Sudan University of Science and Technology

College of Graduate Studies

Assessment of Fertility Hormones among Infertile Men in Red Sea State

قياس مستوى هرمونات الخصوبة لدى الرجال المصابين بالعقم بولاية بالبحر الأحمر

A thesis Submitted in Fulfillment for Master Degree In Medical Laboratory Science and Technology (Clinical Chemistry)

BY

Hussien Elhady Hussien Ahmed

BSc (Honor) in Medical Laboratory Science

Shendi University,1999

Supervisor : Dr. **Badreldein Hassan Elabid**

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كلية الدراسات العليا

بسم الله الرحمن الرحيم

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Approval Page

Name of Candidate: ..Hussien Elhady Hussien Ahmed

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Approved by:

1. External Examiner

Name: ....Isam Alamel mohmd sadik.....

Signature: ....Date: .....

2. Internal Examiner

Name: Dr. Munsoor Mohammed Munsoor.....

Signature: ....Date: 09-06-2014

3. Supervisor

Name: .....C/Dr. Khalda Mirghani Hamza.....

Signature: ....Date: 09/6/2014.....

الآية

بسم الله الرحمن الرحيم

قال تعالى فى كتابه الكريم :

(لِلَّهِ مُلْكُ السَّمَاوَاتِ وَالْأَرْضِ يَخْلُقُ مَا يَشَاء يَهَبُ لِمَنْ يَشَاء إِنَاثًا وَيَهَبُ لِمَن يَشَاء الذُّكُورَ * أَوْ يُزَوِّجُهُمْ ذُكْرَانًا وَإِنَاثًا وَيَجْعَلُ مَن يَشَاء عَقِيمًا إِنَّهُ عَلِيمٌ قَدِيرٌ)

صدق الله العظيم

- سورة الشورى آية رقم (٤٩-٥٠)

Dedication

To my father and mother who give me all hopes,

To my wife Alzeina , my son Yousef and my daughter Hebat Alla ,

my support is difficult and hard.

To my brother and sister .

To my friends .

I dedicate this work

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Finally, I am grateful to those whom samples were taken.

Abstract

Male infertility is a common problem in Red Sea State , Sudan . In the majority the cause is previous exposure to venereal diseases , and in some cases there is no known cause of infertility . This study aimed to asses the sperm count and the plasma levels of fertility hormones among menwith history of infertility in Red Sea State ,Sudan.

Across-sectional study was conducted in Port Sudan Teaching Hospital, Red Sea State , Sudan, during the period from March 2012 to March 2014 , 150 married men with a complain of infertility were assessed for the analysis of plasma levels of fertility hormones and the sperm count , along with 94 apparently health proven fathers as a control group. The test group and the control group were matched for age . SPSS was used for analysis of data . The data was compared using student's □t" test and Pearson's correlation was used for assessment of correlation between different variables.

This results showed that the mean of the sperm count of the test group was significantly reduced compared with that of the control group , (p = 0.000). The study showed a significant increase in the means of plasma levels of Follicle stimulating hormone , Luteinizing hormone and prolactin of the test group compared to control group , (p = 0.000) . There is no significant difference between plasma levels of Testosterone of the test group and control group (p = 0.100) . The results of present study showed no significant correlations between plasma levels of Follicle stimulating hormone , Luteinizing hormone and prolactin hormone with Testosterone , and also no significant correlation between plasma levels of Prolactin and Testosterone to the sperm count. A significant correlations of the Follicle stimulating hormone and Luteinizing hormone to the Sperm count.

The present data indicate that among infertile men in Red Sea State , the sperm count is significantly reduced ,whereas the plasma levels of Follicle Stimulating Hormone, Luteinizing Hormone and Prolactin are significantly increased. Plasma levels of Follicle Stimulating Hormone, Luteinizing Hormone have a significant positive correlations with the sperm count .

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مستخلص الدراسة

العقم عند الرجال من المشاكل الشائعة في ولاية البحر الأحمر بالسودان. في غالبية المرضى يعتقد أن السبب هو التعرض للأمراض التناسلية فى فترات سابقه، وفي بعض الحالات لا يوجد سبب معروف للعقم . تهدف هذه الدراسة لتقييم عدد الحيوانات المنوية ومستويات هرمونات الخصوبة لدى الرجال .

هذه دراسة مقطعيه أجريت في مستشفى بورتسودان التعليمي، ولاية البحر الأحمر بالسودان، خلال الفترة من مارس ٢٠١٢ الى مارس عام ٢٠١٤، على ١٥٠ رجلا متزوجا يشكون من العقم . تم تحليل مستويات هرمونات الخصوبة وهى تشمل الهرمون منشط الجريب (FSH), الهرمون اللوتيئني(LH) ،هرمون البرولاكتين(PRL) وهرمون التستوستيرون (Testosterone) عن طريق جهاز TOSOH AIA360 الالى وعدد الحيوانات المنوية بالطريقة اليدوية عن طريق neubauer improved champer للمشاركين ، تم إختيار ٩٤ من الآباء كمجموعة تحكم . كلا المجموعتان متشابهتان فى الأعمار. تم إستخدام برنامج كمبيوتر SPSS لتحليل البيانات. تم مقارنة البيانات باستخدام "t" وقيمة (P. value)، ثم يستخدم ارتباط بيرسون لتقييم الارتباط بين المتغيرات المختلفة.

متوسط عدد الحيوانات المنوية فى مجموعة المرضى انخفض انخفاضا ذو دلاله إحصائية مقارنة مع المجموعة الضابطة حيث كان الإحتمال (0.000) . أظهرت الدراسة أيضا إرتفاعًا ذات دلاله إحصائيه في متوسط مستويات البلازما لهرمون منشط الجريب (FSH), الهرمون اللوتيئني(LH) وهرمون و البرولاكتين(PRL) في مجموعة الإختبار مقارنة مع المجموعة الضابطة حيث كان الاحتمال هو (0.000). عند مقارنة مستوى هرمون التستوستيرون في مجموعة الإختبار مع المجموعة الضابطة كان الإحتمال هو (0.10) وهى قيمه ليست ذات دلاله إحصائية .

كما أظهرت الدراسة ايضا أن العلاقة بين مستويات الهرمون المنبه للجريب (FSH) , الهرمون اللوتيئني (LH) وهرمون البرولاكتين مع هرمون التستوستيرون ليست علاقات ذات دلالات إحصائية .

كما أوضحت الدراسة عدم وجود إرتباط ذو دلاله إحصائيه بين مستويات البلازما من هرمون تستوستيرون وهرمون البرولاكتين مع عدد الحيوانات المنوية ،كما أظهرت النتائج وجود

إرتباط ذو دلالة إحصائية بين مستويات البلازما للهرمون المنبه للجريب (FSH) و الهرمون اللوتيئني (LH) مع عدد الحيوانات المنوية .

تشير نتائج الدراسه عن العقم أن عدد الحيوانات المنوية يقل عند الرجال المصابين بالعقم ، في حين أن مستويات البلازما من هرمون منشط الجريب (FSH), الهرمون اللوتيئني(LH) وهرمون و البرولاكتين (PRL) ترتفع بعلاقة موجبه وذات دلاله إحصائيه . كما أن هرمون منشط الجريب (FSH), الهرمون اللوتيئني(LH) ترتبط إرتباط بعلاقة موجبه وذات دلاله إحصائيه مع عدد الحيوانات المنوية .

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Abbreviations

FSH Follicle stimulating hormone

LH Luteinizing hormone

PRL prolactin hormone

GnRH gonadotropin-releasing hormone

GH growth hormone

hCG human chorionic gonadotropin

TSH Thyroid releasing hormone

TRH Thyroid stimulating hormone

SHBG sex hormone binding globulin

IHH idiopathic (isolated) hypogonadotropic hypogonadism

CHAPTER ONE

Introduction and Literature Review

Chapter One

Introduction and Literature Review

1.1. Introduction

Infertility can be defined as a failure to conceive after 12 months of unprotected sexual intercourse.(Matorras *et al* 1997) It is classified as primary infertility if no previous pregnancies have occurred, and secondary infertility if it occurred after one or more pregnancies(Matorras *et al* 1997).

Approximately 15% of couples attempting their first pregnancy meet with a failure, and another 10% face secondary infertility. (Matorras *et al* 1997)Data available over the past 20 years reveal that in approximately 30% of the cases of infertility, the pathology is found in the man alone, and in another 20%, the pathology of both the man and the woman are abnormal .(Matorras *et al* 1996)Therefore, the male factor is at least partly responsible for the infertility in about 50% of cases. (Matorras et al 1996)Male fertility depends upon an intact hypothalamopituitary-testicular axis to initiate and maintain quantitatively and qualitatively normal spermatogenesis, The initial evaluation of the male patient should be rapid, noninvasive, and cost-effective, as nearly 70% of conditions that cause infertility in men can be diagnosed with history, physical examination, and hormonal and semen analysis alone. More detailed, expensive, and invasive studies can then be ordered if necessary , A trained expert assesses the man's sperm count, their shape, movement, and other variables. Generally, a higher number of normal-shaped sperm means higher fertility. But exceptions are common. Many men with low sperm counts or abnormal semen are still fertile. And about 15% of infertile men have normal semen and plenty of normal sperm(Carlsen *et al*1992).

Maintain normal secondary sex glands functions and sexual functions.(Carlsen *et al*1992) Thus, it is surprising how infrequent infertile males have a recognizable endocrinopathy, even though up to 20% of male infertility can be attributable to endocrinopathy. (Matorras *et al* 1996) In fact, endocrine disorders which may be associated with significant medical pathology remain an important factor to consider in the etiology of male infertility because they be amenable to treatment. However, in clinical practice, endocrine evaluation is usually done only in patients with severe oligospermia or azoospermia.(Matorras *et al* 1996)The hormones initially evaluated include follicle stimulating hormone (FSH) luteinizing hormone (LH), testosterone and prolactin (Matorras *et al* 1996).

Further studies, like the evaluation of oestradiol, sexhormone binding globulin, thyroid function test amongothers, can be done, depending on the clinical scenario andthe results of the initial studies. (Jacobsen et al 1999)Based on the results ofthe hormonal studies, a precise endocrinological diagnosis such as hypergonadotropic hypogonadism can be made,and the patient managed accordingly. The objective ofthis study was to determine the prevalence and pattern of endocrinological abnormalities in patients investigated formale infertility in environment(Jacobsen *et al* 1999).

Although there were much data on infertility in other African countries, no data exists about infertility in Sudan. Seven hundred and ten Sudanese couples were investigated for the infertility in Khartoum Fertility Center, Sudan: 443 (62.4%) had primary infertility and 267 (37.6%) had secondary infertility. A positive male factor alone was found in 257 (36.2%) couples and a female factor in 350 (49.3%) couples: eleven (1.5%) couples had a combination of male and female factors: and the

cause of infertility was unexplained in 92 (13.0%) couples. Oligozoospermia and asthenozoospermia were factors responsible for 16.8% and 17.5% of male infertility, respectively. Failure of ovulation (60.3%) was the most common cause of female infertility. The study revealed a high proportion of secondary infertility and a greater contribution of the female factors to infertility(Rucker *et al* 1998).

1.2 Literature Review

Infertility

Infertility is defined as the inability to achieve pregnancy after one year of unprotected intercourse. An estimated 15% of couples meet this criterion and are considered infertile, with approximately 35% due to female factors alone, 30% due to male factors alone, 20% due to a combination of female and male factors, and 15% unexplained. Conditions of the male that affect fertility are still generally underdiagnosed and undertreated(Matorras *et al* 1996).

Causes of infertility in men can be explained by deficiencies in sperm formation, concentration (eg, oligospermia [too few sperm], azoospermia [no sperm in the ejaculate]), or transportation. This general division allows an appropriate workup of potential underlying causes of infertility (Carlsen *et al*1992).

The initial evaluation of the male patient should be rapid, noninvasive, and cost-effective, as nearly 70% of conditions that cause infertility in men can be diagnosed with history, physical examination, and hormonal and semen analysis alone. More detailed, expensive, and invasive studies can then be ordered if necessary (Matorras *et al* 1996).

Treatment options are based on the underlying etiology and range from optimizing semen production and transportation with medical therapy or surgical procedures to complex assisted reproduction techniques. Technological advancements make conceiving a child possible with as little as one viable sperm and one egg. Although the workup was traditionally delayed until a couple was unable to conceive for 12 months, evaluation may be initiated at the first visit in slightly older couples (Jacobsen *et al* 1999).

Normal ejaculate volume ranges from 1.5 to 5 mL and has a pH level of 7.05-7.8. The seminal vesicles provide 40-80% of the semen volume, which includes fructose for sperm nutrition, prostaglandins and other coagulating substances, and bicarbonate to buffer the acidic vaginal vault. Normal seminal fructose concentration is 120-450 mg/dL, with lower levels suggesting ejaculatory duct obstruction or absence of the seminal vesicles. The prostate gland contributes approximately 10-30% (0.5 mL) of the ejaculate. Products include enzymes and proteases to liquefy the seminal coagulum. This usually occurs within 20-25 minutes. The prostate also secretes zinc, phospholipids, phosphatase, and spermine.The testicular-epididymal component includes sperm and comprises about 5% of the ejaculate volume (Carlsen *et al* 1992).

In addition to the components above , semen is also composed of secretions from the bulbourethral (Cowper) glands and the (periurethral) glands of Litre, each producing 2-5% of the ejaculate volume, serving mainly to lubricate the urethra and to buffer the acidity of the residual urine. The ordered sequence of release is important for appropriate functioning (Carlsen *et al* 1992).

For conception, sperm must reach the cervix, penetrate the cervical mucus, migrate up the uterus to the fallopian tube, undergo capacitation and the acrosome reaction to digest the zona pellucida of the oocyte, attached to the inner membrane, and releases its genetic contents within the egg. The cervical mucus changes consistency during the ovulatory cycle, being most hospitable and easily penetrated at mid cycle. After fertilization, implantation may then take place in the uterus. Problems with any of these steps may lead to infertility(Carlsen *et al* 1992).

An estimated 10-15% of couples are considered infertile, defined by the World Health Organization (WHO) as the absence of conception after at

least 12 months of unprotected intercourse. poor semen quality, or both account for 90% of cases; however, studies of infertile couples without treatment reveal that 23% of these couples conceive within 2 years, and 10% more conceive within 4 years. Even patients with severe oligospermia (< 2 million sperm/mL) have a 7.6% chance of conception within 2 years (Matorras et al 1996).

Isolated conditions of the female are responsible for infertility in 35% of cases, isolated conditions of the male in 30%, conditions of both the male and female in 20%, and unexplained causes in 15%. Even if one partner has an obvious cause for the infertility, a thorough evaluation of both partners for completeness is prudent. In addition, both partners may be aided by evaluation of their sexual practices.

Patterns of male infertility vary greatly among regions and even within regions. The highest reported fertility rates are in Finland, while Great Britain has a low fertility rate. A combination of social habits, environmental conditions, and genetics is suspected to contribute to this variation.

Recent debate has occurred in the literature regarding a poorer semen quality, decreased sperm counts (113 million/mL in 1940 compared with 66 million/mL in the 1990s), and decreased fertility in men today compared with fertility 50 years ago(Carlsen *et al* 1992). Investigators hypothesize that environmental conditions and toxins have led to this decline; however, others argue that this is solely because of differences in counting methods, laboratory techniques, and geographic variation

The effect of aging on fertility is unclear. As men age, their testosterone levels decrease, while estradiol and estrone levels increase (Carlsen *et al* 1992). Studies have shown that, as men age, their sperm density

decreases. Young men have spermatids present in 90% of seminiferous tubules, which decreases to 50% by age 50-70 years and to 10% by age 80 years. Additionally, 50% of Sertoli cells are lost by age 50 years, 50% of Leydig cells are lost by age 60 years. Despite this, aging men may achieve fertility rates similar to those in younger men, although conception often takes longer (Carlsen *et al* 1992).

Causes of male infertility

Causes of infertility can be divided into pretesticular, testicular, and posttesticular.

Pretesticular causes of infertility

Pretesticular causes of infertility include congenital or acquired diseases of the hypothalamus, pituitary, or peripheral organs that alter the hypothalamic-pituitary axis(Rucker *et al*1998).

Disorders of the hypothalamus lead to hypogonadotropic hypogonadism. If GnRH is not secreted, the pituitary does not release LH and FSH. Ideally, patients respond to replacement with exogenous GnRH or HCG, an LH analogue, although this does not always occur (Rucker *et al* 1998).

Primary testicular causes of infertility

Primary testicular problems may be chromosomal or nonchromosomal in nature. While chromosomal failure is usually caused by abnormalities of the sex chromosomes, autosomal disorders are also observed (Rucker *et al* 1998).

Chromosomal abnormalities

An estimated 6-13% of infertile men have chromosomal abnormalities (compared with 0.6% of the general population). Patients with

azoospermia or severe oligospermia are more likely to have a chromosomal abnormality (10-15%) than infertile men with sperm density within the reference range (1%). A karyotype test and a Y chromosome test for microdeletions are indicated in patients with nonobstructive azoospermia or severe oligospermia (< 5 million sperm/mL), although indications are expanding (Rucker *et al* 1998).

