Rehan , (1975); De Castro & Mastrorocco ,(1984); Bang *et al.*, (2005); Haugen , (2006) and Li *et al.*, (2009)** who pointed out similar results of sperm concentrations in fertile men. **Fekri *et al.*,(2009**)concluded a decline in the sperm count and sperm morphology over a 12 year period among men in infertile relationships from the south of Tunisia, suggesting that the reported world-wide decline in semen quality is also real in certain areas of the African continent**.** Lower sperm concentration in our infertile men than control may explain their infertility. Azoospermic patients have zero concentration of sperm because their testes and seminiferous tubules has ceased to perform spermatogenesis and to produce sperms. In cases of primary hypogonadism, the core defect is in the testis itself while in the secondary hypogonadism, low levels of gonadotropins (FSH and LH) are the signals and causes that explain the cessation of spermatogenesis. The cause of azoospermia could be congenital or acquired. Important acquired causes includes varicocele, gonadotoxins, infections and ductal obstruction. Oligozoospermia may occur due to the same causes of azoospermia. Important factors that can ultimately lead to oligoasthenozoospermia includes varicocele, gonadotoxins, systemic infection, endocrinopathies and cryptorchidism and idiopathic causes (**Walsh , *et al.* , 2003**.).The variation in sperm concentration from other studies could be due to different degrees of

exposure of these factors mentioned above together with the role of genetic, racial and environmental factors. Normal sperm concentration is essential for natural successful fertilization.

5.9.8 Sperm motility:

In this study the sperm motility was significantly lower (P<0.01) in patients groups than control and in patients with oligoasthenozoospermia than patients with oligozoospermia. In our infertile and fertile men groups, the means of grade(a+b) motility were 0±0, 29.19±11.76 , 54.58±3.96 and60.33±6.55 (%) respectively (**Table 4.14**). The results of this study were in accordance with **Portuondo & Calabozo ,(1983); Ayala ,(1996); Ombelet & Bosmans ,(1997) ; Guzick *et al.* , (2001); Menkveld *et al.*, (2001) and Athayde & Cocuzza, (2007)** who stated that sperm motility was lower in infertile men than fertile population under study. The percentages of sperm motility in their fertile and subfertile groups were [52.58±16.06,28.38±10.83],[61.45,41.23],[53.4,45.8],[53.1±15.9,31.9±19.2],[68.5 ±58.7,49±35.5] and [>63,<32] (%) respectively.Our results were also in consistence with those of **Vasqez *et al.*, (1986) ; Bruno *et al.* , (1986)** who mentioned that average sperm motility was lower in patients than in control. We also agreed with **Fekri *et al.* (2009)** who showed results similar to our results in infertile men .Our observations in infertile men sperm motility were in contrast with the reports of **Trummer & Habermann , (2002) and Taskiran *et al.* (2006)** who showed lower values and also in disagreement with **Small *et al.* , (1987) ; Chia & Tay , (1998) ;Meeker & Bailey , (2007);** who demonstrated higher results than ours. Among fertile men, we were in line with **Bang *et al.* ,(2005)** and in disagreement with **Rehan *et al.*, (1975) ;Sobrero & Rehan , (1975); Eskenazi *et al.*, (2003); Li *et al.* ,(2009)** who observed higher means for sperm motility than we did. Important factors that can contribute to asthenozoospermia includes spermatozoal structural defects, prolonged abstinence, antisperm antibodies, partial obstruction. varicocele, infection and idiopathic causes (**Walsh , *et al.* , 2003**.).The difference between our results and other researchers reports is probably due to different exposure to the above risk factors and of course different genetic, racial and environmental backgrounds.Normal sperm motility is vital in the process of fertilization which usually occurs in the ampullary region of Fallopian tubes.

5.9.9 Sperm morphology:

We observed that the percentage of normal sperm morphology was significantly lower (P<0.01) in patients than in control.Also patients with oligoasthenozoospermia expressed lower values than control **(Table 4.14)**.The percentages of normal sperm morphology in our patients and control were 0± 0 , 32.5± 6.77, 34.5± 6.85 and 36.5± 5.79 (%) respectively **(Table 4.14)**. Many researchers reported similar results in infertile versus fertile population (**Portuondo & Calabozo, 1983 ;Menkveld , *et al.*,2001 ; Athayde & Cocuzza, 2007)** .They pointed out that the percentages of normal morphology sperms infertile and infertile men were [54.37±13.2,29.22±10.08],[40.1±14.1,21.7±10.9] and [20±12.28,10±5.22](%) respectively. Our results were in disagreement with **Ombelet & Bosmans , (1997)and Guzick *et al.*, (2001)** who found no difference in the infertile and fertile men groups.Our findings were in contrast with**, Chia & Tay ,(1998);Meeker & Bailey , (2007); Fekri *et al.* (2009)** who demonstrated percentages for normal sperm morphology in infertile men lower than ours and with **Small *et al.* (1987)** who reported higher values.Among fertile men, **Haugen ,(2006)** observed higher percentage than we did. Our results demonstrated that sperm morphology abnormalities may be of lower importance than low count or motility as a contributing factor for male infertility in our sample.However, Sperm Chromatin Structure Assays (SCSA) had demonstrated that sperms can be morphologically normal under light microscope but their DNA may be fragmented and this can be detected by special techniques other than the usual light microscopy. These DNA fragmentations can greatly affect sperm functions including motility **(Avendano ,*et al.*,2009)**.Normal sperm morphology depends on the accuracy of the steps of spermatogenesis from the stage of spermatogonium to the formation of the fully mature spermatozoa. Any defect at any step whether it is spontaneous , genetic or from the exposure to environmental factors could lead to teratozoopermia.It is important to mention that the WHO manual of semen analysis **(WHO,1999)** had proposed the reference value for accepted percentage of normal sperm morphology is 15% .This indicates that the process of spermatogenesis is naturally not a completely perfect process and some sperms with abnormal morphology is an expected finding. For some reason or another, normal sperm morphology is important in the process of fertilization and zygote formation.

5.9.10 Sperm vitality:

This study demonstrated that the percentage of sperm vitality was significantly lower ($P<0.01$) in patients than in control. Furthermore, patients with oligoasthenozoospermia expressed lower values($P<0.05$) than oligozoospermic group **(Table 4.14)**.Despite finding many articles comparing semen parameters in fertile and infertile men(**Portuondo & Calabozo ,(1983); Ayala ,(1996); Ombelet & Bosmans ,(1997) ; Menkveld *et al.*, (2001); ; Guzick *et al.* , (2001) and ; Athayde & Cocuzza, 2007**) ,we found no article presenting sperm vitality measurement although it is one of the component that should be measured whenever sperm motility is below 50% **(WHO,1999)**.

5.9.11 Sperm agglutination:

No significant difference in the frequencies of men with abnormal sperm agglutination was observed neither between patients and control nor among the patients groups **(Table 4.14)**.As the case for sperm vitality measurement, the articles dealing with semen quality measurement did not mention anything regarding sperm agglutination. Low level of agglutination found in our patients is probably indicating low levels of antisperm antibodies which usually causes marked sperm agglutination. Nevertheless, measurement of antisperm antibodies titer by special methods or kits is the most accurate method to investigate the presence of these antibodies.

5.9.12 Semen WBCs Count:

Seminal WBCs count was significantly higher in those with azoospermia and oligoasthenozoospermia than in control **(Table 4.14)**.We disagreed with the observation of **Trummer & Habermann,(2002)** who pointed out that WBCs count in the semen of infertile men was $2.7\pm 3.3 \times 10^6$ cell/ml .Our results also were in contrast with those of **; Athayde & Cocuzza ,(2007)** who found that seminal WBCs count in fertile men was zero and in infertile group to be 0.4×10^6 cell/ml ,i.e., they found lower values than ours.**Fekri *et al.* (2009)** mentioned similar results among infertile men. **Lackner *et al.* (2006)** demonstrated that in the first semen analysis of a group of infertile men , 21% of men had leukocytospermia . High semen WBCs count in our study groups may indicate the presence of concurrent infection which affects both sperm motility and vitality.

5.10 Serum Hormones:

Hormones play a vital role in initiating and maintaining male reproductive function, yet it is not well understood how variability in the levels of some hormones impact semen quality**.** It was reported that circulating levels of specific reproductive hormones in men are associated with semen quality parameters. In particular, inhibin B and FSH are thought to be markers of spermatogenesis and Sertoli cell function, and it has even been suggested that measuring the two hormones in serum could serve as a substitute for measuring semen quality or fecundability in epidemiologic studies **(Meeker & Bailey , 2007) .**

5.10.1 Serum hormones measurement:

This study showed that serum FSH in patients with azoospermia was significantly higher(P<0.01) than that of patients with oligoasthenozoospermia and oligozoospermia as well as higher(P<0.01) than control **(Table 4.15)**. Moreover, the level of S.FSH was significantly higher in the group of oligozoospermic patients than control **(Table 4.15)**.Regarding the level of serum LH & serum Prolactin, both hormones were higher(P<0.05) in patients than in control. Besides, no significant difference within patients subgroup was detected **(Table 4.15)**.Finally, serum Testosterone was markedly lower(P<0.05) in azoospermic patients than patients with oligoasthenozoospermia and oligozoospermia as well as lower(P<0.05) than fertile men **(Table 4.15)**.The pattern of hormonal abnormalities that we found in this study runs in line with that of hypogonadism. In primary hypogonadism , there is reduction in serum Testosterone level and elevation of serum FSH and serum LH values as a result of the negative feedback mechanism of the hypothalamic-pituitary-gonadal axis. In cases of oligozoospermia of whatever cause, again the negative feedback mechanism operates and cause similar hormonal pattern. Regarding serum Prolactin, although it was higher in patients than in control, it does not reached very high levels as seen in pituitary tumors. Mild elevation of prolactin could be the result of stress, exercise or medications and all of these are reversible **(Tanagho & McAninch , 2008)**.

The distribution of patients who had got oligozoospermia in our sample (all oligozoospermic patients with or without asthenozoospermia) according to the level of sperm concentration and serum hormones level had demonstrated that serum FSH was significantly higher(P<0.05) in those with sperm concentration

below 10 million/ml than those with concentration of 10-20 million/ml **(Table 4.16)** .Nevertheless, no significant differences between those with sperm concentration of <5 and 5-10 million/ml.Regarding serum LH, it was significantly higher(P<0.05) in the group of sperm concentration <5 and (5-10 million/ml than the groups of 10-20 million/ml. Serum Testosterone was significantly lower(P<0.05) in the group <5 than the group of 5-10 and 10-20 million/ml. Finally, no significant difference in serum Prolactin was found among the 3 groups of oligozoospermic patients. This point out to an important finding that serum hormones (FSH,LH,Testosterone and Prolactin) should not be requested routinely for every infertile men. Instead, they should be sent for only in cases of azoospermia , moderate to sever oligozoospermia (< 10 million/ml)or when there is clinical indication. In our Iraqi community, hormonal assays are not always available in every hospital and clinicians usually send infertile men to private laboratories in which the hormonal assays are costly. So this study stresses on proper selection of patients with respect to hormonal assays which reduces the costs and burden on the infertile patients.

The findings of hormonal abnormalities among fertile and infertile men from different studies including this study are summarized in **table 5.1** .In other studies that had compared the level of serum hormones in fertile against infertile men, many of these papers pointed out that serum FSH and serum LH was higher in men with infertility than fertile group **(Fossati , *et al.*,1979 ;Pierrepoint ,*et al.* ,1982; Bruno , *et al.* , 1986; Adenkunle , *et al.*,2000; Andersson , *et al.*, 2004 and Ali , *et al.*,2005**).**Pierrpoint *et al.*,(1982)** stated that the levels of the 2 hormones(i.e, serum FSH & serum LH) were significantly different between infertile and fertile groups. Moreover, they concluded that azoospermic patients had got high S.FSH level. **Bruno *et al.*, (1986); Adenkunle *et al.* ,(2000) and Ali *et al.* (2005)** reported similar results. **Check *et al.*, (1995)** detected that the most common hormonal abnormalities in the group of infertile men studied were elevated S.FSH levels. **Fossati *et al.*, (1979)** pointed out that serum FSH levels in fertile versus infertile males were 1.8 ng/ml & 3.8 ng/ml respectively while the levels of serum LH were 2.2 ng/ml & 3.3 ng/ml respectively .We were in good agreement with these papers. Some studies had measured serum FSH serum LH in infertile men only without comparison to healthy fertile men. **Abramsson & Duchek , (1989)** observed high serum FSH and serum LH values in patients with low sperm

concentration in Sweden men. Add to that, they mentioned that patients with azoospermia expressed high levels of these two hormones .**Geidam & Yawe, (2008)** estimated the levels of S.FSH & S.LH in infertile men group in Nigeria to be 20.7 IU/L & 12.8 IU/L respectively. Another researchers found that the levels of the 2 hormones in American men were 9.18 IU/L & 11.2 IU/L respectively (**Meeker & Bailey, 2007**). **Trummer & Habermann, (2002)** also investigated the infertile population and their results about the levels of serum FSH & serum LH were 7.1±7.9 mIE/ml & 4.4±2.8 mIE/ml respectively.In one study conducted in fertile men , the levels were 2.4 mIU/L & 3.6 mIU/L respectively **(Kehinde ,** ***et al.*****,2005)**. **Sina** ***et al.*** **,(1975) ; Bang** ***et al.*** **,(2005) and Dhooge** ***et al.*** **,(2007)** observed that serum FSH and serum LH levels in fertile men were [2.5 ng/ml & 2.2 ng /ml] ,[4 IU/L & 4 IU/L] and [3.6±3.8 IU/L& 3.7±2 IU/L] respectively. High serum FSH in the presence of low sperm concentration is expected from the negative feedback mechanism of the hypothalamic-pituitary gonadal axis

With regard to serum Testosterone, **Ali** ***et al.*** **(2005)** stated that serum Testosterone levels were significantly lower in infertile men than in fertile group in Pakistan. Furthermore, they pointed out that patients with azoospermia had lower levels of that hormone than in those with oligozoospermia. **Abramsson & Duchek ,(1989)** reported that among infertile males, azoospermic patients showed low serum Testosterone levels. **Modebe , (1994)** observed that in Nigerian men group, serum Testosterone was lower in oligozoospermic and hyperprolactinemic groups than in normospermic one.**Trummer & Habermann ,(2002); Meeker & Bailey,(2007) and Geidam & Yawe ,(2008)** investigated the level of S.Testosterone among infertile males and found it to be 4.2 ± 2.1 ng/ml, 419 ng/dl and 1.5 nmol/L respectively. **Kehinde** ***et al.*****,(2005)** observed that serum level of that hormone in fertile population in Kuwait was 12.6 nmol/L. **Sina** ***et al.*** **(1975); Bang** ***et al.*** **(2005) and Dhooge** ***et al.*** **(2007)** detected that S.Testosterone in fertile men in fertile men were 540 ng /dl , 23±6 nmol/L, 13.3 nmol/L respectively .The same researchers above had also estimated the levels of serum Prolactin. **Trummer & Habermann,(2002) and Geidam & Yawe, (2008)** found that serum Prolactin among infertile men was 13.1±7.7 ng/ml and 429.8pmol/L respectively. Besides, **Kehinde** ***et al.*****,(2005)** mentioned that the level of that hormone was 5.7 ng/ml among fertile men group. The low level of serum Testosterone which is accompanying high FSH in our azoospermic patients points

to the primary testicular failure rather than secondary one as the cause of seminal fluid abnormalities. **Bruno *et al.*,(1986)** demonstrated that serum FSH and LH increased when sperm density dropped to less than 5 $\times 10^6$ spermatozoa/ml . Our findings indicates that requesting the 3 hormones (serum FSH, serum LH and serum Testosterone) should not be done routinely for any male infertility patients with any SFA abnormality. Instead, it should preserved for those with moderate (5-10 million /ml) or sever (<5 million /ml) oligozoospermia and of course for those with azoospermia. Many authors and papers had supported this trend **(Walsh, *et al.*, 2003; Tanagho & McAninch ,2008).**

5.10.2 <u>Serum Hormones Correlations:</u>

In this study and in both fertile and infertile groups, there was a negative correlation between age and S.Testosterone as well as a positive correlation between age and each one of serum FSH, serum LH & serum Prolactin **(Table 4.17)** .Nevertheless, none of these correlations had reached a statistically significant level. Regarding the correlation between serum hormones and sperm concentration, we observed a statistically significant(P<0.05) positive correlation between S.Testosterone and sperm concentration in both patients and control (r = 0.91 , r = 0.93 respectively) **(Table 4.18)**.Furthermore, we found a negative correlation between serum FSH, serum LH & serum Prolactin (each separately) and sperm concentration but the only significant one(P<0.05) was that between serum FSH and sperm concentration in patients group(r = – 0.89) (Table 4.18).Furthermore, we concluded that there was a positive correlation between serum Testosterone and sperm motility as well as between serum Prolactin versus sperm motility **(Table 4.19)**.Add to that, we found a negative correlation between serum FSH against sperm motility as well as between serum LH and sperm motility (Table 4.19).It worth to say that none of these correlations between serum hormones and sperm motility was statistically significant.

We were in agreement with some papers which targeted the correlation between age or serum hormones versus semen parameters .**Meeker & Bailey, (2007)** pointed out that serum FSH, serum LH, and serum LH:Testosterone ratio were inversely associated with sperm concentration and morphology. **Pierrpoint *et al.*,(1982)** stated that there was an inverse relationship between serum LH and sperm count in both fertile & infertile men studied. However, they found that

Table

serum FSH was not varied with that parameter. **Vasquez *et al.*, (1986)** observed a reciprocal correlation between serum LH, sperm concentration and sperm motility.Nevertheless, he pointed out no correlation between serum FSH or prolactin with these semen measurements. In a study conducted by **Bruno *et al.* ,(1986)** they observed that there was a negative correlation between serum gonadotropins and sperm count. **Ishikawa *et al.*, (2004)** detected a negative relationship between serum FSH and sperm maturation arrest in Japanese men. **Dhooge *et al.*,(2007)** observed in Belgium a negative correlation between serum FSH, serum LH and serum Testosterone (each separately) versus sperm concentration , but it did not reach the statistical significance level.**Pincus *et al.*, (1997)** , observed in American men a significant negative correlation in age against serum FSH and serum LH ($r = -0.75$).**Yeap , *et al,.* (2007)** found that serum LH and sex hormones -binding globulin (SHBG) increased with aging in a study conducted in Australia on men above 70 years.They reached a conclusion that total serum Testosterone remained stable while free Testosterone decreased with age. The effects of age on male reproductive physiology have recently been reviewed . Aging is associated with decreased total serum and bioavailability testosterone concentrations, decreased testosterone to estradiol ratio, increased sex hormone-binding-globulin (SHBG) leading to increased plasma protein binding of circulating testosterone and decreased testosterone clearance, decreased LH pulse frequency, and diminished accumulation of 5α-reduced steroids in reproductive tissues **(Kandeel , *et al.*,2001).**

Our study demonstrated good correlation between serum Testosterone and sperm concentration as well as between serum FSH and sperm concentration. This observation may indicates that serum Testosterone and serum FSH are the best two hormones to be requested for initial evaluation of male infertility in our Iraqi men and possibly for all infertile men. This selectivity again reduces the hormonal assays costs on the patients. If these two hormones are abnormal, then the full endocrine evaluation could be started.

5.11 Hematological investigations:

We observed that the mean of PCV was significantly lower ($P<0.05$) in infertile men than fertile control **(Table 4.20)**.Besides, patients with azoospermia demonstrated higher($P<0.05$) PCV than those with oligoasthenozoospermia or oligozoospermia. Total WBCs count was significantly higher ($P<0.001$) in patients than in control(Table 4.20).Moreover, patients with oligoasthenozoospermia had got higher($P<0.05$) values than those with azoospermia and both were higher($P<0.05$) in WBCs count than those with oligozoospermia. Erythrocytes sedimentation rate(ESR) mean was higher($P<0.05$) in patients with oligoasthenozoospermia or oligozoospermia than those with azoospermia or control (Table 4.20).Besides, patients with oligoasthenozoospermia had got higher($P<0.05$) ESR level than those with oligozoospermia. The only studies that we found are those that investigated the association between sickle cell anemia and male infertility **(Grigg, 2007 and Berthaut, 2008)**. Long term treatment with testosterone can lead to polycythemia while elevated total WBCs count and ESR are expected in infections and inflammatory conditions. It is important to mention that although there was significant differences between patients and control or among patients groups, the majority of the hematological values for patients and control were within the standard reference ranges. This indicates that our infertile men demonstrated no marked abnormalities in the levels of PCV, total WBCs and ESR.

Chapter Six
Conclusions & Recommendations

6.1 Conclusions:

Our study was one of the Iraqi studies that explored the clinical and hormonal risk factors among Iraqi infertile men and control and yielded many findings:

1. Primary infertility is more predominant than secondary type.
2. Most infertile men are young.
3. Urban infertile men are more than the rural.
4. While many clinical risk factors were prevalent in infertile men in many studies conducted in the Western countries, many of these risk factors appeared to be of low frequencies and possibly of low importance in contributing to male infertility in our own Iraqi patients.
5. Men with infertility have higher proportion of positive history of wifes' abortion.
6. Men with infertility showed higher degree of loss of libido,lower socio-economic status and lower use of contraceptives.
7. Infertile men demonstrated higher positivity for family history of male infertility.
8. Chronis diseases are more frequent in men with infertility.
9. Infertile men have higher proportion of testicular varicocele history.
10. They have higher proportion of scrotal surgery and testicular biopsy.
11. They demonstrated more prevalence in smoking and excessive heat exposure.
12. By physical examination, they demonstrated higher proportions of atrophied testes.
13. The highest proportion of seminal fluid analysis abnormalities was azoospermia followed by oligoasthenozoospermia then oligozoospermia in descending order.
14. Patients and control as well as patients groups (azoospermia, oligoasthenozoospermia and oligozoospermia) were different with regard to abstinence time and liquefaction time (higher values in patients).