Posttesticular causes of infertility

Posttesticular causes of infertility include problems with sperm transportation through the ductal system, either congenital or acquired. Genital duct obstruction is a potentially curable cause of infertility and is observed in 7% of infertile patients. Additionally, the sperm may be unable to cross the cervical mucus or may have ultrastructural abnormalities(Rucker et al1998).

1.2.1. Reproductive hormones

1.2.1.1 Follicle-stimulating hormone

Follicle-stimulating hormone (FSH) is a glycoprotein gonadotropin secreted by the anterior pituitary in response to gonadotropin-releasing hormone (GnRH), which is released by the hypothalamus. The same pituitary cell also secretes luteinizing hormone (LH), another gonadotropin. FSH and LH are composed of alpha and beta subunits. The specific beta subunit confers the unique biological activity. FSH and LH bind to receptors in the testis and ovaries and regulate gonadal function by promoting sex steroid production and gametogenesis (Grover *et al* 2005).

In men, LH stimulates testosterone production from the interstitial cells of the testes (Leydig cells). FSH stimulates testicular growth and

enhances the production of an androgen-binding protein by the Sertoli cells, which are a component of the testicular tubule necessary for sustaining the maturing sperm cell. This androgen-binding protein causes high local concentrations of testosterone near the sperm, an essential factor in the development of normal spermatogenesis. Sertoli cells, under the influence of androgens, also secrete inhibin, a polypeptide, which may help to locally regulate spermatogenesis. Hence, maturation of spermatozoa requires FSH and LH (Grover *et al* 2005).

FSH and LH secretion are affected by a negative feedback from sex steroids. Inhibin also has a negative feedback on FSH selectively. High-dose testosterone or estrogen therapy suppresses FSH and LH. Primary gonadal failure in men and women leads to high levels of FSH and LH, except in selective destruction of testicular tubules with subsequent elevation of only FSH, as in Sertoli-cell-only syndrome. Similarly, any process leading to a low FSH level also simultaneously results in a low LH level, except in rare instances of isolated FSH deficiency or isolated LH deficiency in fertile eunuch syndrome(Grover *et al* 2005).

1.2.1.1.1Causes of low FSH level (hypogonadotropic hypogonadism or secondary hypogonadism)

(i) Congenital: Sexual differentiation is normal. In men, phallic development may be subnormal, resulting in a micropenis. Pubertal development is diminished or even absent, depending on the degree of gonadotropin deficiency.

- Isolated idiopathic hypogonadotropic hypogonadism: This usually results from GnRH deficiency, with absence of any other abnormalities. FSH and LH levels are low. Human G protein-coupled receptor 54

(GPR54) receptor model. Mutations identified in patients with idiopathic hypogonadotropic hypogonadism are indicated (Karges and Roux, 2005).

● Kallmann syndrome: This is characterized by hypogonadotropic hypogonadism and 1 or more nongonadal congenital abnormalities, including anosmia, red-green blindness, midline facial abnormalities (eg, cleft palate), urogenital tract abnormalities, and neurosensory hearing loss. Hypogonadism in this syndrome is a result of deficient hypothalamic secretion of GnRH. Most cases are sporadic, but familial cases also occur. It is caused by mutations in the Kalman filter gene (Karges and Roux, 2005). This is a frequently sampled serum luteinizing hormone

(LH) profile in a male patient with Kallmann syndrome (KS), compared with that in a healthy individual. A lack of LH pulsatility is seen in the former (Karges and Roux, 2005).

● Idiopathic hypogonadotropic hypogonadism associated with mental retardation: Several syndromes (eg, Prader-Willi syndrome) have been described in which hypogonadotropic hypogonadism is associated with retardation and other abnormalities, including obesity.

● Combined pituitary hormone deficiency: This results from a rare mutation in the gene encoding a transcription factor (PROP1), which is necessary for the differentiation of a cell type that is a precursor to somatotroph, lactotroph, thyrotroph, and gonadotroph cells, thus resulting in deficiencies in prolactin, thyroid-stimulating hormone (TSH), growth hormone (GH), FSH, and LH (Dandona *et al* 2008) (Ferhi *et al* 2009).

● Fertile eunuch syndrome: This is thought to represent an incomplete form of GnRH deficiency in men, in which an isolated and partial LH

deficiency is present with low testosterone and normal FSH levels, resulting in preservation of spermatogenesis.

● Abnormal beta subunit of LH: This is a rare mutation in the LH beta subunit gene(Karges and Roux 2005).

● Abnormal beta subunit of FSH: This is a rare mutation in the gene for the beta subunit of FSH, resulting in a low FSH level. This condition is encountered only in women but has been studied in male mice in which the FSH beta subunit gene has been knocked out. These mice have oligospermia but are fertile (Dandona *et al* 2008) (Ferhi *et al* 2009).

(ii) Acquired: This can be caused by any disease that affects the hypothalamic-pituitary axis, impairing the secretion of GnRH, FSH, or LH.

● Mass lesions: These include pituitary adenomas, cysts, and metastatic cancer to the sella (breast in women, lung and prostate in men). These masses may cause temporary or permanent damage by extrinsic compression of pituitary cells. Hypothalamic tumors may lead to delayed puberty, hypogonadism, and obesity, originally called Fröhlich syndrome or adiposogenital dystrophy. The presence of obesity indicates that the appetite-regulating regions of the hypothalamic have been damaged.

● Hypothalamic/pituitary surgery: If sufficient normal tissue is excised inadvertently, symptomatic hypogonadism may ensue initially, followed by dysfunction of other pituitary cells.

● Hypothalamic/pituitary radiation: This may lead to multiple hormonal

deficiencies, including FSH and LH.

● Infiltrative lesions: Hemochromatosis, sarcoidosis, histiocytosis, and lymphoma can cause hypogonadism by involving the hypothalamic/pituitary region.

● Infections: Meningitis, especially tuberculous, is a rare cause of hypogonadism.

● Pituitary apoplexy: Is a sudden and severe hemorrhage into the pituitary, which can result in varying degrees of hypopituitarism, excruciating headaches, visual changes, and altered mental status.

● Trauma: Head trauma of sufficient severity to fracture the skull base can the hypothalamic-pituitary stalk, preventing GnRH from reaching the pituitary, thus decreasing FSH and LH release.

● Glucocorticoid excess: Exogenous or endogenous (Cushing syndrome) glucocorticoid excess can lead to hypogonadotropic hypogonadism. Direct inhibition of testosterone secretion may also occur at the testicular level.

● Hyperprolactinemia: This can result from a pituitary adenoma, renal or liver insufficiency, primary hypothyroidism, or some drugs (eg, neuroleptics). Hyperprolactinemia can suppress GnRH secretion through a central dopamine-related mechanism. In addition to hypogonadism, this condition can also manifest as galactorrhea and as gynecomastia in men.

● Primary hypothyroidism: This can lead to hypogonadism through hyperprolactinemia. A low thyroxine (T4) level results in a high thyrotropin-releasing hormone (TRH) level, which stimulates prolactin secretion(Karges and Roux 2005).

● Critical illness: Surgery, myocardial infarction, or other illness can cause transient hypogonadotropic hypogonadism, with resolution upon recovery(Karges and Roux, 2005).

● Excessive exercise: This can cause a functional hypothalamic hypogonadism in men, analogous to women with functional hypothalamic amenorrhea.

● Sex steroid–secreting tumors: These may be adrenal, testicular, or

ovarian in origin, or, they may result from adrenal rest tumors. The

excessive amount of testosterone or estradiol can inhibit FSH and LH secretion.

● Intentional (iatrogenic) secondary hypogonadism: Prolonged administration of high doses of anabolic steroids (by athletes) or GnRH analogs (for prostate cancer) can cause low FSH or LH levels. Recovery may take many months or years after cessation of the drug. Also, women who discontinue oral contraceptives may have post-pill amenorrhea; recovery of the gonadotropin axis may take up to one year.

● Pituitary infarction: This condition rarely occurs in males; but, when present, it primarily manifests in older patients with vascular insufficiency during coronary artery bypass surgery. In women, it can occur postpartum as Sheehan syndrome, usually after substantial blood loss during childbirth. This condition manifests as partial or complete hypopituitarism, depending on the hormonal deficiencies; a low FSH or LH level causing amenorrhea is the most frequent cause.

● Chronic systemic diseases: Cirrhosis, chronic renal failure, and AIDS may lead to hypogonadism, which has a dual mechanism, ie, primary and secondary(Karges and Roux 2005).

● Anorexia nervosa: In women, significant weight loss, up to 10% below the ideal body weight, may lead to functional hypothalamic amenorrhea.

● Acute alcohol ingestion: This may lead to primary or secondary hypogonadism (Karges and Roux 2005).

● Idiopathic: No cause is identified in some men and women with acquired secondary hypogonadism. The cause may be autoimmune in origin.

● Type 2 diabetes: Research has indicated that low concentrations of testosterone, LH, and FSH are prevalent in patients with type 2 diabetes who are obese.(Dandona *et al* 2008)Evidence suggests that inflammation may play an important part in this phenomenon.

1.2.1.1.2 Causes of high FSH level

Primary hypogonadism: Can be congenital or acquired

(i) Congenital:-

Sexual differentiation in men may vary from pseudohermaphrodism to a male with only a micropenis and lack of full pubertal development. In women, sexual differentiation is normal but puberty is delayed or absent (Ferhi *et al* 2009).

● Klinefelter syndrome: This syndrome is the most common congenital abnormality causing primary hypogonadism in men. The typical genotype is 47,XXY. The clinical presentation includes infertility, small and firm testes, and low testosterone with high FSH and LH levels. Males with Klinefelter syndrome usually present in their prepubertal years (Ferhi *et al* 2009)(Wikstrom and Dunkel 2008). Adolescent male with Klinefelter

syndrome who has female-type distribution of pubic hair, as well as testicular dysgenesis.

● Other chromosomal abnormalities: These result in testicular hypofunction; they include the 46,XY/XO and the 47,XYY karyotypes.

● Mutation in the FSH receptor gene in men: This mutation is rare and results in low sperm count with a high FSH level. Inactivating mutations of the LH receptor in females have been identified. These patients present with a milder phenotype compared to males(Peroff and Fritz 2005).

● Cryptorchidism: This refers to undescended testes, The clinical consequences depend on whether 1 or both testes are cryptorchid. If only 1 testis is affected, the sperm count is subnormal in almost 30% of patients and the FSH level is slightly elevated. If both testes remain undescended, the sperm count is usually severely subnormal with a high FSH level and low serum testosterone. Hypoplastic right hemiscrotum in a patient with an undescended right testis(Sharma *et al* 2007).

● Disorders of androgen biosynthesis: This involves mutations of the genes that encode the enzymes necessary for testosterone biosynthesis. They result in incomplete virilization, low sperm count, low testosterone level, and high LH and FSH levels.

● Sertoli-cell-only syndrome: The characteristic features are complete, or almost complete, absence of germ cells in all seminiferous tubules. Leydig cells are only mildly impaired. These men have azoospermia with high FSH levels. LH and testosterone levels are normal. The cause has not been identified, but it is thought to be a congenital absence or early neonatal loss of the germ cells(Sharma *et al* 2007) (Loret *et al* 2007).

● End organ resistance to androgens: This is due to androgen receptor defects. In its complete form, it is called testicular feminization. Affected individuals are genetically males but phenotypically females. The testes are located in the labia, the inguinal canal, or the abdomen. Testosterone and LH levels are high. The FSH level is normal or slightly increased(Loret *et al* 2007).

(ii) Acquired

● Infections: The most common is mumps orchitis. The seminiferous tubules are almost always severely affected, often resulting in infertility, especially with bilateral testicular involvement. The Leydig cells may also be damaged, resulting in decreased testosterone production with high LH levels.

● Radiation: This mostly damages the seminiferous tubules or the ovaries. The degree of damage is proportionate to the level of radiation exposure.

● Antineoplastic agents: As with cyclophosphamide, chlorambucil, cisplatin, and carboplatin may decrease the sperm count by destruction of the seminiferous tubules. Less commonly, testosterone secretion also declines. Recovery may occur over the long-term. Similarly, in women, chemotherapy may lead to ovarian failure.

● Chemicals: Chemicals such as dibromodichloropropane which was used as a soil fumigant and nematocide on over 40 different crops in the United States can decrease spermatogenesis.

● Glucocorticoids: These can lead to hypogonadism via inhibition of the pituitary and testes.

● Ketoconazole: This is an antifungal drug that inhibits testosterone biosynthesis.

● Suramin: This is an antiparasitic drug that can block testosterone synthesis by the Leydig cells.

● Trauma: Injuries can be sufficiently severe to damage both seminiferous tubules and Leydig cells.

● Testicular torsion: Torsion of more than 8 hours duration may lead to a low sperm count. Even if the torsion involves only1 testis, both testes may be damaged; the mechanism is not known (Loret *et al* 2007).

● Chronic systemic diseases: Cirrhosis, chronic renal failure, and AIDS may lead to hypogonadism, both primary and secondary. Bilateral aortofemoral anastomosis in men may lead to decreased blood supply to the testes, predominantly affecting the seminiferous tubules.

● Autoimmune damage: This is due to antisperm antibodies. It may be part of an autoimmune polyglandular syndrome.

● Idiopathic: Many men and women with primary hypogonadism have idiopathic disease, and the cause is never identified. The cause may be autoimmune in origin(Peroff and Fritz 2005).

1.2.1.2. Luteinizing hormone

LH is a glycoprotein dimer composed of 2 glycosylated noncovalently-linked subunits designated alpha and beta. The alpha subunit is composed of 92 amino acids and is encoded on the long arm of chromosome 6. The beta subunit is 121 amino acids and is encoded on the long arm of chromosome 19.

The alpha subunit of LH is biologically identical to 3 other hormones: FSH, thyroid-stimulating hormone (TSH), and human chorionic gonadotropin (hCG). The beta subunit is unique and determines LH immunologic and biologic activity. The half-life of LH is 20 minutes (Peroff and Fritz 2005).

Hypothalamic-Pituitary-Gonadal Function

Axis gonadotropin-releasing hormone (GnRH) is secreted by neurons in the arcuate nucleus of the hypothalamus and released into the pituitary portal circulation. LH and FSH are produced by gonadotrope cells located in the anterior pituitary gland. The gonadotrope cells release LH and FSH in a pulsatile fashion approximately every hour when stimulated by GnRH. Once released into the systemic circulation, both LH and FSH stimulate the gonads of females and males to release steroid hormones (Speroff *et al* 2005).

In the male, both LH and FSH are required for spermatogenesis. LH stimulates Leydig cells to convert cholesterol to testosterone. Testosterone and FSH, in turn, modulate Sertoli cells, which serve as "nurse" cells for spermatogenesis within the lumen of the seminiferous tubules. Clinically, only FSH is used as a marker of testicular dysfunction (Sharma *et al* 2007).

Luteinizing hormone (LH) deficiency is an uncommon condition that almost always occurs in conjunction with follicle-stimulating hormone (FSH) deficiency because LH and FSH are secreted by the same pituitary gonadotrope cells. LH deficiency can manifest in females or males as delayed puberty, hypogonadism at any age, or reproductive abnormalities that can be dramatic or subtle. LH and FSH play central roles in the hypothalamic-pituitary-gonadal axis, and, thus, conditions related to LH

and FSH deficiency can be caused by pathology of either the hypothalamus or pituitary. Careful analysis of the presenting problem, the patient's overall health, and the hormonal profile is often necessary to determine the cause of LH deficiency and, thus, the most appropriate treatment (Lofrano *et al* 2007).

1.2.1.2.1 Path

1.2.1.2.1.1.Hypothalamic causes of LH deficiency

(i) Kallmann syndrome

○ Kallmann syndrome was first described by Franz Hosef Kallmann in 1944 and refers to congenital secondary hypogonadism (hypogonadotropic hypogonadism) associated with lack of sense of smell (anosmia). The condition occurs sporadically in 60% of patients, but can be genetically transmitted as an X-linked, autosomal dominant or autosomal recessive condition.

○ This condition affects both females and males who usually present with anosmia and delayed puberty. Laboratory evaluation reveals low LH and FSH levels and normal karyotypes.

○ Females present with primary amenorrhea, and some males present with micropenis. Kallmann syndrome results from the congenital absence of GnRH-producing neurons in the hypothalamus. During embryogenesis, olfactory axonal and GnRH neurons from the olfactory placode fail to migrate to the hypothalamus. In the absence of GnRH, the pituitary gonadotrope cells are not signaled to produce LH and FSH, ultimately leading to lack of sex hormone production by the gonads. (Loret *et al* 2007).

○ Hormone replacement therapy (estrogen for females andtestosterone for males) is used to induce sexual maturation and minimize the long-term risk of osteoporosis. When fertility is desired, the treatment consists of either GnRH, given by a subcutaneous pump, or exogenous gonadotropins given by injection. Women with Kallmann syndrome do not ovulate when given clomiphene citrate, which relies on an intact hypothalamic-pituitary-gonadal axis. Likewise, maintenance therapy with clomiphene citrate does not appear to increase testosterone secretion or sperm production in men with Kallmann syndrome.

(ii) LH subunit mutation: Some report gene mutations of the beta-subunit of LH, leading to hypogonadotropic hypogonadism (Lofrano *et al* 2007) (Valdes *et al* 2004)(Phillip *et al* 1998).