15. Infertile men showed lower sperm concentration ,lower motility, lower propotions of normal sperm morphology than fertile control. Within patients groups differences in these parameters are prominent also.
16. Our infertile and fertile men have different SFA parameters and hormones levels from those of Western countries population.
17. Infertile patients have higher serum levels of FSH ,LH and Prolactin and lower values for serum Testosterone.
18. Patients with azoospermia have higher levels of serum FSH and lower levels of serum Testosterone than other patients groups.
19. Oligozoospermic men with sperm concentration below 10 million/ml showed higher serum FSH and higher serum LH than those with sperm concentration of 10-20 million/ml .Furthermore, those with sperm concentration below 5 million/ml demonstrated lower serum Testosterone than those with 5-10 and 10-20 million/ml sperm concentration .
20. Significant correlations between serum Testosterone and sperm concentration as well as serum FSH & sperm concentration were observed.
21. The results of this study confirm that measurements seminal fluid analysis parameters and serum hormones (FSH,LH,Testosterone and Prolactin) all provide useful information for evaluating male infertility
22. Significant differences were obtained between patients and control regarding the values of PCV , WBC and ESR but the majority of values were within the normal reference ranges.

6.2 Recommendations:

Based on the multiple observations in this study we recommend the following:

1. Obtaining careful , detailed and relevant history and examination from men with infertility is the cornerstone to reach a correct diagnosis of the cause and risk factors for male infertility and it is vital step in the evaluation as well as the management.
2. Seminal fluid analysis should be performed in all research centers and medical laboratories using the most recent guidelines recommended by the *WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction* (WHO, 1999) and care should be taken to adhere

to all the details and recommendations as well as quality control techniques to avoid errors as much as possible. While the manual is being now under revision, the latest version of the manual should be always adopted in the future.

3. Performing large-scale studies in Iraq and other regional countries to estimate the cut-off threshold values of normal seminal fluid analysis and normal serum hormones values of our own Iraqi population.
4. It is important that there should be more published reports on the reference ranges of seminal fluid analysis results from various laboratories throughout the world.
5. prospective monitoring of sperm parameters should be carried out in the future to assess the changes in men gonadal function in Iraq.
6. Serum sex hormones should not be requested on routine basis for every patient with male infertility. Instead , it should be preserved for patients with azoospermia and those with moderate to sever oligozoospermia (sperm concentration < 10 million/ml).
7. Serum FSH and serum Testosterone are the best two hormonal investigations for initial hormonal evaluation for male infertility. This is because they showed good correlation with sperm concentration.
8. Conduction of multi-center studies in Iraq and regional countries to further explore and consolidate the evidence provided by this study regarding clinical , hormonal and other laboratory risk factors for male infertility.

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الخلاصة

كان الهدف من هذه الدراسة الضابطة هو معرفة عوامل الخطورة السريرية للعقم الذكري واستكشاف اضطرابات متغيرات السائل المنوي للمرضى الذكور العراقيين المصابين بالعقم ولدى المجموعة الضابطة ايضا.الهدف الاخر للدراسة كان لبيان اضطرابات الهرمونات (FSH , LH وهرمون التستوستررون وهرمون البرولاكتين) في مصل الدم لدى مجاميع الدراسة وارتباط مستوى هذه الهرمونات بمتغيرات السائل المنوي واخيرا استكشاف بعض التغيرات الدموية لدى مجاميع الدراسة.

تم اختيار 81 مريض مصاب بالعقم الذكري و30 شخص غير مصاب بالعقم حسب معايير الاختيار وتم ملء استبيان معد خصيصا للدراسة لتثبيت عوامل الخطورة السريرية(التاريخ المرضي والفحص السريري) لدى المرضى والمجموعة الضابطة وتم اجراء تحليل السائل المنوي حسب معايير منظمة الصحة العالمية وتم تقسيم مجموعة المرضى الى 3 مجاميع فرعية حسب نتائج تحليل السائل المنوي (مجموعة عدم وجود الحيامن ومجموعة نقص عدد وحركة الحيامن ومجموعة نقص الحيامن).كما تم ايضا اجراء فحوص هرمونية لهرمونات (FSH , LH وهرمون التستوستررون وهرمون البرولاكتين) في مصل الدم لمجموعتي المرضى والمجموعة الضابطة بطريقة (الايزا) (ELIZA) المناعية.كما تم اجراء فحوص دموية وهي (حجم الخلية المكدسة وعدد كريات الدم البيضاء الكلي وسرعة تثفل كريات الدم الحمراء).تم اجراء مقارنة بين مجموعة المرضى والمجموعة الضابطة حول عوامل الخطورة السريرية ومتغيرات السائل المنوي ومستوى الهرمونات في مصل الدم والفحوصات الدموية.

اظهرت النتائج انتشار بعض عوامل الخطورة السريرية بين مجموعة مرضى العقم الذكري اكثر من المجموعة الضابطة الغير مصابة بالعقم وكانت هذه الفروقات معنوية وتشمل هذه العوامل تاريخ حدوث الاسقاط لدى الزوجة وقلة الرغبة الجنسية وقلة المستوى المعيشي وقلة استعمال موانع الحمل وتاريخ اصابة احد افراد العائلة بالعقم الذكري والاصابة

بالامراض المزمنة ودوالي الخصية واجراء عمليات في كيس الصفن وعمليات اخذ خزعة من الخصية والتدخين وكثرة التعرض للحرارة العالية واخيرا ضمور الخصى.اظهر المرضى الذكور العراقيون المصابون بالعقم انخفاضا في مستوى متغيرات السائل المنوي اكثر من المجموعة الضابطة الغير مصابة بالعقم وكانت هذه الاختلافات معنوية وكانت هذه التغيرات اكثر وضوحا لدى مجموعة المرضى اللذين لديهم انعدام الحيوانات المنوية في السائل المنوي وخصوصا في مستوى الهرمون FSH وهرمون التستوستيرون .وقد اظهرت المجاميع الفرعية للمرضى ايضا اختلافات في متغيرات السائل المنوي .وفيما يتعلق بمستوى الهرمون FSH والهرمون LH وهرمون التستوستيرون وهرمون البرولاكتين في مصل الدم فقد تم التوصل الى وجود اختلافات معنوية بين مجموعة المرضى والمجموعة الضابطة وبين المجاميع الفرعية للمرضى المصابين بالعقم الذكري.كما تم التوصل ايضا الى وجود علاقة وترابط بين مستوى الهرمون FSH وهرمون التستوستيرون ومستوى تركيز الحيوانات المنوية في السائل المنوي.واخيرا اظهرت نتائج الفحوص الدموية وجود اختلافات معنوية بين مجموعة مرضى العقم الذكري والمجموعة الضابطة ولكن معظم القيم الفردية الخاصة بالمجموعتين كانت ضمن الحدود الطبيعية.

يمكن الاستنتاج بان هنالك اختلاف في عوامل الخطورة السريرية و متغيرات السائل المنوي ومستويات الهرمونات الجنسية في مصل الدم بين المرضى العراقيين الذكور المصابين بالعقم و مجموعة الرجال الغير مصابين بالعقم(المجموعة الضابطة) وتوجد ايضا اختلافات بين المجاميع الفرعية لمجموعة المرضى أيضاً.ومن الأمور المهمة ان هذه العوامل والاختلافات يجب ان تؤخذ بنظر الاعتبار من قبل الطبيب الاخصائي عند تشخيص وعلاج ومتابعة مرضى العقم الذكري.بالإضافة الى ذلك فان كل فحوصات السائل المنوي يجب ان تعمل حسب أخر المعايير التي تعتمدها منظمة الصحة العالمية.ومن الاستنتاجات المهمة الأخرى ان قياس مستوى الهرمونات الجنسية المذكورة اعلاه يجب ان لايتم بشكل روتيني لاي مريض مصاب بالعقم الذكري وانما فقط للمرضى اللذين لديهم انعدام الحيوانات المنوية في السائل المنوي او قلة متوسطة الى شديدة (اقل من 10 مليون حيمن لكل مليلتر) او عند

وجود دواعي سريرية.ومن الاستنتاجات المهمة ايضا ان الهرمون FSH وهرمون التستوسترون هما الهرمونان اللذان يجب ان يعملا اولا عند اجراء تقييم اولي لمستوى الهرمونات الجنسية للمرضى الذكور المصابين بالعقم.واخيرا توصي الدراسة باجراء المزيد من الدراسات حول هذا الموضوع لزيادة تاكيد ودعم النتائج الواردة في هذه الدراسة.

دراسة سريرية و هرمونية للرجال المصابين بالعقم

رسالة تقدم بها

احمد توفيق نعمة
بكالوريوس طب وجراحة عامة

إلى
مجلس كلية الطب / جامعة بابل
وهي جزء من متطلبات نيل درجة ماجستير علوم في الفسلجة الطبية

إشراف
أ.م.د. سعد مرزة الاعرجي
أ.م.د.عماد حسن محمود

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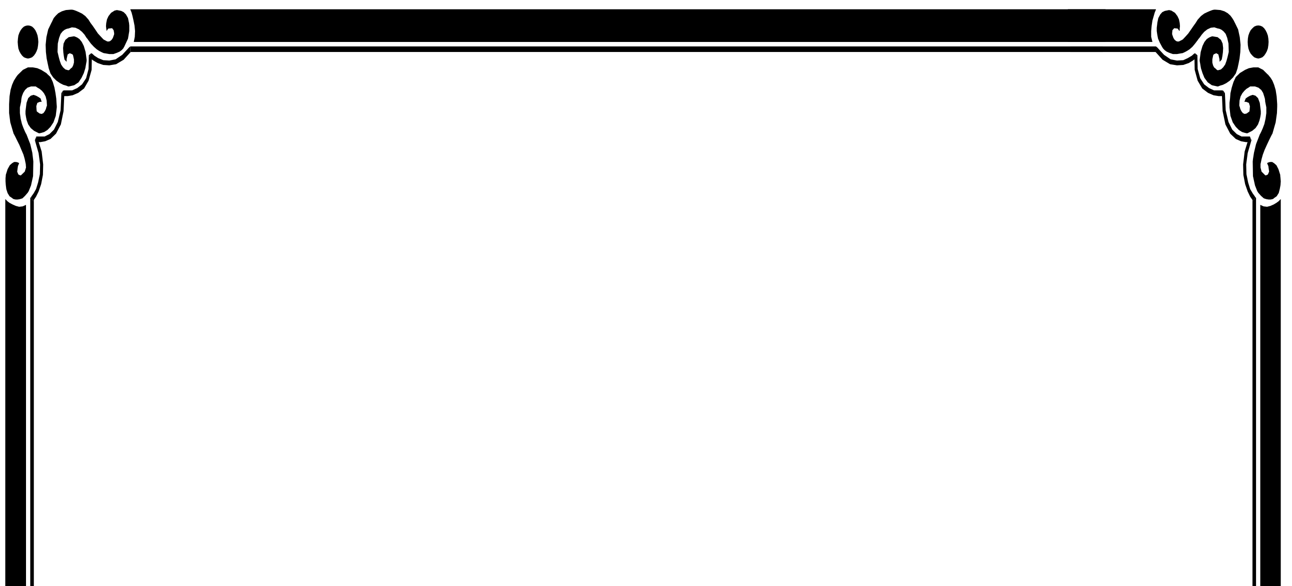
CHAPTER ONE
INTRODUCTION