(iii) Idiopathic hypogonadotropic hypogonadism

○ Adult onset idiopathic (isolated) hypogonadotropic hypogonadism (IHH) refers to complete or partial absence of GnRH-induced release of LH and FSH in the setting of otherwise normal anterior pituitary anatomy and function. This relatively rare condition can occur in both men and women.

○ In men, estrogen (produced by aromatization of testosterone) has a negative feedback effect on hypothalamic secretion of GnRH and thus inhibits pituitary gonadotropin secretion. It has been hypothesized that some cases of IHH result from an acquired defect of enhanced hypothalamic sensitivity to estrogen-mediated negative feedback since maintenance clomiphene citrate therapy can result in complete normalization of pulsatile gonadotropin secretion, serum testosterone

level, and sexual function in men with idiopathic (isolated) hypogonadotropic hypogonadism IHH.

(iv)Stress- hypogonadotropic related hypogonadism

○ Hypothalamic suppression can occur in women under physical or metabolic stress. Stress-related hypothalamic suppression is most commonly related to prolonged strenuous physical exercise and extreme weight loss, particularly in the context of eating disorders, such as anorexia nervosa and bulimia.(Kalantaridou *et al* 2004)These conditions cause an elevation of corticotropin-releasing hormone (CRH), inhibiting pulsatile GnRH release from the hypothalamus. Suppression of GnRH release in women results in decreased secretion of LH and FSH (ie, hypogonadotropic hypogonadism), manifesting as amenorrhea and hypoestrogenemia.(Hergenroeder 1995) Ongoing hypothalamic suppression can lead to serious consequences such as irreversible osteoporosis and bone fractures in these women (Sehu *et al* 2007).

1.2.1.2.1.2 Pituitary dysfunction and LH deficiency

The anterior pituitary produces a number of important peptide hormones, including LH, FSH, TSH, adrenocorticotropic hormone (ACTH), prolactin (PRL), and growth hormone (GH). LH deficiency can result from a myriad of anterior pituitary dysfunctions including pituitary tumors, inflammation, vascular accidents, and pregnancy-related hemorrhagic shock (Sheehan syndrome).

Hyperprolactinemia is a common hormonal abnormality associated with anterior pituitary dysfunction. Women with high levels of serum PRL (>20-25 ng/mL) often develop galactorrhea, and some develop amenorrhea and hypoestrogenemia. The amenorrhea related to

hyperprolactinemia is caused by alterations in the normal release and pulsatility of GnRH as well as subsequent alterations in LH/FSH secretion and the LH surge (Sehu *et al* 2007) .

Causes of hyperprolactinemia include pituitary adenomas, hypothyroidism, hypothalamic dysfunction, and chronic renal insufficiency. Medications such as antipsychotics, estrogen, antihypertensives, metoclopramide, and cimetidine can also cause hyperprolactinemia.

1.2.1.2.2. Frequency of Hypogonadotropic Hypogonadism

• Hypogonadotropic hypogonadism has an overall incidence of approximately 1:10,000 to 1:86,000 men and women respectively . This is associated with anosmia (ie, Kallmann syndrome).

• Stress-related hypogonadotropic hypogonadism accounts for more than 30% of secondary amenorrhea in reproductive-aged women.(Loret *et al* 2007)

1.2.1.2.3. Causes of LH Pthophysiology

- Kallmann syndrome
  - Genetic
- Hypogonadotropic hypogonadism
  - Genetic
  - Idiopathic
  - Prolonged Strenuous Exercise
  - Anorexia Nervosa/Bulimia
  - Starvation
- Pituitary dysfunction

- Pituitary tumors
- Pituitary Infarction

- Luteal phase deficiency
  - Idiopathic

1.2.1.3. Prolactin Hormone

Prolactin is a peptide hormone discovered by Henry Friesen. that is secreted by both men and women. It is released by pituitary gland, an organ responsible for regulating many of body's functions, including reproduction. The pituitary gland is a small, bean-shaped organ located in the middle of the brain.The pituitary gland releases prolactin on a daily basis, both in the morning and throughout the night. Certain activities like sexual intercourse and exercise also cause pituitary gland to release prolactin (Bole *et al* 1998).

Prolactin also acts in a cytokine-like manner and as an important regulator of the immune system. Prolactin has important cell cycle related functions as a growth-, differentiating- and anti-apoptotic factor. As a growth factor binding to cytokine like receptors it has also profound influence on hematopoiesis, angiogenesis and is involved in the regulation of blood clotting through several pathways. In summary, "more than 300 separate actions of PRL have been reported in various vertebrates, including effects on water and salt balance, growth and development, endocrinology and metabolism, brain and behavior,

reproduction, and immune regulation and protection". Prolactin acts in endocrine, autocrine, and paracrine manner through the prolactin receptor and a large number of cytokine receptors (Bole *et al* 1998) .

In breastfeeding, the act of an infant suckling the nipple stimulates the production of oxytocin, which stimulates the "milk let-down" reflex, [Bartholomew etal 2007] which fills the breast with milk via a process called lactogenesis, in preparation for the next feed .

Pituitary prolactin secretion is regulated by endocrine neurons in the hypothalamus, the most important ones being the neurosecretory tuberoinfundibulum (TIDA) neurons of the arcuate nucleus, which secrete dopamine to act on the dopamine-2 receptors of lactotrophs, causing inhibition of prolactin secretion. Thyrotropin-releasing factor (thyrotropin-releasing hormone) has a stimulatory effect on prolactin release(Bartholomew *et al* 2007) .

Vasoactive intestinal peptide and peptide histidine isoleucine help to regulate prolactin secretion in humans (Kulick *et al* 2005).

Prolactin is sometimes classified as a gonadotropin [Marieb etal 2006] although in humans it has only a weak luteotropic effect while the effect of suppressing classical gonadotropic hormones is more important.

1.2.1.3.1. Function of Prolactin

Prolactin has many effects including regulating lactation and stimulating proliferation of oligodendrocyte precursor cells.

It stimulates the mammary glands to produce milk (lactation): Increased serum concentrations of prolactin during pregnancy causes enlargement of the mammary glands of the breasts and prepare for the production of milk. However, the high levels of progesterone during pregnancy suppress the production of milk. Milk production normally starts when the levels of progesterone fall by the end of pregnancy and a suckling stimulus is present. Sometimes, newborn babies (males as well as

females) secrete a milky substance from their nipples known as witch's milk,this is in part caused by maternal prolactin and other hormones (Gregg *et al* 2007).

Prolactin provides the body with sexual gratification after sexual acts: The hormone counteracts the effect of dopamine, which is responsible for sexual arousal. This is thought to cause the sexual refractory period. The amount of prolactin can be an indicator for the amount of sexual satisfaction and relaxation. Unusually high amounts are suspected to be responsible for impotence and loss of libido .

Prolactin also stimulates proliferation of oligodendrocyte precursor cells. These cells differentiate into oligodendrocytes, the cells responsible for the formation of myelin coatings on axons in the central nervous system (Gregg *et al* 2007).

Prolactin also has a number of other effects including contributing to surfactant synthesis of the fetal lungs at the end of the pregnancy and immune tolerance of the fetus by the maternal organism during pregnancy; it also decreases normal levels of sex hormones — estrogen in women and testosterone in men. It is this inhibition of sex steroids that is responsible for loss of the menstrual cycle in lactating women as well as lactation-associated osteoporosis. Prolactin also enhances luteinizing hormone-receptors in Leydig cells, resulting in testosterone secretion, which leads to spermatogenesis (Craven *et al* 2006)It has been shown that Prolactin promotes neurogenesis.

1.2.1.3.2 Production and regulation of Prolactin

In humans, prolactin is produced at least in the pituitary, decidua, myometrium, breast, lymphocytes, leukocytes and prostate (Ben *et al* 1996) (Gerlo *et al* 2006).

Pituitary PRL is controlled by the Pit-1 transcription factor and ultimately dopamine, extrapituitary PRL is controlled by a superdistal promoter and apparently unaffected by dopamine(Gerlo *et al* 2006).

Extrapituitary production of prolactin is thought to be special to humans and primates and may serve mostly tissue specific paracrine and autocrine purposes. It has been hypothesized that in other vetrabrates such as mice a similar tissue specific effect is achieved by a large family of prolactin like proteins controlled by at least 26 paralogous PRL genes not present in primates(Gerlo *et al* 2006).

- dopamine (which blocks prolactin)
- serotonin (which triggers prolactin release)
- thyroid-producing hormone (which also triggers prolactin)

1.2.1.3.3. Variance in Prolactin levels

There is a diurnal as well as an ovulatory cycle in prolactin secretion. In many mammals, there is also a seasonal change in prolactin release.

During pregnancy, high circulating concentrations of estrogen and progesterone inhibit the action of prolactin on milk production. Following delivery, reduced estrogen and progesterone production allows prolactin to induce lactation (Melmed and Jameson 2005).

After childbirth, prolactin levels fall as the internal stimulus for them is removed. Sucking by the baby on the nipple then promotes further prolactin release, maintaining the ability to lactate. The sucking activates mechanoreceptors in and around the nipple. These signals are carried by nerve fibers through the spinal cord to the hypothalamus, where changes in the electrical activity of neurons that regulate the pituitary gland cause increased prolactin secretion. The suckling stimulus also triggers the release of oxytocin from the posterior pituitary gland, which triggers milk let-down: Prolactin controls milk production (lactogenesis) but not the milk-ejection reflex; the rise in prolactin fills the breast with milk in preparation for the next feed.

In usual circumstances, in the absence of galactorrhea, lactation will cease within one or two weeks of the end of demand breastfeeding.

It has also been found that compared to un-mated males, fathers and expectant fathers have increased prolactin concentrations.

High prolactin levels also tend to suppress the ovulatory cycle by inhibiting the secretion of both follicle-stimulating hormone (FSH) and gonadotropic-releasing hormone (GnRH). High prolactin levels can also contribute to mental health issues.

Prolactin levels peak during REM sleep, and in the early morning. Levels can rise after exercise, meals, sexual intercourse, minor surgical procedures , (Melmed and Jameson 2005) or following epileptic seizures (Mellors 2005) .

Hypersecretion of prolactin is more common than hyposecretion. Hyperprolactinemia is the most frequent abnormality of the anterior

pituitary tumors. Clinical signs include inappropriate lactation, lack of menses, infertility in females, and impotence in males (Mellors 2005).

1.2.1.3.4. Prolactin receptors

Prolactin receptors are present in the mamillary glands, ovaries, pituitary glands, heart, lung, thymus, spleen, liver, pancreas, kidney, adrenal gland, uterus, skeletal muscle, skin, and areas of the central nervous system. (Mancini *et al* 2008)

1.2.1.3.5. Diagnostic use of Prolactin

Prolactin levels may be checked as part of a sex hormone workup, as elevated prolactin secretion can suppress the secretion of FSH and GnRH, leading to hypogonadism, and sometimes causing erectile dysfunction in men.

Prolactin levels may be of some use in distinguishing epileptic seizures from psychogenic non-epileptic seizures. The serum prolactin level usually rises following an epileptic seizure (Banerjee *et al* 2004) .

1.2.1.3.6.Conditions associated with elevated prolactin secretion

Hyperprolactinaemia, or excess serum prolactin, is associated with hypoestrogenism, anovulatory infertility, oligomenorrhoea, amenorrhoea, unexpected lactation, and loss of libido in women, and erectile dysfunction and loss of libido in men.

Hyperprolactinemia can result from:

- Prolactinoma

- Excess thyrotropin-releasing hormone (TRH), usually in primary hypothyroidism
- All SSRI(selective serotonin re-uptake inhibitors) and SNRI(Serotonin–norepinephrine reuptake inhibitors) medications
- Many antipsychotic medications
- Emotional stress
- Pregnancy and lactation
- Some sexual disorders (Mancini *et al* 2008)

1.2.1.3.7. Conditions associated with decreased prolactin

Hypoprolactinemia, or serum prolactin deficiency, is associated with ovarian dysfunction in women, (Kauppila *et al* 1988)(Schwärzler *et al* 1997) and metabolic syndrome, anxiety, arteriogenic erectile dysfunction, premature ejaculation,(Corona *et al* 2009) oligozoospermia, asthenospermia, hypofunction of seminal vesicles, and hypoandrogenism (Gonzales etal 1989) in men. In one study, normal sperm characteristics were restored when prolactin levels were brought up to normal values in hypoprolactinemic men (Ufearo and Orisakwe 1995).

Hypoprolactinemia can result from:

- Bulimia
- Excess dopamine (Ufearo and Orisakwe 1995).

1.2.1.4 Testosterone Hormone

Testosterone is a steroid hormone from the androgen group and is found in mammals, reptiles,(Cox and John 2005)birds,(Reed *et al* 2006)and other vertebrates. In mammals, testosterone is primarily secreted in the testes of males and the ovaries of females, although small amounts are

also secreted by the adrenal glands. It is the principal male sex hormone and ananabolic.

In men, testosterone plays a key role in the development of male reproductive tissues such as the testis and prostate as well as promoting secondary sexual characteristics such as increased muscle, bone mass, and the growth of body hair.(Mooradian *et al* 1987) In addition, testosterone is essential for health and well-being (Bassil *et al* 2009) as well as the prevention of osteoporosis (Tuck and Francis 2009).

On average, an adult human male body produces about ten times more testosterone than an adult human female body, but females are more sensitive to the hormone. (Dabbs and Dabbs 2000)Testosterone is observed in most vertebrates.

1.2.1.4.1 Physiological effects of Testosterone

In general, androgens promote protein synthesis and growth of those tissues with androgen receptors. Testosterone effects can be classified as virilizing and anabolic, though the distinction is somewhat artificial, as many of the effects can be considered both. Testosterone is anabolic, meaning it builds up bone and muscle mass.

• Anabolic effects include growth of muscle mass and strength, increased bone density and strength, and stimulation of linear growth andbone maturation.

Androgenic effects include maturation of the sex organs, particularly the penis and the formation of the scrotum in the fetus, and after birth (usually at puberty) a deepening of the voice, growth of the beard and axillary hair. Many of these fall into the category of male secondary sex characteristics(Dabbs and Dabbs 2000).

Testosterone effects can also be classified by the age of usual occurrence. For postnatal effects in both males and females, these are mostly dependent on the levels and duration of circulating free testosterone.

1.2.1.4.2.Prenatal effects of Testosterone

The prenatal androgen effects occur during two different stages. Between 4 and 6 weeks of the gestation.

•Genital virilization (midline fusion, phallic urethra, scrotal thinning and rugation, phallic enlargement); although the role of testosterone is far smaller than that of Dihydrotestosterone.

•Development of prostate and seminal vesicles.During the 2nd trimester androgen level is associated with Gender identity (Swaab and Garcia 2009)This period affects the femininization or masculinization of the fetus and can be a better predictor of feminine or mascular behaviours such as sex typed behaviour than an adult's own levels. A mother's own testosterone level influences behavior more than the daughters's testosterone level during pregnancy(Browne 2000) .

1.2.1.4.3.Early infancy effects of Testosterone

Early infancy androgen effects are the least understood. In the first weeks of life for male infants, testosterone levels rise. The levels remain in a pubertal range for a few months, but usually reach the barely detectable levels of childhood by 4–6 months of age(Forest *et al* 1973) (Corbier *et al* 1992) The function of this rise in humans is unknown. It has been speculated that "brain masculinization" is occurring since no significant changes have been identified in other parts of the body.(Dakin *et al* 2008) Surprisingly, the male brain is masculinized by testosterone being aromatized into estrogen, which crosses the blood-brain barrier and enters the male brain, whereas female fetuses have alpha-fetoprotein

which binds up the estrogen so that female brains are not affected (Dakin *et al* 2008).

1.2.1.4.4. Pre-peripubertal effects of Testosterone

Pre- Peripubertal effects are the first observable effects of rising androgen levels at the end of childhood, o-ccurring in both boys and girls.

- Adult-type body odour
- Increased oiliness of skin and hair, acne
- Pubarche (appearance of pubic hair)
- Axillary hair
- Growth spurt, accelerated bone maturation
- Hair on upper lip and sideburns (Dakin *et al* 2008).

1.2.1.4.5. Pubertal effects of Testosterone

Pubertal effects begin to occur when androgen has been higher than normal adult female levels for months or years. In males, these are usual late pubertal effects, and occur in women after prolonged periods of

heightened levels of free testosterone in the blood.

- Enlargement of sebaceous glands. This might cause acne.
- Phallic enlargement or clitoromegaly
- Increased libido and frequency of erection or clitoral engorgement
- Pubic hair extends to thighs and up toward umbilicus
- Facial hair (sideburns, beard, moustache)
- Loss of scalp hair (Androgenetic alopecia)
- Chest hair, periareolar hair, perianal hair
- Leg hair
- Axillary hair

- Subcutaneous fat in face decreases
- Increased muscle strength and mass (Bhasin *et al* 1996).
- Deepening of voice
- Growth of the Adam's apple
- Growth of spermatogenic tissue in testicles, male fertility
- Growth of jaw, brow, chin, nose, and remodeling of facial bone contours
- Shoulders become broader and rib cage expands
- Completion of bone maturation and termination of growth. This occurs indirectly via estradiol metabolites and hence more gradually in men than women (Bhasin *et al* 1996).

1.2.1.4.6. Role of Testosterone on adults

Adult testosterone effects are more clearly demonstrable in males than in females, but are likely important to both sexes. Some of these effects may decline as testosterone levels decrease in the later decades of adult life.

- Testosterone is necessary for normal sperm development. It activates genes in Sertoli cells, which promote differentiation of spermatogonia.
- Regulates acute HPA (Hypothalamic–pituitary–adrenal axis) response under dominance challenge (Mehta *et al* 2008).
- Mental and physical energy
- Maintenance of muscle trophism
- Testosterone regulates the population of thromboxane A2 receptors on megakaryocytes and platelets and hence platelet aggregation in humans (Ajayi and Halushka 2005) (Ajayi *et al* 1995).
- Testosterone does not cause or produce deleterious effects on prostate cancer. In people who have undergone testosterone deprivation

therapy, testosterone increases beyond the castrate level have been shown to increase the rate of spread of an existing prostate cancer. (Morgentaler and Traish 2009) (Rhoden *et al* 2008) .

Recent studies have shown conflicting results concerning the importance of testosterone in maintaining cardiovascular health. (Haddad etal 2007) (Jones and Saad 2009)Nevertheless, maintaining normal testosterone levels in elderly men has been shown to improve many parameters which are thought to reduce cardiovascular disease risk, such as increased lean body mass, decreased visceral fat mass, decreased total cholesterol, and glycemic control. (Stanworth and Jones 2008) .

- Under dominance challenge, may play a role in the regulation of the fight-or-flight response (Mehta and Josephs 2006).
- Falling in love decreases men's testosterone levels while increasing women's testosterone levels. It is speculated that these changes in testosterone result in the temporary reduction of differences in behavior between the sexes. (Marazziti and Canale 2004) It has been found that when the testosterone and endorphins in the ejaculated semen meet the cervical wall after sexual intercourse, females receive a spike in testosterone, endorphin, and oxytocin levels, and males after orgasm during copulation experience an increase in endorphins and a marked increase in oxytocin levels. This adds to the hospitable physiological environment in the female internal reproductive tract for conceiving, and later for nurturing the conceptus in the pre-embryonic stages, and stimulates feelings of love, desire, and paternal care in the male (this is the only time male oxytocin levels rival a female's (Marazziti and Canale 2004).

- Recent studies suggest that testosterone levels play a major role in risk-taking during financial decisions. (Sapienza *et al* 2009) (Apicella *et al* 2008) .

The administration of testosterone makes men selfish and more likely to punish others for being selfish towards them (Zak *et al* 2009).

Fatherhood also decreases testosterone levels in men, suggesting that the resulting emotional and behavioral changes promote paternal care.(Berg and Wynne 2001).

- Men whose testosterone levels are slightly above average are less likely to have high blood pressure, less likely to experience a heart attack, less likely to be obese, and less likely to rate their own health as fair or poor. However, high testosterone men are more likely to report one or more injuries, more likely to consume five or more alcoholic drinks in a day, more likely to have had a sexually transmitted infection, and more likely to smoke. (Booth *et al* 1999).

1.2.1.4.7. Role of Testosterone on Brain

As testosterone affects the entire body (often by enlarging; males have bigger hearts, lungs, liver, etc.), the brain is also affected by this "sexual" differentiation;(Swaab and Garcia 2009) the enzyme aromatase converts testosterone into estradiol that is responsible for masculinization of the brain in male mice. In humans, masculinization of the fetal brain appears, by observation of gender preference in patients with congenital diseases of androgen formation or androgen receptor function, to be associated with functional androgen receptors. (Wilson 2001).

A study conducted in 1996 found no immediate short term effects on mood or behavior from the administration of supraphysiologic doses of testosterone for 10 weeks on 43 healthy men. (Bhasin *et al* 1996) Another study(Reed etal 2006) found a correlation between testosterone and risk tolerance in career choice among women.

Literature suggests that attention, memory, and spatial ability are key cognitive functions affected by testosterone in humans. Preliminary evidence suggests that low testosterone levels may be a risk factor for cognitive decline and possibly for dementia of the Alzheimer's type, a key argument in life extension medicine for the use of testosterone in anti-aging therapies. Much of the literature, however, suggests a curvilinear or even quadratic relationship between spatial performance and circulating testosterone, (Moffat and Hampson 1996) where both hypo- and hyperse-cretion (deficient- and excessive-secretion) of circulating androgens have negative effects on cognition and cognitively modulated aggressivity, as detailed above. (Moffat and Hampson 1996)

Contrary to what has been postulated in outdated studies and by certain sections of the media, aggressive behaviour is not typically seen in hypogonadal men who have their testosterone replaced adequately to the eugonadal/normal range. In fact, aggressive behaviour has been associated with hypogonadism and low testosterone levels and it would seem as though supraphysiological and low levels of testosterone and hypogonadism cause mood disorders and aggressive behaviour,] with eugondal/normal testosterone levels being important for mental well-being. Testosterone depletion is a normal consequence of aging in men. One possible consequence of this could be an increased risk for the development of Alzheimer's disease. (Pike etal 2006) (Rosario etal 2004).

1.2.1.4.8. Aggression effect of Testosterone

The positive correlation between testosterone levels and aggression in humans has been demonstrated in many studies, but about half of studies fail to find a link. (Wright *et al* 2009) While testosterone itself is not shown to be the direct cause of aggression in males, the testosterone derivative estradiol is known to correlate with aggression in male mice. (Soma *et al* 2008) .

1.2.1.4.9. Ethnic differences of Testosterone

Different ethnic groups have different incidences of prostate cancer. Differences in sex hormones including testosterone have been suggested as an explanation for these differences. A study find ethnical differences between blacks and whites in the testosterone to sex hormone binding globulin ratio in blood from the umbilical cord in infants (Rohrmann *et al* 2009) (McIntosh 1997) (Calistro 2010).

1.2.1.4.10. Biosynthesis effect of Testosterone

Like other steroid hormones, testosterone is derived from cholesterol The largest amounts of testosterone (>95%) are produced by the testes in men. (Mooradian *et al* 1987) It is also synthesized in far smaller quantities in women by the thecal cells of the ovaries, by the placenta, as well as by the zona reticularis of the adrenal cortex and even skin in both sexes. In the testes, testosterone is produced by the Leydig cells. (Brooks 1975) The male generative glands also contain Sertoli cells which require testosterone for spermatogenesis. Like most hormones, testosterone is supplied to target tissues in the blood where much of it is transported bound to a specific plasma protein, sex hormone binding globulin (SHBG) .

1.2.1.4.11. Insufficiency effect of Testosterone

Testosterone insufficiency (also termed hypotestosteronism or hypotestosteronemia) is an abnormally low testosterone production. It may occur because of testicular dysfunction (primary hypogonadism) or hypothalamic-pituitary dysfunction (secondary hypogonadism) and may be congenital or acquired. (Gould and Petty 2000) An acquired form of hypotestosteronism is a decline in testosterone levels that occurs by aging, sometimes being called "andropause" in men, as a comparison to the decline in estrogen that comes with menopause in women.Recent analysis shows average testosterone levels receding in men of all ages. (Travison *et al* 2007) (Dindyal 2007) Several theories from increases in obesity to exposure to endocrine disruptors have been proposed as an explanation for this reduction. (Bhasin 2007).

1.3 Objectives:

i - General Objective:

To assess the fertility hormones of infertile males in Red Sea State.

ii- Specific Objectives :-

1- To compare sperm count of infertile men and healthy fertile volunteers.

2- To measure plasma levels of follicle stimulating hormone (FSH), Luteinizing hormone (LH), Prolactin (PRL) and Testosterone in infertile males and compare them with hormonal levels of healthy fertile males.

3- To assess the relationship between the plasma levels of FSH, LH, PRL and Testosterone and the sperm count.

1.4 Rationale

The number of infertile men in Red Sea State visit to Port Sudan Teaching Hospital and clinics in Red State is high, the exact cause is unknown for the majority of these cases.

As far as few data was available in the assessment of infertility hormones and the correlation to infertility among patients who are diagnosed as infertile men in Red Sea State.

This study is to try to answer the question" Are there any significant of A low or high level of these hormones is commonly suspected to be the cause of this infertility, this study is designed to prove or exclude this possibilities and in treatment of this problem.

CHAPTER TWO

MATERIALS and METHODS

Chapter Two

2- Materials and Methods:

2.1.Materials

2.1.1 Study Design :-

This is a cross - sectional study.

2.1.2. Study area:-

The study was conducted in Port Sudan Teaching Hospital , Port Sudan is the capital of the Red Sea State, located in the eastern Sudan 815 km from Khartoum the capital of Sudan. Port Sudan Teaching Hospital and clinics serves Port Sudan City with a population of 3,500,000 inhabitants. There is also great number of patients who came from other nearby states and towns such as Swakin and Sinkat .The study was during the period March 2012 to March 2014.

2.1.3. Camed out study population:-

One hundred and fifty men diagnosed as infertile as test group and not treated, and 94 healthy fertile men served as a control group.

2.1.4.Ethical consideration:

- Permission of this study was obtained from the local health authorities in the area of study and the medical director of Port Sudan Teaching Hospital.
- The objectives of the study were explained to all individuals participating in the study and the blood sample was obtained after their agreement .
- A written consert was obtained from all participants .

2.1.5. Blood samples:

Five ml of venous blood from each participant was collected in heparin container for measurement of follicle stimulating hormone (FSH), luteinizing hormone (LH), prolactin (PRL) and testosterone .

2.1.6.Data collection:

Interviews with the test group and control group were done, A questionnaire ,(Appendix 1) was designed to obtain the personal and clinical data.

2.1.7. Blood and seminal sampling

A local antiseptic for skin(70% alcohol) was used and 5 ml of venous blood was collected from the arm in a heparin vacotainor from each participant , and centrifuged immediately to obtain plasma

Seminal was collected after three days of abestance of intercourse in sterile container .

2.2.Methods:

For seminal analysis samples were collected by masturbation and the sample reach the lab within half an hour , the sample allowed to liquefies at room temperature and checked for pH by pH paper ,volume with graduated pesteur pepitte, consistency and color . For microscope examination samples were mixed first and determination of sperm motility microscopically , and the sperm count was done by counting champer after dilution of sample by fixative (formal carbonate wich composed of 50 g of sodium bicarbonate (NaHCO3) and 10 ml of 35% formalin in 1000 ml of purified water) (Sayed , 2002).

Full automation technique was used Tosoh's AIA-360 (Automated Immunoassay) The AIA-Pack technology utilities an original concept patented by Tosoh based on individual test cups each containing lyophilized and ready to use reagents for measurement of plasma

hormone levels of follicle stimulating hormone, Luteinizing hormone, Prolactin hormone, Testosterone hormone . The TOSOH AIA 360 Analyzer perform all samples and reagents handling operations automatically .The tosoh AIA system analyzers was read the rate of fluorescence produced by the reaction and automatically convert the rate to test concentration in its unit , this was calculated from the curve of the target test .

Fluorescence Enzyme Immunoassays rely on the ability of an antibody to recognize and bind a specific macro molecule in what might be a complex mixture of macro molecules(Antigen antibody reaction). In immunology the particular macromolecule bound by an antibody is referred to as an antigen and the area on an antigen to which the antibody binds is called an epitope. (Smith and Osikowicz 1993).

2.2.1. Measurement of plasma FSH:-

Principle

Full automation technique is used Tosoh's AIA-360(Automated Immunoassay) the ST AIA-PACK FSH is a two-site Fluorescence Enzyme Immunoassay assay which is performed entirely in the AIA-PACK test cup. FSH present in the sample bound with monoclonal antibody immobilized on magnetic solid phase and enzyme-labeled monoclonal antibody in the AIA-pack test cup . the magnetic beads was washed to remove unbound enzyme –labeled monoclonal antibody and then react with fluorogenic substrate 4-methylumbelliferyl phosphate (4MUP).The amount of enzyme –labeled monoclonal antibody that binds to the beads is directly proportional to the FSH concentration in the test sample (Smith and Osikowicz 1993).

Calculations :-

A standard curve was constructed using calibrators with known concentrations , an unknown sample concentration was calculated using this curve.

Reagent composition and reagent preparation :-

(Appendix 2)

Reference Values :-

Male 2.1 – 18.6 mIU/ml (Appendix 2)

Linearity limit :- Up to 200 mIU/ml (Appendix 2)

Sensitivity limit:-

The lowest measurable concentration in specimens is 1.0 mIU/ml (Appendix 2).

2.2.2 Measurement of plasma LH:-

Principle

Full automation technique was used Tosoh's AIA-360(Automated Immunoassay),the ST AIA-PACK LH is a two-site Fluorescence Enzyme Immunoassay assay which is performed entirely in the AIA-PACK test cup. LH present in the sample is bound with monoclonal antibody immobilized on magnetic solid phase and enzyme-labeled monoclonal antibody in the AIA-pack test cup . The magnetic beads were washed to remove unbound enzyme –labeled monoclonal antibody and were then react with fluorogenic substrate 4-methylumbelliferyl phosphate (4MUP).The amount of enzyme –labeled monoclonal antibody that binds to the beads is directly proportional to the LH concentration in the test sample (Smith and Osikowicz 1993).

Calculations :-

A standard curve was constructed by using calibrators with known concentrations , an unknown sample concentration are calculated using this curve.

Reagent composition and reagent preparation :

(Appendix 3)

Reference Values :-

Male 1.7 – 11.2 mIU/ml (Appendix 3)

Linearity limit :- Up to 200 mIU/ml (Appendix 3)

Sensitivity limit:-

The lowest measurable concentration in specimens is 0.2 mIU/ml. (Appendix 3)

2.2.3. Measurement of plasma PRL:-

Principle of the method

Full automation technique was used Tosoh's AIA-360(Automated Immunoassay),the ST AIA-PACK PRL is a two-site Fluorescence Enzyme Immunoassay assay which is performed entirely in the AIA-PACK test cup. PRL present in the sample is pound with monoclonal antibody immobilized on magnetic solid phase and enzyme-labeled monoclonal antibody in the AIA-pack test cup . the magnetic beads are washed to remove unbound enzyme –labeled monoclonal antibody and are then incubated with fluorogenic substrate 4-methylumbelliferyl phosphate (4MUP).The amount of enzyme –labeled monoclonal antibody that binds to the beads is directly proportional to the PRL concentration in the test sample (Smith and Osikowicz 1993).

Calculations :-

A standard curve is constructed by using calibrators with known concentrations , an unknown sample concentration are calculated using this curve.

Reagent composition and reagent preparation :-

(Appendix 4) .

Reference Values :-

Male 3.6 – 16.3 ng/ml (Appendix 4) .

Linerity limit :- Up to 200 ng/ml (Appendix 4) .

Sensitivity limit:- The lowest measurable concentration in specimens is 1.0 ng/ml. (Appendix 4)

2.2.4. Measurement of plasma Testosterone :-

Principle of the method

Full automation technique is used Tosoh's AIA-360(Automated Immunoassay),the ST AIA-PACK Testosterone is a competitive Fluorescence Enzyme Immunoassay which is performed entirely in the ST AIA-PACK Testosterone test cup. testosterone present in the sample competes with enzyme-labeled testosterone limited number of binding sites on the testosterone specific monoclonal antibody immobilized on a magnetic solid phase. The magnetic beads are washed to remove unbound enzyme-labeled testosterone and are then incubated with a fluorogenic substrate ,

substrate 4-methylumbelliferyl phosphate (4MUP),the amount of enzyme-labeled testosterone that bind to the beads is inversely proportional to the testosterone concentration in the test sample. A standard curve is constructed , and unknown sample concentration are calculated using this curve (Smith and Osikowicz 1993).

Calculations of results :-

A standard curve witch constructed by using calibrators with known concentrations , an unknown sample concentration are calculated using this curve.

Reagent composition and reagent preparation :-

(Appendix 5) .

Refrence Values :-

Male 262 – 870 ng/dl (Appendix 5) .

Linerity limit :-

Up to 2000 ng/dl (Appendix 5) .

Sensitivity limit:-

The lowest measurable concentration in specimens is 7.0 ng/dl. (Appendix 5)

2.2.5. Quality control:-

The precision and accuracy of all methods used in this study were checked at least once per day with commercially available control, was done at least two levels of control(normal and abnormal).

2.2.6. Statistical analysis:-

The data collected in this study were analyzed using SSPS computer program . The means and standard deviations of plasma levels of FSH,LH,PRL,Testosterone were obtained

for both the test group and the control group and calculated the t-test was used for comparison (p value ≤ 0.05 was considered to be significant)

Linear regression analysis was used to assess correlation between FSH , LH , PRL and Testosterone.