LITERATURE REVIEW

CHAPTER THREE

MATERIALS AND METHODS

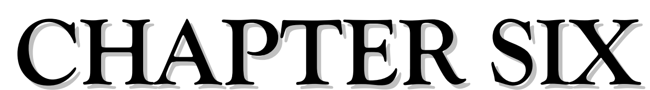


CHAPTER FOUR

RESULTS

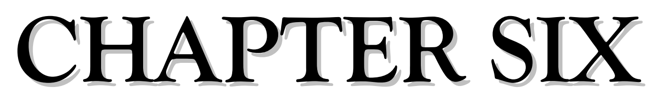
CHAPTER FIVE

DISCUSSION



CHAPTER SIX

CONCLUSIONS & RECOMMENDATIONS



CONCLUSIONS & RECOMMENDATIONS

CHAPTER SEVEN

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Table(5.1) : The results of serum hormonal assays for infertile and fertile men from multiple studies

Authors	Year	Serum FSH		Serum LH		Serum Testosterone		Serum Prolactin	
		Infertile	Fertile	Infertile	Fertile	Infertile	Fertile	Infertile	Fertile
Sina *et al.*	**1975**		2.5 ng/ml		2.2 ng /ml	-	540 ng /dl	-	-
Fossati *et al.*	**1979**	3.8 ng/ml	1.8 ng/ml	3.3 ng/ml	2.2 ng/ml	-	-	-	-
Trummer & Habermann	**2002**	7.1±7.9 mIE/ml		4.4±2.8 mIE/ml	-	4.2 ± 2.1 ng/ml	-	13.1±7.7 ng/ml	-
Bang, *et al.*	**2005**	**-**	3.6±3.8 IU/L	-	3.7±2 IU/L	-	23±6 nmol/L	-	-
Kehinde , *et al.*	**2005**	**-**	2.4 mIU/L	-	3.6 mIU/L	-	12.6 nmol/L	-	5.7 ng/ml
Dhooge, *et al.*	**2007**	**-**	4 IU/L	-	4 IU/L	-	13.3nmol/L	-	-
Meeker & Bailey	**2007**	9.18 IU/L		11.2 IU/L	-	419 ng/dl	-	-	-
Geidam & Yawe	**2008**	20.7 IU/L	-	12.8 IU/L	-	1.5 nmol/L	-	429.8pmol/L	-
Present Study	**2009**	22.63±21.5* 10.41±8.4** 11.22±7.41† mIU/ml	2.81±2.22 mIU/ml	14.84±14.33* 12.04±11.1** 13.55±12.66† mIU/ml	3.80±2.67 mIU/ml	2.39±2.34* 4±0.71** 3.8±1.64† ng/ml	5.1±1.07 ng/ml	7.12±4.34* 7.56±6.15** 9.28±8.97† ng/ml	4.01±1.83 ng/ml

***in infertile men with azoospermia**
****in infertile men with oligoasthenozoospermia**
†in infertile men with oligozoospermia

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Appendix-I

Questionnaire

Patient No.
( )

- Patient name:
- Age : **y***
- Occupation :
- Residence (urban◌ , rural◌)
- Duration of infertility : **/y**
- Have children? **(yes◌, no◌)**if (yes) then what is number of children **(Males◌,Females◌) / No. ( ),**
  previous wife children ?**(Males◌,Females◌)/ No. ( )**
- History of abortions : **(yes◌, no◌) / Times : ( ) /Trimester :**
- Frequency of sexual intercourse : **/week /m , Timing:**
- Use of lubricants ?◌ **Type: ,every coitus?: (yes◌, no◌)**
- History of contraception in wife? **(yes◌, no◌) ,Type:**
  Duration:
- Onset of puberty : **/y**
- Family history of infertility(husband)**(yes◌, no◌) relationship**:

- **<u>Erectile or ejaculatory dysfunction history:</u>**

- Impotence **(yes◌, no◌)**
- Premature ejaculation **(yes◌, no◌)**(before intercourse or they feel it premature)
- Retrograde ejaculation **(yes◌, no◌)** (small volume semen, Normal orgasm,semen in urine)
- Ejaculatory impotence (ejaculate by masturbation but not by intercourse) **(yes◌, no◌)**
- Lack of ejaculation **(yes◌, no◌)**

- Painful intercourse **(yes◯, no◯)**
- Libido : **(decreased◯, N◯)**
- Stressful environment **(yes◯, no◯)**
- Relationship with wife **(very good◯,good ◯ , bad◯)**

Past medical history

Infections :
(yes◯,no◯)

(orchitis, epididymitis,
prostatitis,STD,syphilis,gonorrhea,etc..)
TB,leprosy,brucellosis, ,typhoid
fever, influenza , smallpox)
mumps (post-puberty)
fever (before 3 m)
recurrent respiratory tract infections

systemic & chronic diseases:
(yes◯, no◯)

renal diseases, live diseases,
hypertension , diabetes, celiac disease
heart diseases, vascular diseases,
sickle cell anemia, anemia,
neurological disorders, hemochromatosis
brain tumors, other malignancies

endocrine disorders:
(yes◯,no◯)

gonads, thyroid, pituitary, adrenal,
galactorrhea, sever headache,visual field
abnormalities

congenital disorders:
(yes◯,no◯)

undescended testes, absent or obstructed
vas deference, Hypospadias, Klinefelter
syndrome, cystic fibrosis , polycystic
kidney disease, Kartagener syndrome

History of surgical operations and injuries

Injuries: testicular or pelvic injuries, testicular torsion,
(yes◌,no◌) excessive heat applied to testes,testes irradiation(e.g. X-ray), spinal cord injury, head trauma, radiotherapy, Chemotherapy

History of varicocele with or without treatment

(yes◌,no◌) if yes before **…… /y** diagnosed, duration **……/y**, treatment was before …… /**y** and by ……………… and result was **………………………..**

Previous surgical history : inguinal surgery (e.g. repair of hernia) (especially use of mesh)

(yes◌,no◌) scrotal surgery (e.g. hydrocele, vasectomy, vasectomy reversal, varicocele ,orchidopexy) prostate, bladder neck, retroperitoneal surgery, abdominal surgery

Social and exposure history

- Smoking **(yes◌,no◌)** (**quantity, duration**)(………...., …………)
- Alcohol **(yes◌,no◌)**(**quantity, duration**) (………...., …………)
- Intense exercise **(yes◌,no◌) duration /y**
- Pesticides exposure **(yes◌,no◌) duration /y**
- Lead exposure (paints, batteries workers) **(yes◌,no◌)duration /y**
- Long distance runners **(yes◌,no◌) duration /y**
- Long distance bicycling **(yes◌,no◌) duration /y**
- excessive heat exposure jobs **(yes◌,no◌) duration /y**

Family history:

- testicular atrophy
- hypogonadotropic hypogonadism
- cryptorchidism
- Hypospadias
- intersex(androgen abnormalities)
- congenital medline defect

Drugs affect sexual function

- H2 blockers (cimetidine) **(yes◯,no◯) Duration /y Dose:**
- Ketokenazole **(yes◯,no◯) Duration /y Dose:**
- Spironolactone **(yes◯,no◯) Duration /y Dose:**
- Ca-channel blockers **(yes◯,no◯) Duration /y Dose:**
- Allopurinol (zyloric) **(yes◯,no◯) Duration /y Dose:**
- Colchicine **(yes◯,no◯) Duration /y Dose:**
- Antibiotics e.g. nitrofuran, erythromycin,gentamicin
  (yes◯,no◯). Duration /y Dose:
- Corticosteroids **(yes◯,no◯) Duration /y Dose:**
- Phenytoin **(yes◯,no◯) Duration /y Dose:**
- Na valproate **(yes◯,no◯) Duration /y Dose:**
- Methadone **(yes◯,no◯) Duration /y Dose:**
- Methotrexate **(yes◯,no◯) Duration /y Dose:**
- Salazopyrine **(yes◯,no◯) Duration /y Dose:**
- caffaine **(yes◯,no◯) Duration /y Dose:**

Drugs affect ejaculation

Anti-hypertensive: guanithedine, prazocin, phenoxibenzamine,
phentolamine, reserpine, thiazides

sex steroids for body build
anti-depressants: amitryptiline, imipramine, fluoxetine.sertraline

anti-psychotic : chloropromazine, haloperidol , Librium (chlordiazipoxide)

<u>Previous investigations for infertility & its results (male & Female)</u>

<u>Previous treatments for infertility & its duration</u>

- Drugs : **(yes◯,no◯) type: duration:**
- Surgery : **(yes◯,no◯) type: before:** y
  results:
- ART (assisted reproductive techniques) : **(yes◯,no◯)**
  Type:

PART-II Examination:

A -General examination:

- secondary sexual characteristics (facial, axillary and pubic hair, voice, musculature, **(present◯,absent◯)**
- body build **(overweight◯ or not◯)**
- gynecomastia **(present◯,absent◯)**
- features of Klinefelter syndrome **(present◯,absent◯)**
- features of endocrine disorders(e.g. hypothyroidism & acromegally) **(present◯,absent◯)**
- lymph nodes enlargement **(present◯,absent◯) Site: ………**
- Goiter **: (present◯,absent◯)**

B-scrotal examination:

<u>testes:</u> undescended testes,ectopic, retractile **(present◯,absent◯)**
size **(small atrophied ◯ , normal ◯)**
consistency **(firm◯ , soft ◯)**
tenderness **(yes◯,no◯)**
swelling **(yes◯,no◯)**
varicocele **(yes◯,no◯)** (especially **Left** side)
mass **(yes◯,no◯)**
absence of vas & epididymis **(yes◯,no◯)**

hydrocele **(yes◯,no◯)**
hernia **(yes◯,no◯)**

Penis : length (infantile) **(yes◯,no◯)**
congenital abnormalities (e.g. Hypospadias , phimosis)
(yes◯,no◯)
If (yes) then mention: ____________________

*** y: year**

Appendix-II
WHO form for Seminal Fluid Analysis (WHO,1999)

Patient name:
Patient no. :
Date of sample: / /200
Duration of abstinence : **…….** days
Time between sample arrival and test performance:

Parameter	Value	Reference Value
Appearance : **Color :**	(normal, abnormal) ( )	
Liquefaction time:	min	≤60 min
Volume :	ml	≥ 2 ml
PH :		≥ 7.2
Viscosity :	(normal, abnormal)	
Motility :	A (%) (rapid) B (%) (slow) C (%) (sluggish) D (%) (immotile)	≥ (50 %) grade A+B
Agglutnation :	(-) (+) (++) (+++)	
Vitality :	(%)	≥ (50 %) / 200 sperms
Concentration :	$\times 10^6$/ml	$\geq 20\times10^6$/ml
Morphology :	Normal (%) Abnormal (%)	In 100 sperms
WBC	$\times 10^6$ /ml	$\leq 1\times10^6$ /ml

Appendix-III
Sperm vitality by dye exclusion

Reagents:

EosinY stain ; A (5 g/l) solution of EosinY (Colour Index, C.I. 45380) was dissolved in a (9 g/l) aqueous sodium chloride solution.

Procedure:

One drop of fresh semen was mixed with one drop of the Eosin solution on a microscope slide, covered with a cover slip and examined after 30 seconds at 400 X with a light microscope. These slides were assessed immediately: live spermatozoa were unstained **(white)** while dead cells were stained **(red) (WHO, 1999)**.

Appendix-IV
Assessment of sperm concentration

The dilution is determined (1:5, 1:10, 1:20, 1:50) from the preliminary estimation of sperm concentration **(Table 3.4)**. For example, a 1:20 dilution was made by diluting 5 μl of liquefied semen with 95 μl of diluent. The diluent was prepared by adding 50 g of $NaHCO_3$ to 10 ml of (40 %) saturated formaldehyde solution. Then the constituents were dissolved in distilled water and diluted to 1000 ml. A positive displacement type of pipette was used for loading chamber **(WHO, 1999)**.

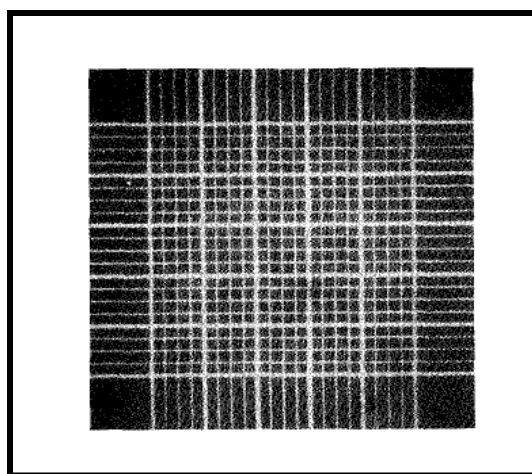

Fig.(IV-I) The central grid of the improved Neubauer haemocytometer contain 25 squares in which the spermatozoa are to be counted (WHO, 1999).

The coverslip was secured on the counting chambers of the improved Neubauer haemocytometer .After that 10 μl of the thoroughly mixed diluted specimen was transferred to each of the counting chambers of the haemocytometer. This was done by carefully touching the edge of the cover glass with the pipette tip and allowing each chamber to fill by capillary action. Care was taken to ensure that chambers were not over or underfilled and the cover glass was not moved. The haemocytometer was allowed to stand for about five minutes . The cells sediment during this time and were then counted at a magnification of 400X. The count was made of complete spermatozoa (heads with tails) **(WHO, 1999)**.

The procedure for counting the spermatozoa in the haemocytometer chamber was as follows: The central square of the grid in an improved Neubauer haemocytometer (which is usually used for RBCs count) contains 25 large squares, each containing 16 smaller squares **(Fig.3.2)**. For samples containing fewer than ten spermatozoa per large square, spermatozoa in the whole grid of 25 large squares were assessed; for samples containing 10 to 40 spermatozoa per large square, 10 large squares were assessed; and, for samples containing more than 40 spermatozoa per large square, spermatozoa in 5 large squares were assessed(**Table 3.4**). If a spermatozoa lies on the line dividing 2 adjacent squares, it were counted only if it was on the upper or the left side of the square being assessed. In order to determine the concentration of spermatozoa in the original semen sample in millions/ml, the average number of spermatozoa was divided by the appropriate conversion factor shown in **(Table 3.4)**. For example, for an average count of 230 on a 1 : 20 dilution and ten squares counted per chamber, the conversion factor is 2 and the sperm concentration 115×10^6/ml **(WHO, 1999)**.

Chambers, other than the haemocytometer, are available for determining sperm concentration, e.g., the Makler chamber and the Microcell . Such chambers, while convenient in that they can be used without dilution of the specimen, may lack the accuracy and precision of the haemocytometer technique. When used, it is recommended that their validity be established by comparison with the haemocytometer method **(WHO, 1999)**.

Appendix-V
Method for detecting leukocytes in semen

The traditional method for counting leukocytes in human semen is to use a histochemical procedure to identify the peroxidase enzyme that characterizes polymorphonuclear granulocytes. This technique has the advantage of being relatively easy to perform(**WHO, 1999**).

Peroxidase stain using Ortho-toluidine stain:

Reagents:

1. Saturated NH_4Cl solution (250 g/l)
2. Na_2EDTA, 50 g/l in phosphate buffer (pH 6.0)
3. Ortho- toluidine stain (0.25 mg/ml)
4. H_2O_2 (30 %) in distilled water

The working solution consists of: 1 ml of reagent 1; 1 m1 of reagent 2; 9 m1 of reagent 3; and one drop of reagent 4.

Procedure:

1. We withdrew 0.1 m1 semen and it was mixed with (0.9) m1 working solution.
2. This is followed by shaking for 2 minutes.
3. The mixture was left for 20-30 minutes at room temperature.
4. This is followed by shaking again.
5. Peroxidase-positive cells were stained brown, while peroxidase-negative cells were unstained.
6. Leukocytes were counted in a haemocytometer chamber to obtain their concentration.(**WHO,1999**).

Appendix-VI
Serum Testosterone measurement

I-Principle of the assay:

The Testosterone ELIZA assay is based on the principle of competitive binding between Testosterone in the test specimen and Testosterone-HRP conjugate for a constant amount of rabbit anti- Testosterone. In the incubation, goat anti-rabbit IgG-coated wells are incubated with 10 µl of Testosterone standards, controls, patient samples, 100 µl Testosterone-HRP conjugate reagent and 50 µl rabbit anti-Testosterone reagent at 37°C for 90 minutes. During the incubation, a fixed amount of HRP-labeled Testosterone competes with the endogenous Testosterone in the standard, sample, or quality control serum for a fixed number of binding sites of the specific Testosterone antibody. Thus, the amount of Testosterone peroxidase conjugate immunologically bound to the well progressively decreases as the concentration of Testosterone in the specimen increases. Unbound Testosterone peroxidase conjugate is then removed and the wells washed. Next, a solution of TMB Reagent is then added and incubated at room temperature for 20 minutes, resulting in the development of blue color. The color development is stopped with the addition of 1N HCl, and the absorbance is measured spectrophotometrically at 450 nm. The intensity of the color formed is proportional to the amount of enzyme present and is inversely related to the amount of unlabeled Testosterone in the sample. A standard curve is obtained by plotting the concentration of the standard versus the absorbance. The Testosterone concentration of the specimens and controls run concurrently with the standards can be calculated from the standard curve.

II-Reagents:

A-Materials were provided with the kit:

- Goat Anti-Rabbit IgG-coated microtiter wells, 96 wells
- Testosterone Reference Standards: 0, 0.1, 0.5, 2.0, 6.0 and 18.0 ng/ml. Liquids, 0.5 ml each, ready to use.
- Rabbit Anti-Testosterone Reagent (pink color), 7 ml
- Testosterone-HRP Conjugate Reagent (blue color), 12 ml

- TMB Reagent (One-Step) 11 ml.
- Stop Solution (1N HCl), 11 ml.

B-Materials were used but not provided with the kit:

- Micropipettes (100 µl)
- Disposable pipette tips.
- Distilled water.
- A squeeze bottle for washing .
- Absorbent Paper for blotting the microplate wells.
- ELIZA Microplate Reader with 450nm and 620nm wavelength absorbance capability.
- Timer.
- Linear-linear graph paper.

III-Assay procedure:

All reagents, serum references and samples were brought to room temperature .

1. The desired number of coated wells were secured in the holder.
2. (10 µl) of standards and specimens was dispensed into appropriate wells.
3. (100 µl) of Testosterone-HRP Conjugate Reagent was added into each well.
4. (50 µl) of rabbit anti-Testosterone reagent was transferred to each well.
5. This is followed by thorough mixing for 30 seconds.
6. Then incubation at 37°C for 90 minutes.
7. The microwells were washed and dried 5 times with distilled water.
8. 100 µl of TMB Reagent was dispensed into each well followed by gentle mixing for 10 seconds.
9. Then incubation at room temperature for 20 minutes.
10. The reaction was stopped by adding (100 µl) of Stop Solution to each well.
11. Gently mixing was done for 30 seconds. It was made sure that all the blue color changes to yellow color completely.

12. Absorbance was read at 450 nm with an ELIZA microtiter well reader within 15 minutes.

IV- Calculations of the results:

1. The mean absorbance value (A450) was obtained for each set of reference standards and samples.
2. A standard curve was constructed by plotting the mean absorbance obtained for each reference standard against its concentration in ng/ml on a linear-linear graph paper, with absorbance values on the vertical or Y axis, and concentrations on the horizontal or X axis **(Fig.(VI-I))**.It is highly recommended by the ELIZA kits producing companies to avoid obtaining the standard curve for ELIZA test by using linear regression method to get the best-fit linear standard curve because this increase error due to assumption.
3. The mean absorbance values for each specimen was used to determine the corresponding concentration of Testosterone in ng/ml from the standard curve.

V-Kits reference values for serum testosterone:

Each research center and laboratory should establish its own normal range based on the patient population. The Testosterone ELIZA was performed on randomly selected outpatient clinical laboratory samples by the kit company. The results of these determinations were as follows:

Males: prepubertal (late) 0.1 – 0.2 ng/ml

Adult 3.0 – 10.0 ng/ml

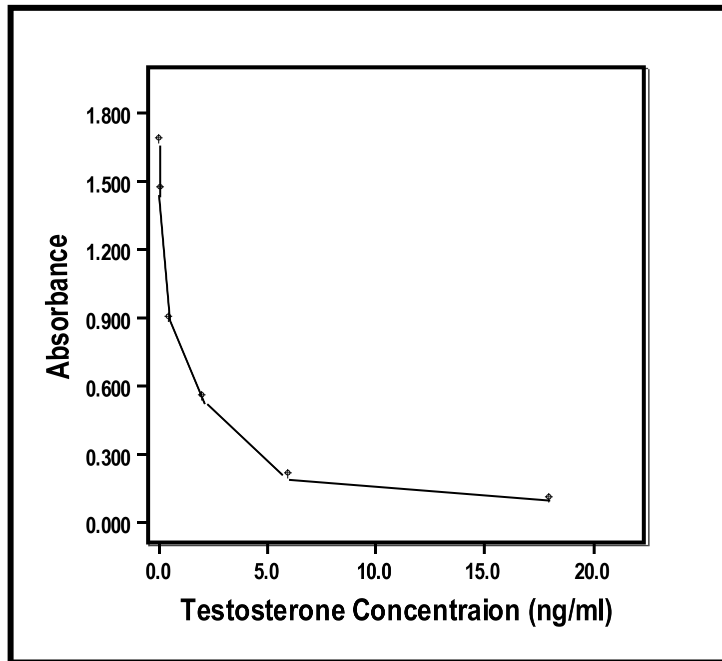

Fig.(VI-I):Testosterone standard curve.