CHAPTER THREE

RESULTS

Chapter Three

3. Results

A total of 150 Sudanese patients from red sea state who were diagnosed as infertile males were investigated in this study as test group, age of test group ranged between 20 and 56 years , 94 healthy fertile volunteers as a control group were enrolled in this study. In this study the mean of duration of married was 6.34 years , also there was 14% of test group had a past sexual transmitted diseases and 86% were not infected , also there was 4% were diabetic and 96% non diabetic , also it found that 7.3% were infected by mumps and the other 92.7% had no previous infection , among the population of the study there were 14% had testicular pain and 86% did not complain from testicular pain , also there was 12.7% had past history of testicular surgery but 87.3% had no past history of testicular surgery . 2% of study population had a congenital defects but 98% were normal . 26% were smoker but 74% were not smokers , 29.3% snough users but 70.7% don't use it . 2% drink alcohol and 98% not use alcohol . In the current study 66.3% of were oligospermic , 36.7% were azoospermic . The mean of seminal volume of azoospermic was 2.42 ±1.4 ml,and of oligospermic was 2.4±1.4 ml . Table (3 -1) showed a significant decrease of the sperm count of the test group when compared to the control group , ($p = 0.000$). It showed a significant increase in the mean of plasma levels of Follicle stimulating hormone , Luteinizing hormone and prolactin of the test group compared to that of the control group , ($p = 0.00$). No significant difference between plasma levels of testosterone hormone of the test group compared to control , ($p = 0.100$) .

Fig. (3-1): Showed a scatter plot with no significant correlation between the plasma levels of Follicle stimulating hormone and Testosterone (r= - 0.11 , p 0.088)

Fig. (3-2): Showed a scatter plot with no significant correlation between the plasma levels of Luteinizing hormone and Testosterone (r= - 0.11 , p 0.165).

Fig. (3-3): Showed a scatter plot with no significant correlation between the plasma levels of prolactin hormone and Testosterone (r= - 0.03 , p 0.362).

Fig. (3-4): Showed a scatter plot with no significant correlation between the plasma levels of Testosterone and the sperm count (r= 0.04 , p 0.658).

Fig. (3-5): Showed a scatter plot with a significant correlation between the plasma levels of Follicle stimulating hormone and the sperm count (r= - 0.17 , p 0.018).

Fig. (3-6): Showed a scatter plot with a significant week correlation between the plasma levels of Luteinizing hormone and the sperm count (r= - 0.21 , p 0.004).

Fig. (3-7): Showed a scatter plot with no significant correlation between the plasma levels of prolactin hormone and the sperm count (r= - 0.03 , p 0.371).

Table (3 -1): Plasma levels the of FSH , LH , PRL and Testosterone of the study group compared to control groups.

Variable	Test group (n=150)	Control (n=94)	p-value
Sperm count (million/ml)	4.41± 0.96	82.13 ± 15.3	0.000
Follicle Stimulating Hormone(mIU/mL)	14.79 ± 2.8	5.31 ± 2.5	0.000
Luteinizing Hormone(mIU/mL)	8.49 ± 2.0	5.01 ± 2.8	0.000
Prolactin Hormone(ng/mL)	17.15 ± 0.6	14.04 ± 4.5	0.001
Testosterone Hormone (ng/dL)	533.39 ±253.29	555.53 ± 239.4	0.100

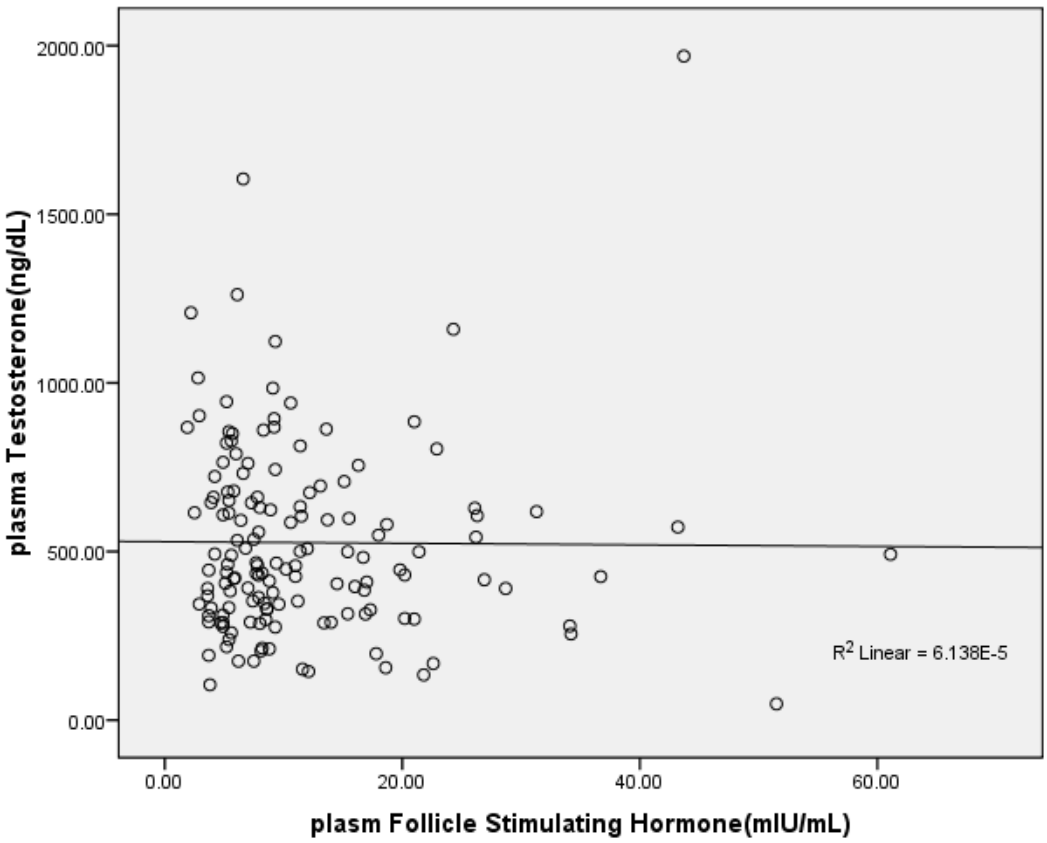

Fig. (3-1): A scatter plot shows the correlation between the plasma levels of Follicle stimulating hormone and Testosterone (r= - 0.11 , P 0.088) among the test group which is insignificant .

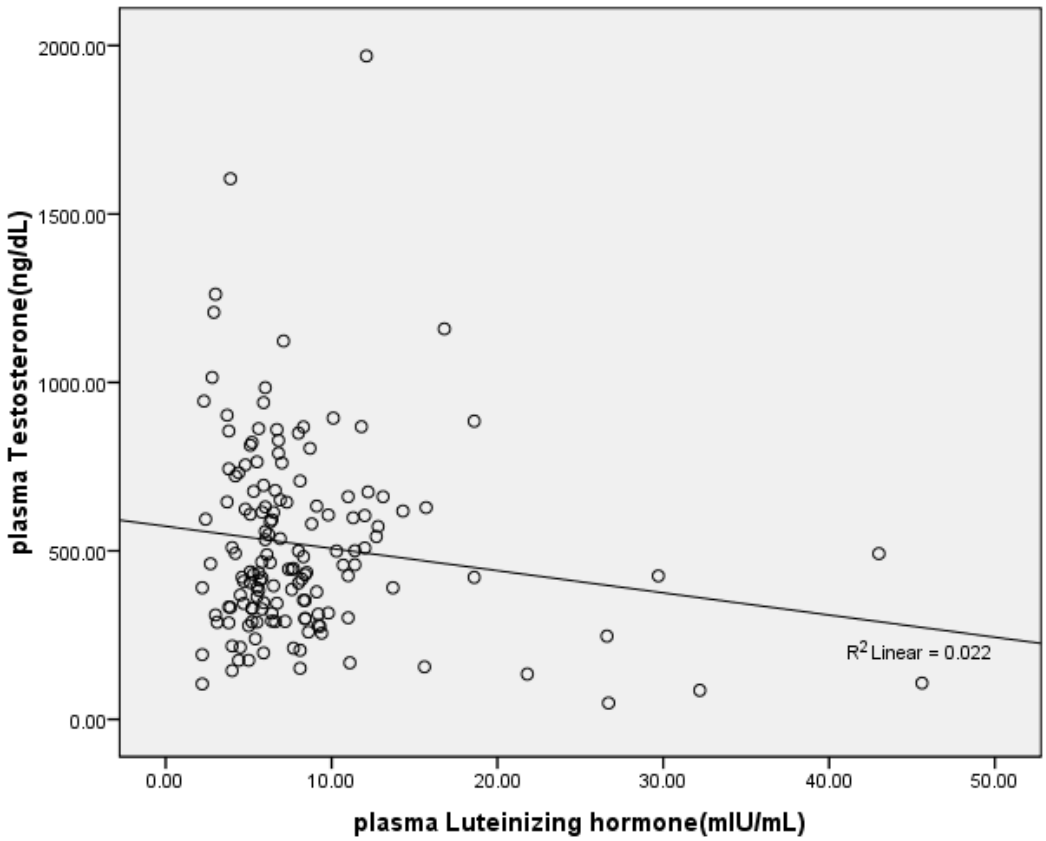

Fig. (3-2): A scatter plot shows the correlation between the plasma levels of Luteinizing hormone and Testosterone (r= - 0.11 , P 0.165) among the test group which is insignificant .

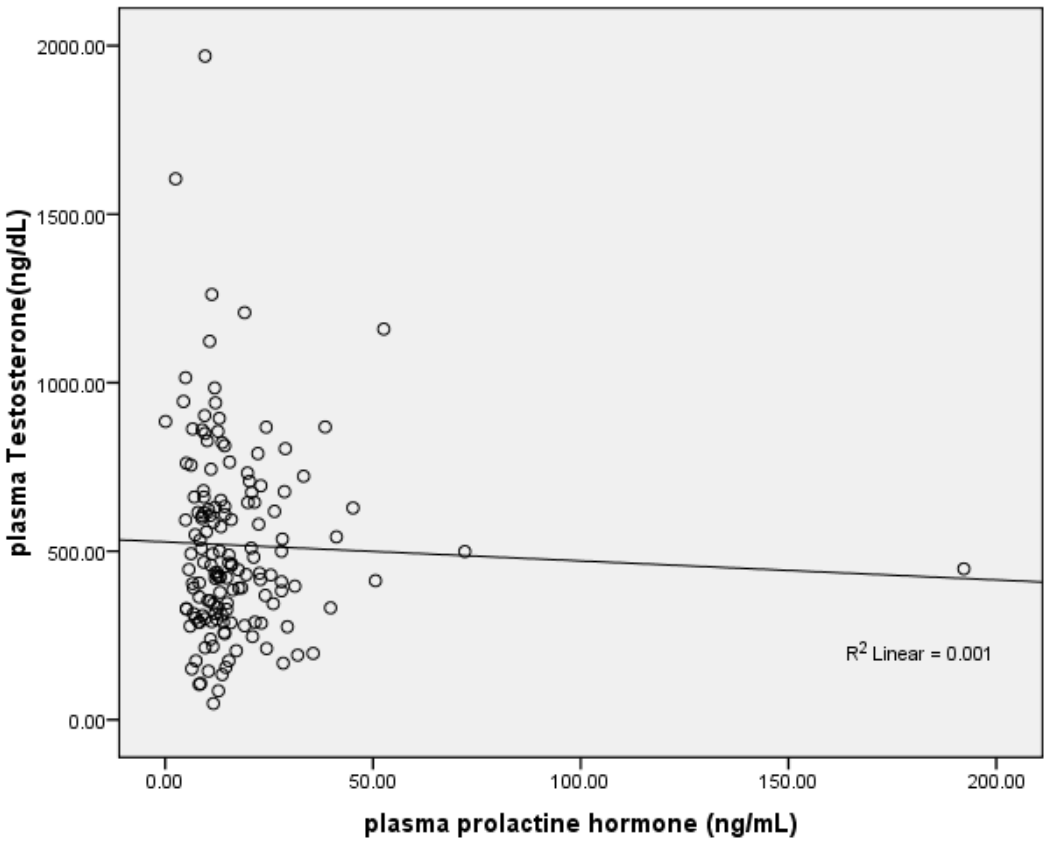

Fig. (3-3): A scatter plot shows the correlation between the plasma levels of prolactin hormone and Testosterone (r= - 0.03 , P 0.362) among the test group which is insignificant .

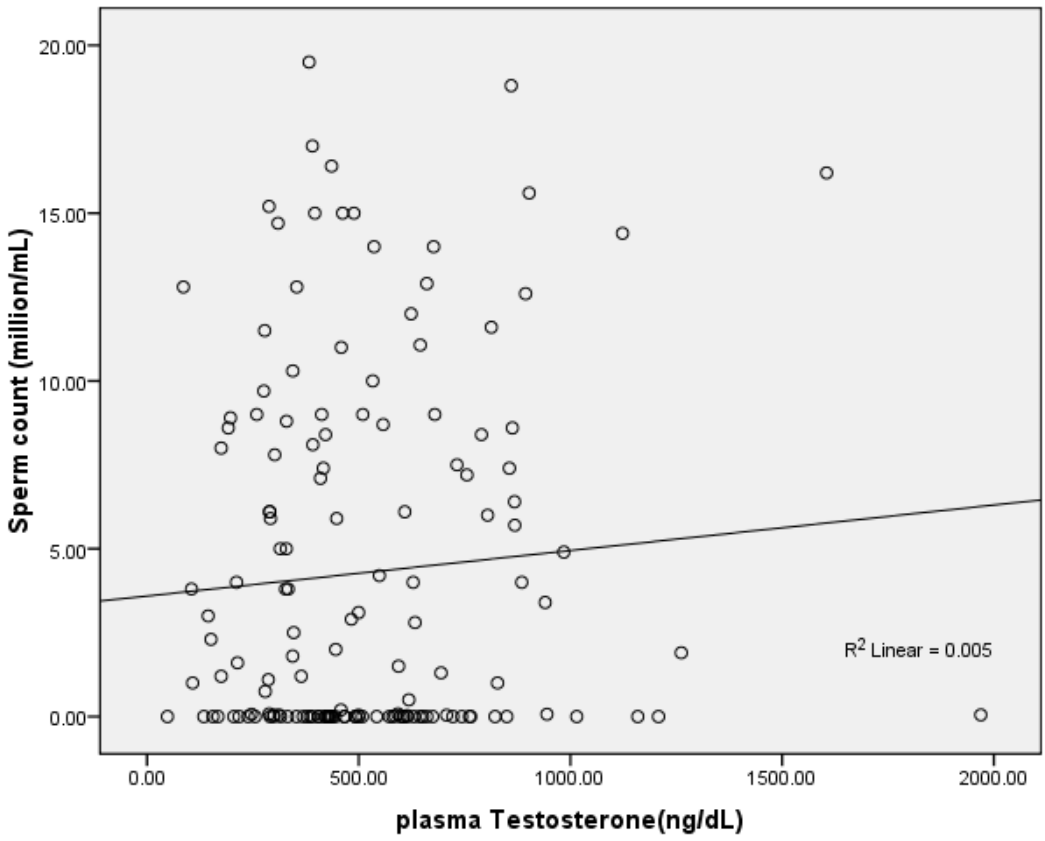

Fig. (3-4): A scatter plot shows the correlation between the plasma levels of Testosterone and the sperm count (r= 0.04 , P 0.658) among the test group which is insignificant .

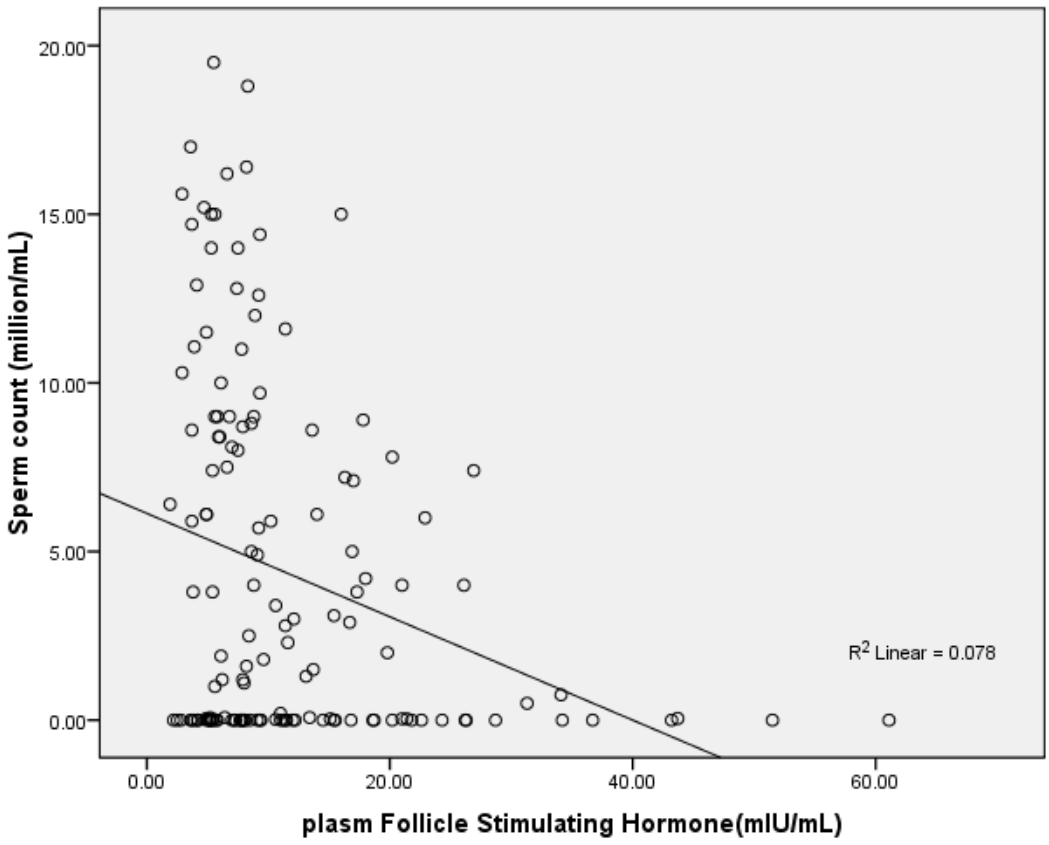

Fig. (3-5): A scatter plot shows the correlation between the plasma levels of Follicle stimulating hormone and the sperm count (r= - 0.17 , P 0.018) among the test group which is significant .

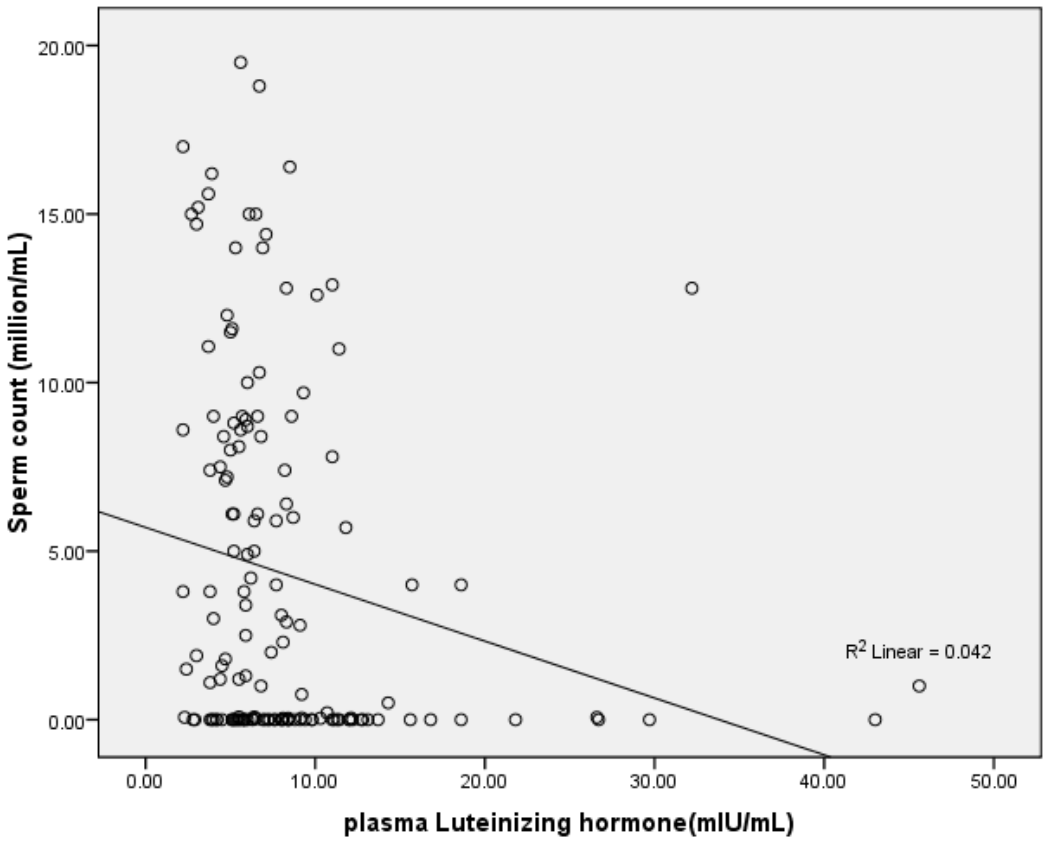

Fig. (3-6): A scatter plot shows the correlation between the plasma levels of Luteinizing hormone and the sperm count (r= - 0.21 , P 0.004) among the test group which is significant .

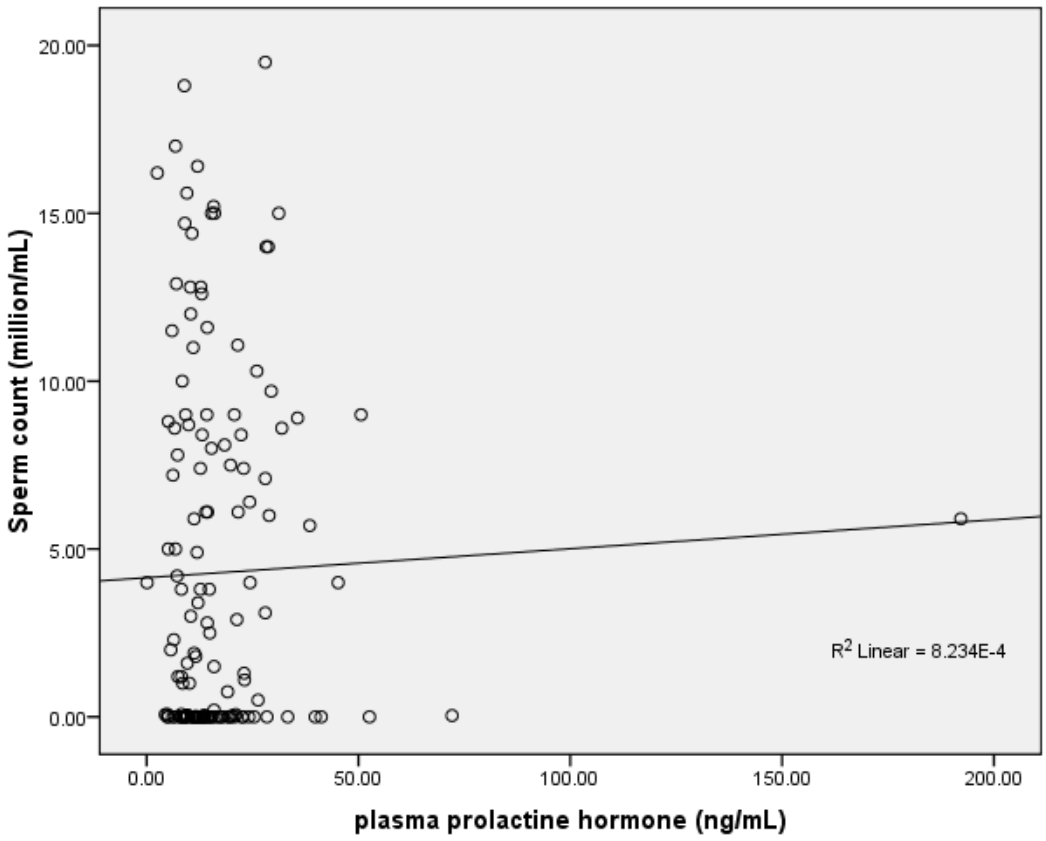

Fig. (3-7): A scatter plot shows the correlation between the plasma levels of prolactin hormone and the sperm count (r= - 0.03 , P 0.371) among the test group which is insignificant .

CHAPTER FOUR

DISCUSSION, CONCLUSION

and

RECOMENDATIONS

Chapter Four

4. Discussion, Conclusion and Recommendations

4.1. Discussion

This study aimed to determine plasma levels of Follicle stimulating hormone , Luteinizing hormone , Prolactin and Testosterone of patients diagnosed as infertile males in red sea state , age of the patients ranged from 20 to 56 year.with mean of 37.8 ±7.2 year, 80% had primary infertility . and 20% had secondary infertility.

The results of the sperm count in this study showed a significant reduction when the mean of the sperm count of the test group when compared with that of the control group , (p = 0.000), in test group there were 36.7% of them were found azoospermia and 63.3% were oligospermia .

In this study there were 14% had a past sexual transmitted disease and 86% not infected never and the correlation between the sperm count of test group and sexually transmitted diseases (p =0. 011) which indicating significant meaning that these diseases affect the sperm production .

In the study the correlation between the sperm count of patients who are diabetic and who are not diabetic (p =0. 010) the results showed that Diabetes mellitus cause a decrease in sperm production , also the correlation between the sperm count of patients who was infected by mumps and who hadn't previous infection was (p =0. 109) which is insignificant mean that mumps doesn't affect the production of sperm in test group , in the correlation between the sperm count of patients who had Tests pain and who hadn't a pain was (p =0. 074) which is insignificant mean that the tests pain may not affect the production of

sperm , also the correlation between the sperm count of patients who had past history of tests surgery and who hadn't was (p =0. 004) which is significant and the Tests surgery may cause of low sperm count . The correlation between the sperm count of patients who had a congenital defects and who hadn't was (p =0. 462) which is insignificant because the most of this patients the defect was they had one test which can do the job.

According to social habits the correlation between the sperm count of patients that who were smoke and who weren't smoke (p =0. 350) which is insignificant . The correlation between the sperm count of patients that who were use snough and who weren't use it (p =0.278) which is insignificant , and also the correlation between the sperm count of patients that who were drink alcohol and who weren't drink it (p =0.911) which is insignificant .

The present study showed a significant increase in the mean value of the plasma levels of Follicle stimulating hormone (FSH) of the test group compared with control group as shown in table (4-1) , (p = 0.00) . These results agrees with a study done by (Geidam *et al* 2008) and (Burney 2002) who reported that the level of FSH usually correlates inversely with spermatogensis . Elevation of FSH occur in men with intact hypothalamic – pituitary-adrenal axis when there is severe damage of germinal epithelium , and high FSH value are compatible with primary testicular failure . It has been known for a long time that low levels of inhibin causes raised levels of FSH , a significant positive correlation between the two variables showed in Fig. (4-5) .

The current study also showed a significant increase in plasma levels of Luteinizing hormone (LH) of the test group compared to control group, (p = 0.00) ,this agreed with a study done by Geidam (Geidam *et al*

2008) , and who reported that the LH in the test group is higher than normal range , in Fig. (4-6) . The present study also showed a significant weak correlation between that plasma levels of LH and the sperm count . This suggested that a secondary increase of LH occur as a result of reduction in the sperm count .

The results also showed a significant increase of the levels of serum Prolactin hormone of the test group when compared to control group (p = 0.001) , as (Burney 2002) study reported that PRL is elevated in patients with normal count of sperm but infertile and this study find that PRL is elevated in test group patients whom were known to have low sperm count , as the (Carter *et al* 1978) reported, as it may signify prolactin-secreting pituitary adenoma, which is amenable to therapy , however, as prolactin elevation may be induced by some abnormalities of the thyroid gland, further evaluation of these patients should include pituitary imaging and thyroid function test .

No significant correlation between the plasma levels of Prolactin hormone and the sperm count in the test group.

The current study also shows no significant difference between the mean of the plasma levels of testosterone hormone of the test group and to that of the control group (p = 0.100) . These results agreed with the results of Geidam (Geidam *et al* 2008) and El- Migadi (El-Migdadi etal 2005)

No significant correlation between the plasma levels of testosterone and the sperm count in the test group.

The results showed insignificant negative correlations between FSH , LH , PRL and Testosterone .

4.2. Conclusion

1. The sperm count is significantly decreased in test group compare to control group .

2. Plasma levels of Follicle Stimulating hormone , Luteinizing hormone and Prolactin significantly increased in test group .

3. No significant difference in Testosterone plasma levels of test group compare to control .

4. No significant correlation of Follicle Stimulating Hormone , Luteinizing Hormone and Prolactin plasma levels and that of Testosterone , and also no significant correlation of Testosterone ,Prolactin and the sperm count .

5.There is a significant correlation between the plasma levels of Follicle Stimulating hormone ,Luteinizing hormone and the sperm count.

4.3 Recommendations :

1. Beside seminal analysis , FSH , LH , PRL and testosterone investigation is recommended this may help in infertility diagnosis

2. Further study should be done to asses thyroid function, oestradiol ,and gonadotropin releasing hormone in infertile men ,since these hormones have a role in normal reproductive females .

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APPENDIX

Appendix - 1

بسم الله الرحمن الرحيم

جامعة السودان للعلوم والتكنولوجيا ـ كلية الدراسات العليا

Sudan University of Science and Technology

College of Graduate Gtudies & Scientific Research

Assessment of Fertility Hormones among Infertile Men in Red Sea State

Questionnaire no :

5.1 Personal Data:

Name :-

Age :-

Laboratory Number:-

Hospital or clinic: -

Duration of married:-

5.2 Clinical Presentation:-

Has child	Ys	No
Past history of STD	Ys	No
Diabetes mellitus	Ys	No
Hypertension	Ys	No
Mumpus in life	Ys	No
Testicular pain	Ys	No
Testicular, surgery	Ys	No

Congenital defect | YS | No

5.3.Habits

Smoke | YS | No

Alcohol | YS | No

Snough | YS | No

5.4.Laboratory Findings:

A) Blood Test :

FHS Level	LH level	PRL level	Testosterone level

B) Seminal tests :

Seminal volume

Sperm count:-

Azoospermia	Oligospermia	Normal
		.

Appendix - 2

ST AIA-PACK FSH

For Quantitative Measurement of follicle-stimulating hormone (FSH) in Serum or Heparinized Plasma

NAME AND INTENDED USE

ST AIA-PACK FSH is designed for IN VITRO DIAGNOSTIC USE ONLY for the quantitative measurement of follicle-stimulating hormone (FSH) in human serum or heparinized plasma on TOSOH AIA System Analyzers.

SUMMARY AND EXPLANATION OF TEST

Follicle-stimulating hormone (FSH) is a glycoprotein which, like LH, hCG and TSH, consists of alpha and beta chains. The alpha chain is virtually identical in all four hormones, whereas the beta chains are different and determine both the specific biological activity and immunological characteristics of each hormone *(1,2)*. The FSH molecule contains approximately 16% carbohydrate and has a molecular weight of approximately 28,000 to 30,000 daltons *(3)*. FSH stimulates ovarian follicle growth and estrogen production in women, and testicular spermatogenesis in men *(4-7)*.

Hypothalamic control of both FSH and LH secretion by the anterior pituitary appears to be by a common releasing hormone, gonadotropin-releasing hormone (GnRH) with negative feed back at the hypothalamic level by estrogen in the female and testosterone in the male.

Determination of FSH concentrations is essential in assessment and monitoring of patients with suspected infertility. Other physiological disorders associated with abnormal FSH secretion have been reported *(3, 8-11)*.

PRINCIPLE OF THE ASSAY

The ST AIA-PACK FSH is a two-site immunoenzymometric assay which is performed entirely in the AIA-PACK test cups. FSH present in the test sample is bound with monoclonal antibody immobilized on a magnetic solid phase and enzyme-labeled monoclonal antibody in the AIA-PACK test cups. The magnetic beads are washed to remove unbound enzyme-labeled monoclonal antibody and are then incubated with a fluorogenic substrate 4-methylumbelliferyl phosphate (4MUP). The amount of enzyme-labeled monoclonal antibody that binds to the beads is directly proportional to the FSH concentration in the test sample. A standard curve is constructed and unknown sample concentrations are calculated using this curve.

MATERIAL PROVIDED (ST AIA-PACK FSH, Cat. No. 0025265)

Plastic test cups containing lyophilized twelve magnetic beads coated with anti-FSH mouse monoclonal antibody and 100 µL of anti-FSH mouse monoclonal antibody (to human FSH) conjugated to bovine alkaline phosphatase with sodium azide as a preservative.

MATERIALS REQUIRED BUT NOT PROVIDED

The following materials are not provided but are required to perform follicle-stimulating hormone analysis using the ST AIA-PACK FSH (Cat. No. 0025265) on the TOSOH AIA System Analyzers. They are available separately from TOSOH.

Materials			Cat. No.
AIA Nex-IA or AIA-21			0018539
AIA Nex-IA or AIA-21 LA			0018540
AIA-1800 ST			0019836
AIA-1800 LA			0019837
AIA-2000 ST			0022100
AIA-2000 LA			0022101
AIA-600 II			0019014
AIA-600 II BCR			0019328
AIA-360			0019945
AIA-PACK SUBSTRATE SET II			0020968
AIA-PACK SUBSTRATE REAGENT II			
AIA-PACK SUBSTRATE RECONSTITUENT II			
AIA-PACK FSH CALIBRATOR SET			0020365
ZERO CALIBRATOR	0	mIU/mL	
POSITIVE CALIBRATOR	100	mIU/mL (approx.)	
AIA-PACK FSH SAMPLE DILUTING SOLUTION			0020565
AIA-PACK WASH CONCENTRATE			0020955
AIA-PACK DILUENT CONCENTRATE			0020956
SAMPLE CUPS			0018581
AIA-PACK DETECTOR STANDARDIZATION TEST CUP			0020970
AIA-PACK SAMPLE TREATMENT CUP			0020971
Additional Requirements for AIA Nex-IA / AIA-21 only:			
PIPETTE TIPS			0018552
PRELOADED PIPETTE TIPS			0018583
Additional Requirements for AIA-600 II, AIA-1800 and AIA-2000:			
PIPETTE TIPS			0019215
TIP RACK			0019216
PRELOADED PIPETTE TIPS			0022103

Only materials obtained from TOSOH should be used. Materials obtained elsewhere should not be substituted since assay performance is characterized based strictly on TOSOH materials.