Appendix-VII
Serum Follicle Stimulating Hormone (FSH) measurement

I-Principle of the assay:

In this method, FSH calibrator, patient specimen or control is first added to a streptavidin coated well. Biotinylated monoclonal and enzyme labeled antibodies (directed against distinct and different epitopes of FSH) are added and the reactants mixed. Reaction between the various FSH antibodies and native FSH forms a sandwich complex that binds with the streptavidin coated to the well. After the completion of the required incubation period, the enzyme-Follicle Stimulating Hormone antibody bound conjugate is separated from the unbound enzyme-follicle stimulating hormone conjugate by aspiration or decantation. The activity of the enzyme present on the surface of the well is quantitated by reaction with a suitable substrate to produce color. The employment of several serum references of known Follicle Stimulating Hormone levels permits construction of a dose response curve of activity and concentration. From comparison to the dose response curve, an unknown specimen's activity can be correlated with Follicle Stimulating Hormone concentration.

II-Reagents:

A-Materials were provided with the kit:

- FSH Callibrators – 1 ml/vial : Six vials of references for FSH Antigen at levels 0(A). 5(B).10(C).25(D) .50 (E)and 100 (F) mIU/ml. A preservative has been added .
- FSH Enzyme Reagent – 13 ml/vial : One vial containing enzyme labeled antibody , biotinylated monoclonal mouse lgG in buffer , dye , and preservative .
- Streptavidin Coated Microplate – 96 wells :One 96-well microplate coated with streptavidin and packaged in an aluminum bag with a drying agent .
- Wash Solution Concentrate – 20 ml :One vial containing a surfactant in buffered saline . A preservative has been added .

- Substrate A -7.0ml /vial :One bottle containing tetramethybenzidine (TMB) in buffer .
- Substrate B -7.0 ml/vial :One bottle containing hydrogen peroxide (H_2O_2) in buffer .
- Stop Solution – 8ml/vial :One bottle containing a strong acid (1N HCI) .

B-Materials were used but not provided with the kit:

- Micropipette capable of delivering (50 µl) volumes .
- Micropipette for repetitive deliveries of (0.100) ml and (0.300) ml volumes .
- Disposable pipette tips.
- Distilled water.
- A squeeze bottle for washing .
- Absorbent Paper for blotting the microplate wells.
- ELIZA Microplate Reader with 450nm and 620nm wavelength absorbance capability.
- Timer.
- Linear-linear graph paper.

Reagent preparation:

1. Wash Buffer :Contents of Wash Concentrate were diluted to 1000 ml with distilled water in a suitable storage container.
2. Working Substrate Solution: The contents of the vial labeled Solution 'A' were added into the vial labeled Solution 'B' followed by mixing.

III-Assay procedure:

All reagents, serum references and samples were brought to room temperature .

1. (50 µl) of the appropriate serum reference or specimen was pipetted into the assigned well.
2. (100 µl) of FSH-Enzyme Reagent solution was added to all wells.

3. The microplate was swirled gently for 20-30 seconds to mix then it was covered.
4. This is followed by incubation for 60 minutes at room temperature.
5. The contents of the microplate were discarded by decantation .Then the plate was dried with absorbent paper.
6. (300 µl) of wash buffer was added followed by decantion (tap and blot). This was repeated for two (2) additional times for a total of three (3) washes.
7. (l00 µl) of working substrate solution was added to all wells . Always reagents were added in the same order to minimize reaction time differences between wells .
8. This is followed by incubation at room temperature for (15) minutes.
9. (50 µl) of stop solution was added to each well and gently mixed for (15-20 seconds).
10. The absorbance in each well was read at 450nm (using a reference wavelength of 620 nm to minimize well imperfections) in an ELIZA microplate reader. The results were read within (15) minutes of adding the stop solution.

<u>IV- Calculations of the results:</u>

A dose response curve was used to ascertain the concentration of Follicle Stimulating Hormone in unknown specimens.

1. The mean absorbance value (A450) was obtained for each set of reference standards, controls and samples
2. The absorbance for each serum reference versus the corresponding FSH concentration in mIU/ml was plotted on linear graph paper (where hormone concentration on the X-axis while absorbance on the Y-axis).
3. The standard curve was drawn through the plotted points **(Fig.(VII-I))**.
4. To determine the concentration of FSH for an unknown, the average absorbance was located for each unknown on the vertical axis of the graph, the intersecting point on the curve was found,

and the concentration (in mIU/ml) was read from the horizontal axis of the graph.

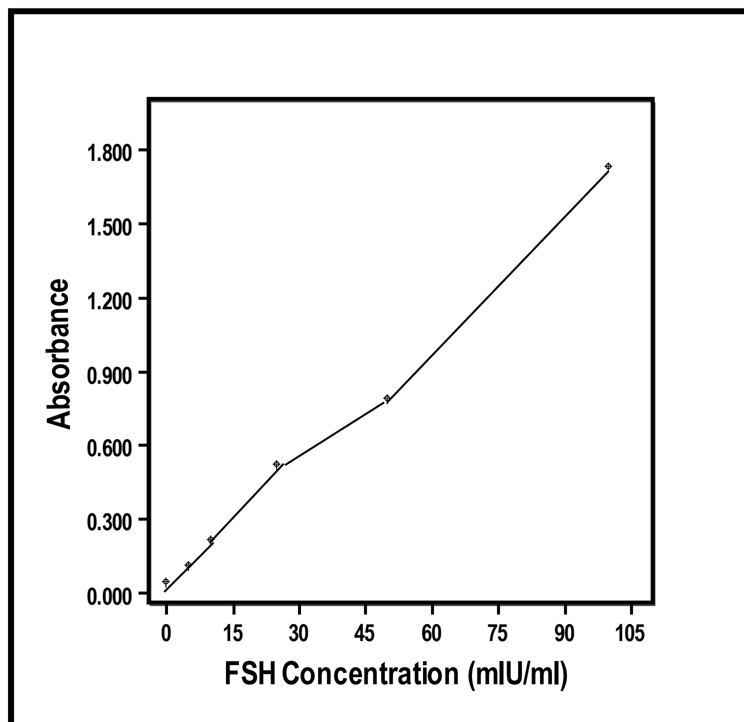

Fig.(VII-I):Follicle-Stimulating Hormone (FSH) standard curve.

V-Kit reference values for serum FSH:

A study of an apparent normal adult population was undertaken by the kit producing company to determine expected values for the FSH ELISA Microplate Test System. The expected values for men was from (1.0-14.0 mIU/ml)

Appendix-VIII
Serum Luteinizing Hormone (LH)measurement

I-Principle of the assay:

In this method, LH calibrator, patient specimen or control is first added to a streptavidin coated well. Biotinylated monoclonal and enzyme labeled antibodies (directed against distinct and different epitopes of LH) are added and the reactants mixed. Reaction between the various LH antibodies and native LH forms a sandwich complex that binds with the streptavidin coated to the well. After the completion of the required incubation period, the enzyme-luteinizing hormone antibody bound conjugate is separated from the unbound enzyme- luteinizing hormone conjugate by aspiration or decantation. The activity of the enzyme present on the surface of the well is quantitated by reaction with a suitable substrate to produce color. The employment of several serum references of known Luteinizing Hormone levels permits construction of a dose response curve of activity and concentration. From comparison to the dose response curve, an unknown specimen's activity can be correlated with Luteinizing Hormone concentration.

II-Reagents:

A-Materials were provided with the kit:

- LH Callibrators – 1 ml/vial : Six vials of references for LH Antigen at levels 0(A) . 5 (B), 25(C). 50(D) .100 (E)and 200 (F) mIU/ml. A preservative has been added .
- LH Enzyme Reagent – 13 ml/vial : One vial containing enzyme labeled affinity purified antibody , biotinylated monoclonal mouse lgG in buffer , dye , and preservative .
- Streptavidin Coated Microplate – 96 wells :One 96-well microplate coated with streptavidin and packaged in an aluminum bag with a drying agent .
- Wash Solution Concentrate – 20 ml :One vial containing a surfactant in buffered saline . A preservative has been added .

- Substrate A-7.0ml /vial :One bottle containing tetramethybenzidine (TMB) in buffer .
- Substrate B-7.0 ml/vial :One bottle containing hydrogen peroxide (H_2O_2) in buffer .
- Stop Solution – 8ml/vial :One bottle containing a strong acid (1N HCI) .

B-Materials were used but not provided with the kit:

- Micropipette capable of delivering (50 µl) volumes .
- Micropipette for repetitive deliveries of 0.100 ml and 0.300 ml volumes .
- Disposable pipette tips.
- Distilled water.
- A squeeze bottle for washing .
- Absorbent Paper for blotting the microplate wells.
- ELIZA Microplate Reader with 450nm and 620nm wavelength absorbance capability.
- Timer.
- Linear-linear graph paper.

Reagent preparation:

1. Wash Buffer :Contents of Wash Concentrate were diluted to 1000 ml with distilled water in a suitable storage container.
2. Working Substrate Solution: The contents of the vial labeled Solution 'A' were added into the vial labeled Solution 'B' followed by mixing.

III-Assay procedure:

All reagents, serum references and samples were brought to room temperature .

1. (50 µl) of the appropriate serum reference or specimen was pipetted into the assigned well.
2. (100 µl) of LH-Enzyme Reagent solution was added to all wells.

3. The microplate was swirled gently for 20-30 seconds to mix then it was covered.
4. This is followed by incubation for 60 minutes at room temperature.
5. The contents of the microplate were discarded by decantation .Then the plate was dried with absorbent paper.
6. (300 μl) of wash buffer was added followed by decantion (tap and blot). This was repeated for two (2) additional times for a total of three (3) washes.
7. (l00 μl) of working substrate solution was added to all wells . Always reagents were added in the same order to minimize reaction time differences between wells .
8. This is followed by incubation at room temperature for (15) minutes.
9. (50 μl) of stop solution was added to each well and gently mixed for (15-20 seconds).
10. The absorbance in each well was read at 450nm (using a reference wavelength of 620 nm to minimize well imperfections) in an ELIZA microplate reader. The results were read within (15) minutes of adding the stop solution.

IV- Calculations of the results:

A dose response curve was used to ascertain the concentration of LH in unknown specimens.

1. The mean absorbance value (A450) was obtained for each set of reference standards, controls and samples
2. The absorbance for each serum reference versus the corresponding LH concentration in mIU/ml was plotted on linear graph paper (where hormone concentration on the X-axis while absorbance on the Y-axis).
3. The standard curve was drawn through the plotted points **(Fig.(VIII-I))**.
4. To determine the concentration of LH for an unknown, the average absorbance was located for each unknown on the vertical axis of the graph, the intersecting point on the curve

was found, and the concentration (in mIU/ml) was read from the horizontal axis of the graph .