WARNINGS AND PRECAUTIONS

1. The ST AIA-PACK FSH is intended for in vitro diagnostic use only.
2. Test cups from different lots or different assays should not be mixed within a tray.
3. The ST AIA-PACK FSH contains sodium azide, which may react with lead or copper plumbing to form potentially explosive metal azides. When disposing of such reagents, always flush with large volumes of water to prevent azide build-up.
4. Human serum is not used in the preparation of this product; however, since human specimens will be used for samples and other quality control products in the lab may be derived from human serum, please use standard laboratory safety procedures in handling all specimens and controls.
5. Do not use beyond the expiration date.
6. The ST AIA-PACK FSH has been designed so that the high dose "hook effect" is not a problem for the vast majority of samples. Samples with FSH concentrations between 200 and 20,000 mIU/mL will read > 200 mIU/mL. The "hook effect" phenomenon may occur at FSH concentrations > 20,000 mIU/mL.
7. TOSOH AIA-1200 series or AIA-600 Immunoassay Analyzers can NOT be used to perform the ST AIA-PACK FSH assay.

STORAGE AND STABILITY

All unopened materials are stable until the expiration date on the label when stored at the specified temperature.

Materials	Cat. No.
2-8°C:	
ST AIA-PACK FSH	0025265
AIA-PACK FSH CALIBRATOR SET	0020365
AIA-PACK FSH SAMPLE DILUTING SOLUTION	0020565
AIA-PACK SUBSTRATE SET II	0020968
AIA-PACK WASH CONCENTRATE	0020955
AIA-PACK DILUENT CONCENTRATE	0020956
1-30°C:	
AIA-PACK DETECTOR STANDARDIZATION TEST CUP	0020970
AIA-PACK SAMPLE TREATMENT CUP	0020971

ST AIA-PACK FSH test cups may be stored at 18-25°C for up to 1 day. Calibrators and Sample Diluting Solution should be used within 1 day and 7 days of opening, respectively, provided the vials are kept tightly sealed and refrigerated at 2-8°C. Reconstituted substrate solution is stable for 3 days at 18-25°C or 7 days at 2-8°C. Working diluent and wash solutions are stable for 30 days at 18-25°C. Reagents should not be used if they appear cloudy or discolored.

SPECIMEN COLLECTION AND HANDLING

- Serum or heparinized plasma is required for the assay. EDTA and citrated plasma SHOULD NOT BE USED.
- If using serum, a venous blood sample is collected aseptically without additives. Store at 18-25°C until a clot has formed (usually 15-45 minutes), then centrifuge to obtain the serum specimen for assay.
- If using heparinized plasma, a venous blood sample is collected aseptically with designated additive. Centrifuge and separate plasma from the packed cells as soon as possible.
- Specimen types should not be used interchangeably during serial monitoring of an individual patient. Measured concentrations may vary slightly between sample types in certain patients.
- Samples may be stored at 2-8°C for up to 24 hours prior to analysis. If the analysis cannot be done within 24 hours, the sample should be stored frozen at –20°C or below for up to 60 days.
- Repeated freeze-thaw cycles should be avoided. Turbid serum samples or samples containing particulate matter should be centrifuged prior to testing. Prior to assay, bring frozen samples to 18-25°C slowly and mix gently.
- The sample required for analysis is 50 µL.

PROCEDURE

For the AIA Nex-IA / AIA-21, AIA-600 II, AIA-1800, AIA-2000 and AIA-360, please refer to their Operator's Manual for detailed instructions.

I. Reagent Preparation

A) Substrate Solution

Bring all reagents to 18-25°C before preparing the working reagent. Add the entire contents of the AIA-PACK SUBSTRATE RECONSTITUENT II (100 mL) to the lyophilized AIA-PACK SUBSTRATE REAGENT II, mix thoroughly to dissolve the solid material.

B) Wash Solution

Add the entire contents of the AIA-PACK WASH CONCENTRATE (100 mL) to approximately 2.0 L of CAP Class I or NCCLS Type I Reagent Grade water, mix well, and adjust the final volume to 2.5 L.

C) Diluent

Add the entire contents of the AIA-PACK DILUENT CONCENTRATE (100 mL) to approximately 4.0 L of CAP Class I or NCCLS Type I Reagent Grade water, mix well, and adjust the final volume to 5.0 L.

II. Calibration Procedure

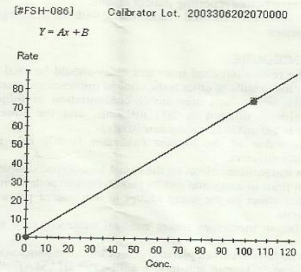
A) Calibration Curve

The calibrators for use with the ST AIA-PACK FSH have been standardized on WHO 2nd IRP 78/549 (1980).

The calibration curve for ST AIA-PACK FSH is stable for up to 90 days. Calibration stability is monitored by quality control performance and is dependent on proper reagent handling and TOSOH AIA System maintenance according to the manufacturer's instructions.

Recalibration may be necessary more frequently if controls are out of the established range for this assay or when certain service procedures are performed (e.g. temperature adjustment, sampling mechanism changes, maintenance of the wash probe or detector lamp adjustment or change). For further information regarding instrument operation, consult the TOSOH AIA System Operator's Manual.

A sample calibration curve from AIA-1800 follows and shows the algorithm used for calculating results.



B) Calibration Procedure

1. Refer to the appropriate TOSOH AIA System Operator's Manual for the procedural instructions.
2. Verify that both the calibrator lot and concentration numbers have been correctly entered into the software.
3. Calibrators for ST AIA-PACK FSH are provided ready for use. TOSOH recommends that all calibrators should be run in triplicate.

C) Calibration Acceptability Criteria

1. The mean rate for the ZERO CALIBRATOR should be < 3.0 nmol/(L·s).
2. Since there is a direct relationship between concentration and rate, the rate should increase as the concentration increases.
3. The replicate values should be within a 10% range.

D) Calibration Review and Acceptance

1. Review the calibration curve carefully, using the criteria listed above.
2. Edit the calibration if necessary, then accept the calibration.

For further information regarding calibration, consult the TOSOH AIA System Operator's Manual.

III. Quality Control Procedure

A) Commercially Available Controls

Commercially available controls should be run at least once per day. It is recommended that at least two levels of controls, normal and abnormal, be used. Laboratory policy for this particular assay designates the following:

Control Material: _____

Frequency: _____

Lot number of control material, acceptable limits, and corrective action to be taken if controls do not meet laboratory criteria will be found in a separate quality control document maintained by the laboratory.

B) Quality Control Procedure

1. Assay quality control specimens as instructed in the specific Operator's Manual for your analyzer. In addition, refer to the TOSOH AIA System Operator's Manual for detailed instructions on defining and editing the files.
2. Quality control material to be run with this assay is defined by individual laboratory policy.

IV. Specimen Processing

A) Preparation

Following specific instructions in the Operator's Manual for the analyzer, place samples on the instrument appropriately. Barcoded primary tubes as well as sample cups can be run on the AIA Nex-IA / AIA-21, AIA-600 II, AIA-1800, AIA-2000 and AIA-360.

B) Assay Procedure

1. Ensure a sufficient quantity of ST AIA-PACK FSH test cups for the number of samples to be run.
2. Load patient samples as instructed in the Operator's Manual and proceed with analysis. Note: The AIA Nex-IA / AIA-21, AIA-600 II, AIA-1800 and AIA-2000 will require AIA-PACK SAMPLE TREATMENT CUPs if onboard dilutions are utilized.

PROCEDURAL NOTES

1. Lyophilized substrate must be completely dissolved.
2. Ligand assays performed by the TOSOH AIA System Analyzers require that the laboratory use water designated by the College of American Pathologists as Class I or by NCCLS as Type I. Water should be tested at least once per month and should be free of particulate matter including bacteria. The pH of the water should also be routinely tested. For further information, consult the NCCLS document "Preparation and Testing of Reagent Water in the Clinical Laboratory," NCCLS Document C3-A3, Volume 11 No. 13, originally approved as a guideline by NCCLS in October 1997.
3. If a specimen follicle-stimulating hormone concentration is found to be greater than the upper limit of the assay range, 200 mIU/mL, the specimen should be diluted with the AIA-PACK FSH SAMPLE DILUTING SOLUTION and reassayed according to the Assay Procedure. The recommended dilution for specimens containing greater than 200 mIU/mL is 1:10 or 1:100. It is desirable to dilute the specimen so that the diluted specimen reads between 5 and 200 mIU/mL. The dilution factor should be entered into the software. For further information on the dilution of specimens, refer to the TOSOH AIA System Operator's Manual.
4. The TOSOH AIA System Analyzers can store two different calibration curves for each analyte at one time. Therefore, up to two different lots of ST AIA-PACK FSH test cups can be used during the same run.
5. If the assay specifications for this test are not ready in the system software, the specifications must be entered under test code **068**.

CALCULATION OF RESULTS

The TOSOH AIA System Analyzers perform all sample and reagent handling operations automatically. The TOSOH AIA System Analyzers read the rate of fluorescence produced by the reaction and automatically convert the rate to follicle-stimulating hormone concentration in mIU/mL.

For samples requiring dilution, the AIA Nex-IA / AIA-21, AIA-600 II, AIA-1800 and AIA-2000 will automatically perform dilutions and calculate results if the dilution factors are entered into the software. Dilution factors may be entered into the Test File, or pre-defined dilution factors may be selected in Specimen Processing.

EVALUATION OF RESULTS

Quality Control

In order to monitor and evaluate the precision of the analytical performance, it is recommended that commercially available control samples should be assayed according to the local regulations.

The minimum recommendations for the frequency of running internal control material are:

After calibration, three levels of the internal control are run in order to accept the calibration curve.

The three levels of controls are repeated when certain service procedures are performed (e.g. temperature adjustment, sampling mechanism changes, maintenance of the wash probe or detector lamp adjustment or change).

After daily maintenance, at least two levels of the control should be run in order to verify the overall performance of the TOSOH AIA System Analyzers.

If one or more control sample value(s) is out of the acceptable range, it is necessary to investigate the validity of the calibration curve before reporting patient results.

Standard laboratory procedures should be followed in accordance with the strict regulatory agency under which the laboratory operates.

LIMITATIONS OF THE PROCEDURE

- For diagnostic purposes, the results obtained from this assay should be used in conjunction with other data (e.g. symptoms, results of other tests, clinical impressions, therapy, etc.).
- Using ST AIA-PACK FSH, the highest measurable concentration of follicle-stimulating hormone in specimens without dilution is 200 mIU/mL, and the lowest measurable concentration in specimens is 1.0 mIU/mL (assay sensitivity).
- Although the approximate value of the highest calibrator is 100 mIU/mL, the exact concentration may be slightly different.
- Although hemolysis has an insignificant effect on the assay, hemolyzed samples may indicate mistreatment of a specimen prior to assay and results should be interpreted with caution.
- Lipemia has an insignificant effect on the assay except in the case of gross lipemia where spatial interference may occur.
- Specimens from patients taking medicines and/or medical treatment may show erroneous results.
- Specimens from patients who have received preparations of mouse monoclonal antibodies for diagnosis or therapy may contain human anti-mouse antibodies (HAMA). Such specimens may show falsely elevated values when tested for follicle-stimulating hormone.
- For a more complete understanding of the limitations of this procedure, please refer to the SPECIMEN COLLECTION AND HANDLING, WARNINGS AND PRECAUTIONS, STORAGE AND STABILITY, and PROCEDURAL NOTES sections in this insert sheet.

EXPECTED VALUES

Each laboratory should determine a reference interval which corresponds to the characteristics of the population being tested. As with all diagnostic procedures, clinical results must be interpreted with regard to concomitant medications administered to the patient *(12)*.

Reference Ranges

The interval given here was determined in serum samples from apparently healthy Asian individuals.

Category	Range
Male (n=132)	2.1 - 18.6 mIU/mL
Ovulating Females (n=143)	
- Follicular Phase (n=52)	4.5 - 11.0 mIU/mL
- Mid-Cycle (n=49)	3.6 - 20.6 mIU/mL
- Luteal Phase (n=42)	1.5 - 10.8 mIU/mL
Postmenopausal Females (n=21)	36.6 - 168.8 mIU/mL

Conversion Factors

FSH concentrations in this application are in units of mIU/mL. Conversion to SI units of IU/L may be made using the following equations:

IU FSH /L = mIU FSH /mL x 1.0

PERFORMANCE CHARACTERISTICS

ACCURACY

a. Recovery: Three serum pools were spiked with three different levels of FSH and assayed before and after spiking.

Sample	Initial Value (mIU/mL)	FSH Added (mIU/mL)	Expected Value (mIU/mL)	Measured Value (mIU/mL)	Percent Recovery (%)
Serum A1	4.70	3.74	8.44	8.07	95.5
	4.70	7.48	12.18	12.07	99.0
	4.70	14.96	19.66	19.75	100.4
Serum B1	4.60	3.74	8.34	8.21	98.4
	4.60	7.48	12.08	12.26	101.5
	4.60	14.96	19.56	19.05	97.4
Serum C1	13.87	3.74	17.61	17.42	98.9
	13.87	7.48	21.35	21.20	99.3
	13.87	14.96	28.83	28.31	98.2

b. Dilution: Three serum samples containing high concentrations of FSH were serially diluted with the AIA-PACK FSH SAMPLE DILUTING SOLUTION and assayed.

Sample	Dilution Factor	Expected Value (mIU/mL)	Measured Value (mIU/mL)	Percent Recovery (%)
Serum A2	none		115.8	
	7.5/10	86.9	86.1	99.1
	5.0/10	57.9	58.5	100.9
	2.5/10	29.0	29.8	103.0
	1.0/10	11.6	11.0	94.8
Serum B2	none		166.0	
	7.5/10	124.5	123.3	99.0
	5.0/10	83.0	83.2	100.2
	2.5/10	41.5	41.1	99.0
	1.0/10	16.6	15.3	92.0
Serum C2	none		184.7	
	7.5/10	138.5	136.5	98.6
	5.0/10	92.4	91.8	99.4
	2.5/10	46.2	43.3	93.7
	1.0/10	18.5	17.0	92.0

PRECISION

a. Within run precision was determined using three controls in a total of 20 runs. Within each run, one set of duplicates per control was assayed. The mean of each duplicate was used to obtain the pooled standard deviation (SD), which was then used to calculate the coefficient of variation (CV).

Sample	Mean (mIU/mL)	Pooled SD (mIU/mL)	CV (%)
Serum A3	4.96	0.128	2.6
Serum B3	16.37	0.290	1.8
Serum C3	60.15	0.886	1.5

b. Total precision was determined by the duplicate assay of three controls in 20 separate runs. The means of each run were used to calculate the pooled standard deviation (SD) and coefficient of variation (CV).

Sample	Mean (mIU/mL)	Pooled SD (mIU/mL)	CV (%)
Serum A3	4.96	0.276	5.6
Serum B3	16.37	0.774	4.7
Serum C3	60.15	2.573	4.3

CORRELATION

The correlation between serum (x) and heparinized plasma (y) on ST AIA-PACK FSH was carried out using 205 patient specimens.

Slope	0.983
y-Intercept	-0.118
Correlation Coefficient	0.999
Range of Samples	1.0-197.1
Number of Samples	205

SPECIFICITY

The following substances were tested for cross-reactivity. The cross-reactivity (%) is the percentage of the compound which will be identified as FSH. If these compounds are present in the specimen at the same concentration as FSH, the final result will be increased by these percentages.

Compound	Cross-reactivity (%)
FSH	100
TSH	0.31
LH	0.15
hCG	0.03

SENSITIVITY

The minimal detectable concentration (MDC) of follicle-stimulating hormone is estimated to be 1.0 mIU/mL. The MDC is defined as the concentration of FSH which corresponds to the rate of fluorescence that is two standard deviations from the mean rate of fluorescence of 20 replicate determinations of a ZERO CALIBRATOR.

INTERFERENCE

Interference is defined, for the purposes of this study, as recovery outside of 10% of the known specimen mean concentration after the following substances are added to human specimens.

- Hemoglobin (up to 390 mg/dL), free bilirubin (up to 17 mg/dL) and conjugated bilirubin (up to 18 mg/dL) do not interfere with the assay.
- Lipemia, as indicated by triglyceride concentration (up to 1,600 mg/dL), does not interfere with the assay.
- Ascorbic acid (up to 20 mg/dL) does not interfere with the assay.
- Protein, as indicated by human albumin concentration (up to 5.0 g/dL), does not interfere with the assay.
- Heparin (up to 100 U/mL) does not interfere with the assay.

REFERENCES

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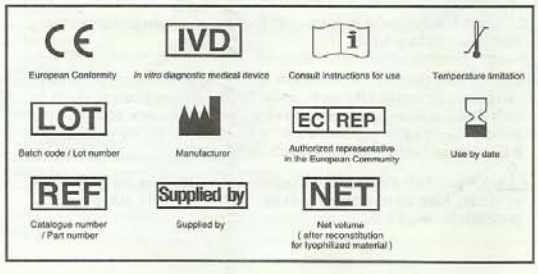
TOSOH CORPORATION
Shiba-koen First Bldg.
3-8-2 Shiba, Minato-ku, Tokyo 105-8623
Japan
Phone :+81-3-5427-5181
Fax :+81-3-5427-5220

EC REP
TOSOH EUROPE N.V.
Transportstraat 4
B-3980 Tessenderlo, Belgium
Phone :+32-13-66 88 30
Fax :+32-13-66 47 49

Supplied by
TOSOH BIOSCIENCE, INC.
6000 Shoreline Ct., Suite 101
South San Francisco, CA94080, USA
Phone :+1-650-615-4970
Fax :+1-650-615-0415
Phone :(800)248-6764
Fax :(800)685-7595



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Appendix - 3

ST AIA-PACK LH II

For Quantitative Measurement of luteinizing hormone (LH) in Serum or Heparinized Plasma

NAME AND INTENDED USE

ST AIA-PACK LH II is designed for IN VITRO DIAGNOSTIC USE ONLY for the quantitative measurement of luteinizing hormone (LH) in human serum or heparinized plasma on TOSOH AIA System Analyzers.