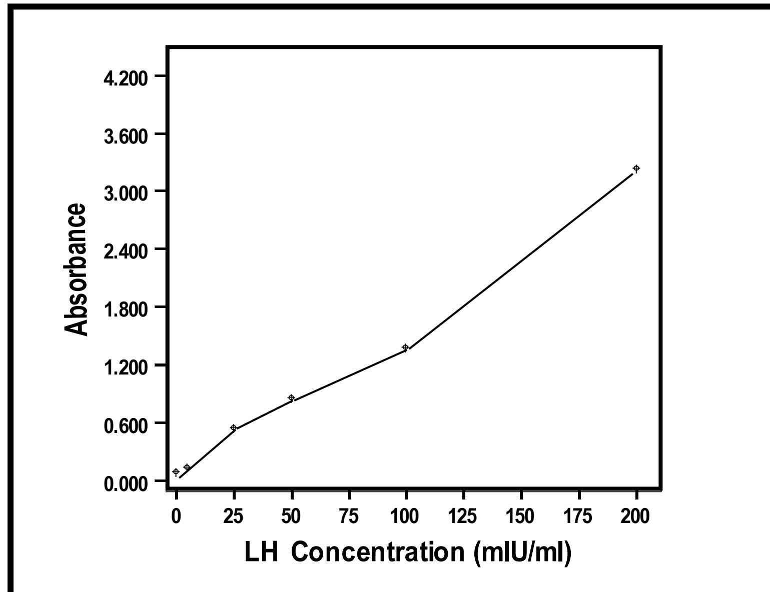

Fig.(VIII-I): Luteinizing Hormone (LH) standard curve.

V-Kit reference values for serum LH:

A study of an apparent normal adult population was undertaken by the kit producing company to determine expected values for the LH ELISA Microplate Test System. The expected values for men was from (0.7-7.4 mIU/ml)

Appendix-IX
Serum Prolactin Hormone measurement

I-Principle of the assay:

In this method, Prolactin calibrator, patient specimen or control is first added to a streptavidin coated well. Biotinylated monoclonal and enzyme labeled antibodies (directed against distinct and different epitopes of Prolactin) are added and the reactants mixed. Reaction between the various Prolactin antibodies and native Prolactin forms a sandwich complex that binds with the streptavidin coated to the well. After the completion of the required incubation period, the enzyme-Prolactin antibody bound conjugate is separated from the unbound enzyme- Prolactin conjugate by aspiration or decantation. The activity of the enzyme present on the surface of the well is quantitated by reaction with a suitable substrate to produce color. The employment of several serum references of known Prolactin levels permits construction of a dose response curve of activity and concentration. From comparison to the dose response curve, an unknown specimen's activity can be correlated with Prolactin concentration.

II-Reagents:

A-Materials were provided with the kit:

- Prolactin Callibrators – 1 ml/vial : Six vials of references for Prolactin Antigen at levels 0(A) . 5 (B), 10(C). 25(D) .50(E)and 100 (F) mIU/ml. A preservative has been added .
- Prolactin Enzyme Reagent – 13 ml/vial : One vial containing enzyme labeled antibody , biotinylated monoclonal mouse lgG in buffer , dye , and preservative .
- Streptavidin Coated Microplate – 96 wells :One 96-well microplate coated with streptavidin and packaged in an aluminum bag with a drying agent .
- Wash Solution Concentrate – 20 ml :One vial containing a surfactant in buffered saline . A preservative has been added .
- Substrate A-7.0ml /vial :One bottle containing tetramethybenzidine (TMB) in buffer .

- Substrate B-7.0 ml/vial :One bottle containing hydrogen peroxide (H_2O_2) in buffer .
- Stop Solution – 8ml/vial :One bottle containing a strong acid (1N HCI) .

B-Materials were used but not provided with the kit:

- Micropipette capable of delivering (25 and 50 µl) volumes .
- Micropipette for repetitive deliveries of 0.100 ml and 0.300 ml volumes .
- Disposable pipette tips.
- Distilled water.
- A squeeze bottle for washing .
- Absorbent Paper for blotting the microplate wells.
- ELIZA Microplate Reader with 450nm and 620nm wavelength absorbance capability.
- Timer.
- Linear-linear graph paper.

Reagent preparation:

1. Wash Buffer :Contents of Wash Concentrate were diluted to 1000ml with distilled water in a suitable storage container.
2. Working Substrate Solution: The contents of the vial labeled Solution 'A' were added into the vial labeled Solution 'B' followed by mixing.

III-Assay procedure:

All reagents, serum references and specimen were brought to room temperature

1. (25 µl) of the appropriate serum reference or specimen was pipetted into the assigned well.
2. (l00 µl) of Prolactin -Enzyme Reagent solution was added to all wells.
3. The microplate was swirled gently for 20-30 seconds to mix then it was covered.
4. This is followed by incubation for 60 minutes at room temperature.
5. The contents of the microplate were discarded by decantation .Then the plate was dried with absorbent paper.

6. (300 μl) of wash buffer was added followed by decantion (tap and blot). This was repeated for two (2) additional times for a total of three (3) washes.
7. (l00 μl) of working substrate solution was added to all wells . Always reagents were added in the same order to minimize reaction time differences between wells .
8. This is followed by incubation at room temperature for (15) minutes.
9. (50 μl) of stop solution was added to each well and gently mixed for (15-20 seconds).
10. The absorbance in each well was read at 450nm (using a reference wavelength of 620 nm to minimize well imperfections) in an ELIZA microplate reader. The results were read within (15) minutes of adding the stop solution.

IV- Calculations of the results:

A dose response curve was used to ascertain the concentration of Prolactin in unknown specimens.

1. The mean absorbance value (A450) was obtained for each set of reference standards, controls and samples
2. The absorbance for each serum reference versus the corresponding Prolactin concentration in (ng/ml) was plotted on linear graph paper (where hormone concentration on the X-axis while absorbance on the Y-axis).
3. The standard curve was drawn through the plotted points **(Fig.(IX-I))**.
4. To determine the concentration of Prolactin for an unknown, the average absorbance was located for each unknown on the vertical axis of the graph, the intersecting point on the curve was found, and the concentration in (ng/ml) was read from the horizontal axis of the graph .

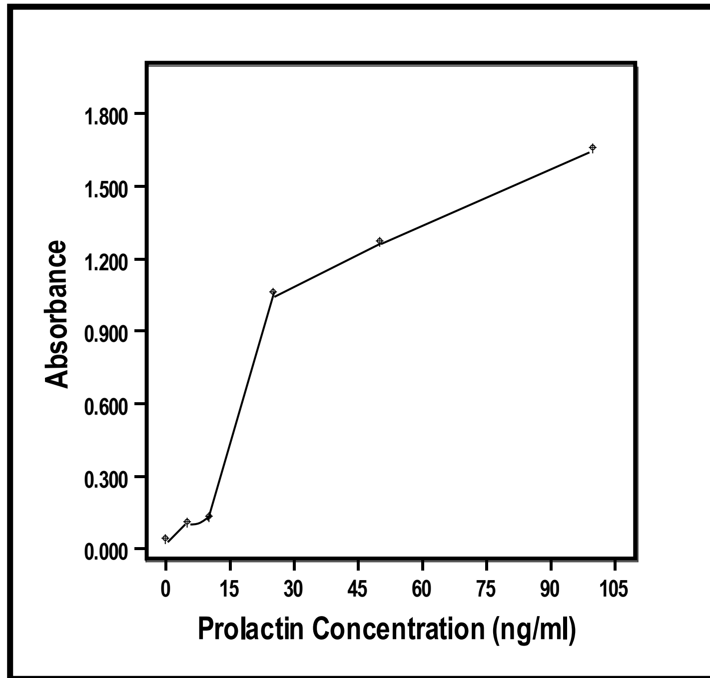

Fig.(IX-I):Prolactin standard curve.

<u>V-Kit reference values for serum Prolactin:</u>

A study of an apparent normal adult population was undertaken by the kit producing company to determine expected values for the Prolactin ELISA Microplate Test System. The expected values for adult men was from (1.8-17.0 ng/ml)

(Appendix-X)
Determination of Packed Cells Volume (PCV)

Microhematocrit method was applied to determine PCV. Heparinized capillary tubes used, and blood was filled to approximately three quarters of their lengths then the unmarked end is closed with modeling clay and put in the microhematocrit centrifuge. After centrifugation for 5 minutes, the red blood cells were separated from plasma and remain a band of buffy coat at the interface between them consisting of leukocytes and blood platelets .After centrifugation, the proportion of the RBCs column to the total column (i.e., the PCV) was determined using special graded circular ruler especially designed for PCV measurement.Reference range of PCV for adult male is (0.45±0.05) **(Lewis, et al,2006).**

Appendix-XI
Total White Blood Cells count (WBCs count)

Blood was diluted with Turk's solution (in this study a preprepared solution was used consisting of acetic acid (2%) colored with gention violet (1%)) .A 1 in 20 dilution of blood was made by adding 0.1 ml of well-mixed anticoagulated blood (with EDTA) to 1.9 ml of diluent in a glass tube.After sealing the tube with lid, the diluted blood was mixed by hand for 2 minutes by tilting the tube to an angle of 120° combined with rotation.Then the counting chamber (Neubauer hemocytometer) with its coverslip already in position was filled by capillary action . It is waited for three minutes to let the cells to settle down and then the chamber is examined under 40X objective lens of the microscope to count WBCs in the four corners secondary squares .The calculation of the WBCs was performed using the formula mentioned below.Refernce range for total WBCs count for adult male is ($4.0\text{-}10.0 \times 10^9$/l)**(Lewis, et al,2006).**

$$\text{Total WBCs count (cell/l)} = \frac{\text{No. of cells counted} \times \text{dilution} \times 10^6}{\text{volume counted } (\mu l)}$$

(No. of secondary squares counted× 0.1)

Appendix-XII
Erythrocyte Sedimentation Rate (ESR) measurement

The dilution solution for ESR measurement that was used was Trisodium citrate (3.8 %).One volume of diluent was added to 4 volumes of the EDTA blood (we used 0.5 of the diluent added to 2 ml of blood).The blood was mixed thoroughly then it was withdrawn into Westergren tube to the 200 mm mark by mean of special withdrawing instrument. The tube was put in exactly vertical position for 60 minutes.The level of the top of the RBCs column then was read and the results were expressed in mm/hour.Reference value for ESR in male (17-50 years) is ≤10 mm/hour **(Lewis, *et al*.,2006).**

Appendix-XIII
Study Documentation

Fig.(XIII-I):Chemical materials used in the study.

1. **Ortho-toluidine stain**
2. **NH4Cl**
3. **H2O2**
4. **Oil ointment**

The 1st 3 items used in the preparation of the Ortho-toluidine special stain used for semen WBCs staining.

Fig.(XIII-II):

1. Eosin Yellow stain (used for sperm vitality test)

2. NaEDTA (used in the preparation of Orthotoluidine stain).

Fig.(XIII-III):

1. Ethanol (95 %):used for fixation

2. NaHCO3 solution: used to immobilize sperms for counting on chamber .

Fig.(XIII-IV): Another photograph for some of the chemical materials used.

Fig.(XIII-V): Sterile wide-mouth semen container used for semen collection.

Fig.(XIII-VI):Graded PH paper (PH 1-14) used for semen PH measurement.

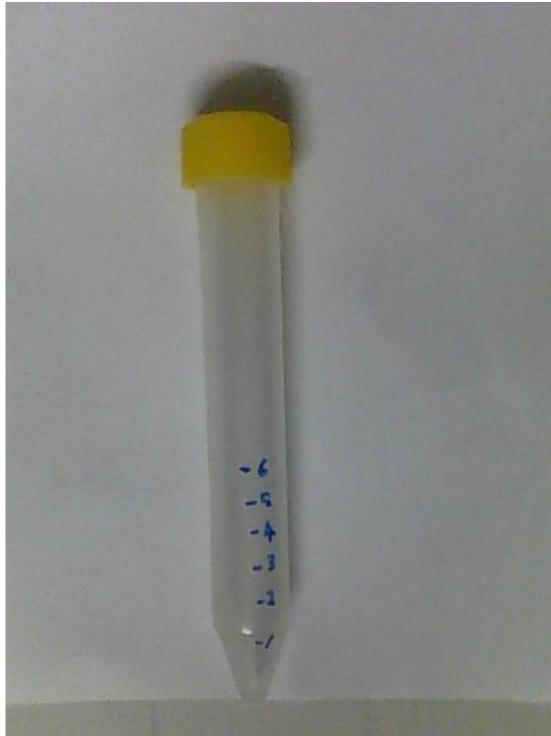

Fig.(XIII-VII):Graded disposable plastic tube used for semen volume measurement (the scales were highlighted).

Fig.(XIII-VII):Woody sticks were used for semen viscosity assessment.

Fig.(XIII-IX):Micropipettes and other materials used during the study.

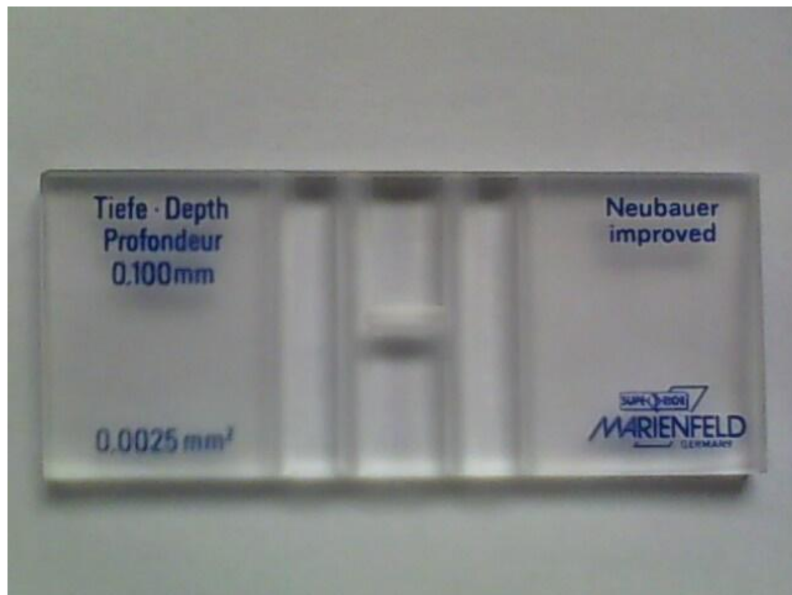

Fig.(XIII-X):Improved Neubauer Chamber was used for sperms counting and for WBCs count.

Fig.(XIII-XI):Andrology Lab at Babylon Hospital for Obstetrics and Pediatrics.

Fig.(XIII-XII):Incubator at the Andrology Lab.

Fig.(XIII-XIII):Electronic centrifuge used for semen centrifugation and for separation of serum samples.

Fig.(XIII-XIV):Biochemistry and hematology lab. at Babylon Hospital.

Fig.(XIII-XV):Patients and controls serum samples in Amedrof's tubes at the deep freeze storage (-20C).

Fig.(XIII-XVI):Patients and controls serum samples in Amedrof's tubes and test tubes.

Fig.(XIII-XVII):Patients and controls serum samples in Amedrof's tubes and test tubes.

Fig.(XIII-XVIII):Preparation of seminal fluid slide at the Andrology Lab. at Babylon Hospital.

Fig.(XIII-XIX):Personal picture at the Andrology Lab.

Fig.(XIII-XX):Hematocrit centrifuge

Fig.(XIII-XXI):EDTA tube shaker

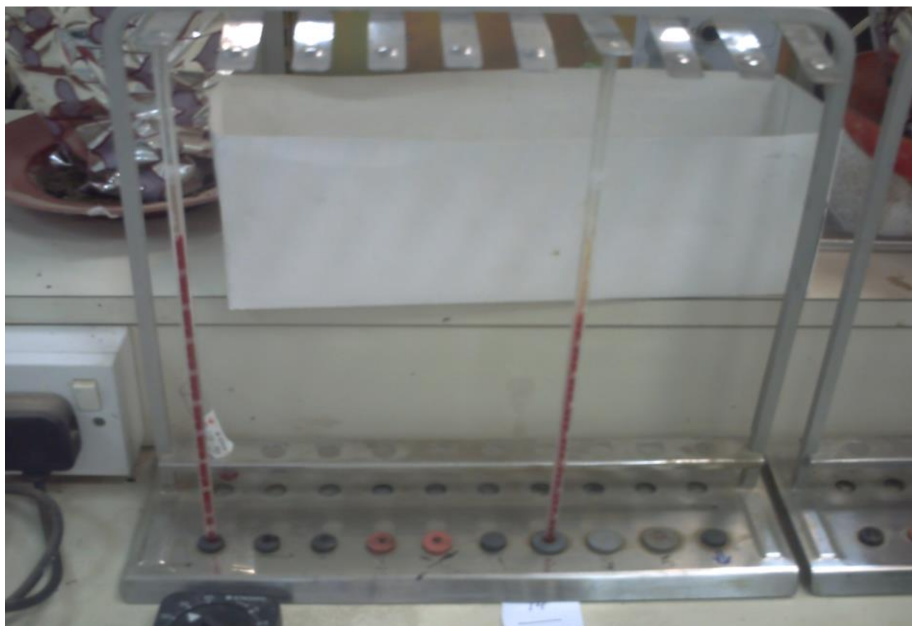

Fig.(XIII-XXII):Westergreen tube for ESR measurement.

Fig.(XIII-XXIII):Some chemical materials used in hematological studies

Fig.(XIII-XXIV):Some components of LH ELIZA kit (Monobind Inc.)

Fig.(XIII-XXV):Other components of LH ELIZA kit (Monobind Inc.)

Fig.(XIII-XXVI):LH ELIZA kit (Monobind Inc.) (N.B. All ELIZA kits from Monobind Company have the same containers appearance exept labeling).

Fig.(XIII-XXVII):FSH ELIZA kit (Monobind Inc.)

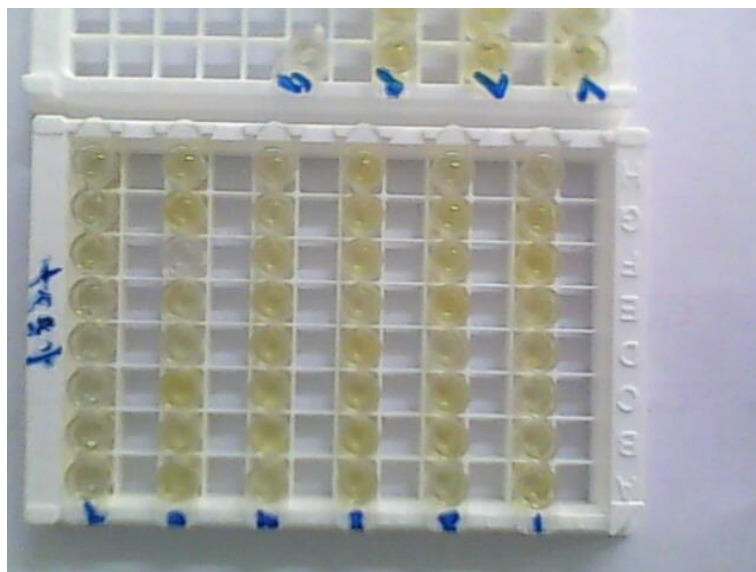

Fig.(XIII-XXVIII):Microwells plate for Testosterone ELIZA hormonal assay containing the study groups serum samples after the final addition of (Stop Solution) and being ready for reading.

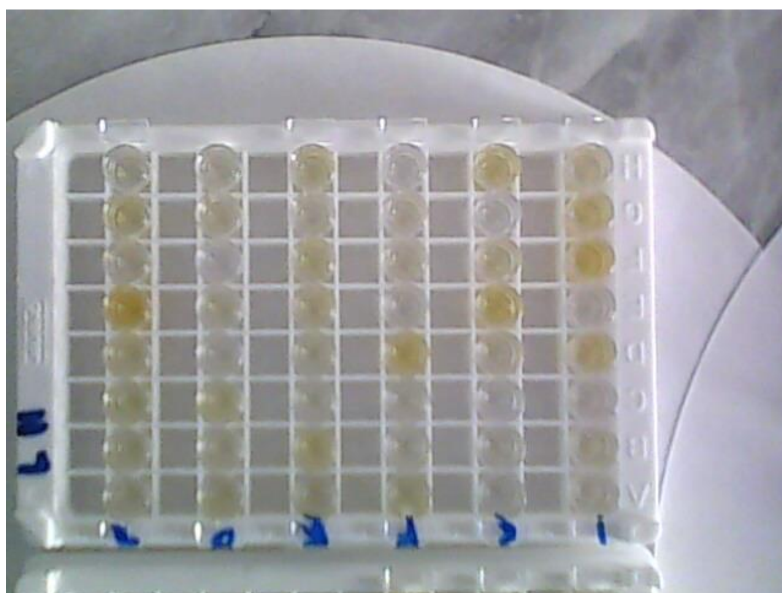

Fig.(XIII-XXIX):Microwells plate for LH ELIZA hormonal assay containing the study groups serum samples after the final addition of (Stop Solution) and being ready for reading.

Fig.(XIII-XXX):ELIZA Microwells reader (Beckman Inc.)

Fig.(XIII-XXXI):Public Health Laboratory at Hilla Teaching Hospital where hormonal assays were done.

Fig.(XIII-XXXII):The addition of study groups serum samples to the microwells plate at ELIZA Assays Unit at Public Health Laboratory

Fig.(XIII-XXXIII):The addition of study groups serum samples to the microwells plate.

Fig.(XIII-XXXIV):Mr.Fareed Kadim Hamzah (ELIZA Assays Unit member)(Biologist) who helped me during hormonal assays procedures.

Fig.(XIII-XXXV):Personal picture with my supervisor Assis.Prof.Dr.Saad Merza Hussein Al-araji .