SUMMARY AND EXPLANATION OF TEST

Human luteinizing hormone (LH) is a glycoprotein which, like FSH, HCG and TSH, consists of alpha and beta chains. The alpha chain is virtually identical in all four hormones, whereas the beta chains are different and determine both the specific biological activity and immunological characteristics of each hormone *(1,2)*.

The LH molecule contains 15-18% carbohydrate and has a molecular weight of approximately 29,000 daltons *(3,4)*. LH induces ovulation and thereafter maintains the corpus luteum and progesterone production in women *(5-8)*. In men, LH stimulates testosterone production by the Leydig cells *(5,6,9)*.

Hypothalamic control of both LH and FSH secretion by the anterior pituitary appears to be by a common releasing hormone, gonadotropin-releasing hormone (GnRH) or luteinizing hormone-releasing hormone (LHRH), with negative feedback at the hypothalamic level by estrogen in female and testosterone in male.

Through the use of monoclonal antibody technology which provides the necessary specificity and sensitivity, determination of LH concentrations is critically important in assessment and monitoring of patients with suspected infertility *(10,11)*. The raised levels of LH and LH: FSH ratio is frequently found in polycystic ovary syndrome (PCOS), a common cause of infertility in women *(12,13)* and other physiological disorders associated with abnormal LH secretion *(12-15)*.

PRINCIPLE OF THE ASSAY

The ST AIA-PACK LH II is a two-site immunoenzymometric assay which is performed entirely in the AIA-PACK test cups. LH present in the test sample is bound with monoclonal antibody immobilized on a magnetic solid phase and enzyme-labeled monoclonal antibody in the AIA-PACK test cups. The magnetic beads are washed to remove unbound enzyme-labeled monoclonal antibody and are then incubated with a fluorogenic substrate 4-methylumbelliferyl phosphate (4MUP). The amount of enzyme-labeled monoclonal antibody that binds to the beads is directly proportional to the LH concentration in the test sample. A standard curve is constructed, and unknown sample concentrations are calculated using this curve.

MATERIAL PROVIDED (ST AIA-PACK LH II, Cat. No. 0025296)

Plastic test cups containing lyophilized twelve magnetic beads coated with mouse anti-LH monoclonal antibody and 100 µL of mouse anti-LH monoclonal antibody (to human LH) conjugated to bovine alkaline phosphatase with sodium azide as a preservative.

MATERIALS REQUIRED BUT NOT PROVIDED

The following materials are not provided but are required to perform luteinizing hormone analysis using the ST AIA-PACK LH II (Cat. No. 0025296) on the TOSOH AIA System Analyzers. They are available separately from TOSOH.

Materials			Cat. No.
AIA Nex-IA or AIA-21			0018539
AIA Nex-IA or AIA-21 LA			0018540
AIA-1800 ST			0019836
AIA-1800 LA			0019837
AIA-2000 ST			0022100
AIA-2000 LA			0022101
AIA-600 II			0019014
AIA-600 II BCR			0019328
AIA-360			0019945
AIA-PACK SUBSTRATE SET II			0020968
AIA-PACK SUBSTRATE REAGENT II			
AIA-PACK SUBSTRATE RECONSTITUENT II			
ST AIA-PACK LH II CALIBRATOR SET			0025396
CALIBRATOR (1)	0	mIU/mL	
CALIBRATOR (2)	5	mIU/mL (approx.)	
CALIBRATOR (3)	20	mIU/mL (approx.)	
CALIBRATOR (4)	50	mIU/mL (approx.)	
CALIBRATOR (5)	100	mIU/mL (approx.)	
CALIBRATOR (6)	220	mIU/mL (approx.)	
AIA-PACK LH II SAMPLE DILUTING SOLUTION			0020596
AIA-PACK WASH CONCENTRATE			0020955
AIA-PACK DILUENT CONCENTRATE			0020956
SAMPLE CUPS			0018581
AIA-PACK DETECTOR STANDARDIZATION TEST CUP			0020970
AIA-PACK SAMPLE TREATMENT CUP			0020971
Additional Requirements for AIA Nex-IA / AIA-21 only:			
PIPETTE TIPS			0018552
PRELOADED PIPETTE TIPS			0018583
Additional Requirements for AIA-600 II, AIA-1800 and AIA-2000:			
PIPETTE TIPS			0019215
TIP RACK			0019216
PRELOADED PIPETTE TIPS			0022103

Only materials obtained from TOSOH should be used. Materials obtained elsewhere should not be substituted since assay performance is characterized based strictly on TOSOH materials.

DO NOT USE IN COMBINATION WITH AIA-PACK LH II CALIBRATOR SET (CAT. NO. 0020396)

ASSAY PREPARATION

1. Set up the TOSOH AIA System Analyzers for luteinizing hormone analysis. REFER TO THE APPROPRIATE TOSOH AIA SYSTEM ANALYZERS OPERATOR'S MANUAL.
2. Bring all test samples and controls to 18-25°C, and mix well.

WARNINGS AND PRECAUTIONS

1. The ST AIA-PACK LH II is intended for in vitro diagnostic use only.
2. Test cups from different lots or different assays should not be mixed within a tray.
3. The ST AIA-PACK LH II contains sodium azide, which may react with lead or copper plumbing to form potentially explosive metal azides. When disposing of such reagents, always flush with large volumes of water to prevent azide build-up.
4. Human serum is not used in the preparation of this product; however, since human specimens will be used for samples and other quality control products in the lab may be derived from human serum, please use standard laboratory safety procedures in handling all specimens and controls.
5. Do not use beyond the expiration date.
6. The ST AIA-PACK LH II has been designed so that the high dose "hook effect" is not a problem for the vast majority of samples. Samples with LH concentrations between 200 and 10,000 mIU/mL will read > 200 mIU/mL. The "hook effect" phenomenon may occur at LH concentrations > 10,000 mIU/mL.
7. TOSOH AIA-1200 series or AIA-600 Immunoassay Analyzers can NOT be used to perform the ST AIA-PACK LH II assay.

STORAGE AND STABILITY

All unopened materials are stable until the expiration date on the label when stored at the specified temperature.

Materials	Cat. No.
2-8°C:	
ST AIA-PACK LH II	0025296
ST AIA-PACK LH II CALIBRATOR SET	0025396
AIA-PACK LH II SAMPLE DILUTING SOLUTION	0020596
AIA-PACK SUBSTRATE SET II	0020968
AIA-PACK WASH CONCENTRATE	0020955
AIA-PACK DILUENT CONCENTRATE	0020956
1-30°C:	
AIA-PACK DETECTOR STANDARDIZATION TEST CUP	0020970
AIA-PACK SAMPLE TREATMENT CUP	0020971

ST AIA-PACK LH II test cups may be stored at 18-25°C for up to 1 day. Calibrators and Sample Diluting Solution should be used within 1 day and 7 days of opening or reconstituting, respectively, provided the vials are kept tightly sealed and refrigerated at 2-8°C. Reconstituted substrate solution is stable for 3 days at 18-25°C or 7 days at 2-8°C. Working diluent and wash solutions are stable for 30 days at 18-25°C. Reagents should not be used if they appear cloudy or discolored.

SPECIMEN COLLECTION AND HANDLING

- Serum or heparinized plasma is required for the assay. EDTA and citrated plasma SHOULD NOT BE USED.
- If using serum, a venous blood sample is collected aseptically without additives. Store at 18-25°C until a clot has formed (usually 15-45 minutes), then centrifuge to obtain the serum specimen for assay.
- If using heparinized plasma, a venous blood sample is collected aseptically with designated additive. Centrifuge and separate plasma from the packed cells as soon as possible.
- Specimen types should not be used interchangeably during serial monitoring of an individual patient. Measured concentrations may vary slightly between sample types in certain patients.
- Samples may be stored at 2-8°C for up to 24 hours prior to analysis. If the analysis cannot be done within 24 hours, the sample should be stored frozen at –20°C or below for up to 60 days.
- Repeated freeze-thaw cycles should be avoided. Turbid serum samples or samples containing particulate matter should be centrifuged prior to testing. Prior to assay, bring frozen samples to 18-25°C slowly and mix gently.
- Diluted serum or heparinized plasma should be used within 24 hours and should not be stored.
- The sample required for analysis is 40 µL.

PROCEDURE

For the AIA Nex-IA / AIA-21, AIA-600 II, AIA-1800, AIA-2000 and AIA-360, please refer to their Operator's Manual for detailed instructions.

I. Reagent Preparation

A) Substrate Solution

Bring all reagents to 18-25°C before preparing the working reagent. Add the entire contents of the AIA-PACK SUBSTRATE RECONSTITUENT II (100 mL) to the lyophilized AIA-PACK SUBSTRATE REAGENT II, mix thoroughly to dissolve the solid material.

B) Wash Solution

Add the entire contents of the AIA-PACK WASH CONCENTRATE (100 mL) to approximately 2.0 L of CAP Class I or NCCLS Type I Reagent Grade water, mix well, and adjust the final volume to 2.5 L.

C) Diluent

Add the entire contents of the AIA-PACK DILUENT CONCENTRATE (100 mL) to approximately 4.0 L of CAP Class I or NCCLS Type I Reagent Grade water, mix well, and adjust the final volume to 5.0 L.

II. Calibration Procedure

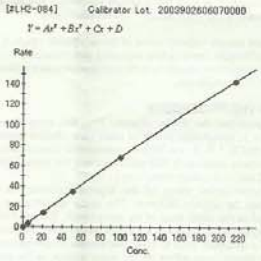
A) Calibration Curve

The calibrators for use with the ST AIA-PACK LH II have been standardized on WHO 2nd IS 80/552.

The calibration curve for ST AIA-PACK LH II is stable for up to 90 days. Calibration stability is monitored by quality control performance and is dependent on proper reagent handling and TOSOH AIA System maintenance according to the manufacturer's instructions.

Recalibration may be necessary more frequently if controls are out of the established range for this assay or when certain service procedures are performed (e.g. temperature adjustment, sampling mechanism changes, maintenance of the wash probe or detector lamp adjustment or change). For further information regarding instrument operation, consult the TOSOH AIA System Operator's Manual.

A sample calibration curve from AIA-1800 follows and shows the algorithm used for calculating results.



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Appendix - 3

ST AIA-PACK LH II

For Quantitative Measurement of luteinizing hormone (LH) in Serum or Heparinized Plasma

NAME AND INTENDED USE

ST AIA-PACK LH II is designed for IN VITRO DIAGNOSTIC USE ONLY for the quantitative measurement of luteinizing hormone (LH) in human serum or heparinized plasma on TOSOH AIA System Analyzers.

SUMMARY AND EXPLANATION OF TEST

Human luteinizing hormone (LH) is a glycoprotein which, like FSH, HCG and TSH, consists of alpha and beta chains. The alpha chain is virtually identical in all four hormones, whereas the beta chains are different and determine both the specific biological activity and immunological characteristics of each hormone *(1,2)*.

The LH molecule contains 15-18% carbohydrate and has a molecular weight of approximately 29,000 daltons *(3,4)*. LH induces ovulation and thereafter maintains the corpus luteum and progesterone production in women *(5-8)*. In men, LH stimulates testosterone production by the Leydig cells *(5,6,9)*.

Hypothalamic control of both LH and FSH secretion by the anterior pituitary appears to be by a common releasing hormone, gonadotropin-releasing hormone (GnRH) or luteinizing hormone-releasing hormone (LHRH), with negative feedback at the hypothalamic level by estrogen in female and testosterone in male.

Through the use of monoclonal antibody technology which provides the necessary specificity and sensitivity, determination of LH concentrations is critically important in assessment and monitoring of patients with suspected infertility *(10,11)*. The raised levels of LH and LH: FSH ratio is frequently found in polycystic ovary syndrome (PCOS), a common cause of infertility in women *(12,13)* and other physiological disorders associated with abnormal LH secretion *(12-15)*.

PRINCIPLE OF THE ASSAY

The ST AIA-PACK LH II is a two-site immunoenzymometric assay which is performed entirely in the AIA-PACK test cups. LH present in the test sample is bound with monoclonal antibody immobilized on a magnetic solid phase and enzyme-labeled monoclonal antibody in the AIA-PACK test cups. The magnetic beads are washed to remove unbound enzyme-labeled monoclonal antibody and are then incubated with a fluorogenic substrate 4-methylumbelliferyl phosphate (4MUP). The amount of enzyme-labeled monoclonal antibody that binds to the beads is directly proportional to the LH concentration in the test sample. A standard curve is constructed, and unknown sample concentrations are calculated using this curve.

MATERIAL PROVIDED (ST AIA-PACK LH II, Cat. No. 0025296)

Plastic test cups containing lyophilized twelve magnetic beads coated with mouse anti-LH monoclonal antibody and 100 µL of mouse anti-LH monoclonal antibody (to human LH) conjugated to bovine alkaline phosphatase with sodium azide as a preservative.

MATERIALS REQUIRED BUT NOT PROVIDED

The following materials are not provided but are required to perform luteinizing hormone analysis using the ST AIA-PACK LH II (Cat. No. 0025296) on the TOSOH AIA System Analyzers. They are available separately from TOSOH.

Materials			Cat. No.
AIA Nex·IA or AIA-21			0018539
AIA Nex·IA or AIA-21 LA			0018540
AIA-1800 ST			0019836
AIA-1800 LA			0019837
AIA-2000 ST			0022100
AIA-2000 LA			0022101
AIA-600 II			0019014
AIA-600 II BCR			0019328
AIA-360			0019945
AIA-PACK SUBSTRATE SET II			0020968
AIA-PACK SUBSTRATE REAGENT II			
AIA-PACK SUBSTRATE RECONSTITUENT II			
ST AIA-PACK LH II CALIBRATOR SET			0025396
CALIBRATOR (1)	0	mIU/mL	
CALIBRATOR (2)	5	mIU/mL (approx.)	
CALIBRATOR (3)	20	mIU/mL (approx.)	
CALIBRATOR (4)	50	mIU/mL (approx.)	
CALIBRATOR (5)	100	mIU/mL (approx.)	
CALIBRATOR (6)	220	mIU/mL (approx.)	
AIA-PACK LH II SAMPLE DILUTING SOLUTION			0020596
AIA-PACK WASH CONCENTRATE			0020955
AIA-PACK DILUENT CONCENTRATE			0020956
SAMPLE CUPS			0018581
AIA-PACK DETECTOR STANDARDIZATION TEST CUP			0020970
AIA-PACK SAMPLE TREATMENT CUP			0020971
Additional Requirements for AIA Nex·IA / AIA-21 only:			
PIPETTE TIPS			0018552
PRELOADED PIPETTE TIPS			0018583
Additional Requirements for AIA-600 II, AIA-1800 and AIA-2000:			
PIPETTE TIPS			0019215
TIP RACK			0019216
PRELOADED PIPETTE TIPS			0022103

Only materials obtained from TOSOH should be used. Materials obtained elsewhere should not be substituted since assay performance is characterized based strictly on TOSOH materials.

DO NOT USE IN COMBINATION WITH AIA-PACK LH II CALIBRATOR SET (CAT. NO. 0020396)

ASSAY PREPARATION

1. Set up the TOSOH AIA System Analyzers for luteinizing hormone analysis. REFER TO THE APPROPRIATE TOSOH AIA SYSTEM ANALYZERS OPERATOR'S MANUAL.
2. Bring all test samples and controls to 18-25°C, and mix well.

WARNINGS AND PRECAUTIONS

1. The ST AIA-PACK LH II is intended for in vitro diagnostic use only.
2. Test cups from different lots or different assays should not be mixed within a tray.
3. The ST AIA-PACK LH II contains sodium azide, which may react with lead or copper plumbing to form potentially explosive metal azides. When disposing of such reagents, always flush with large volumes of water to prevent azide build-up.
4. Human serum is not used in the preparation of this product; however, since human specimens will be used for samples and other quality control products in the lab may be derived from human serum, please use standard laboratory safety procedures in handling all specimens and controls.
5. Do not use beyond the expiration date.
6. The ST AIA-PACK LH II has been designed so that the high dose "hook effect" is not a problem for the vast majority of samples. Samples with LH concentrations between 200 and 10,000 mIU/mL will read > 200 mIU/mL. The "hook effect" phenomenon may occur at LH concentrations > 10,000 mIU/mL.
7. TOSOH AIA-1200 series or AIA-600 Immunoassay Analyzers can NOT be used to perform the ST AIA-PACK LH II assay.

STORAGE AND STABILITY

All unopened materials are stable until the expiration date on the label when stored at the specified temperature.

Materials	Cat. No.
2-8°C:	
ST AIA-PACK LH II	0025296
ST AIA-PACK LH II CALIBRATOR SET	0025396
AIA-PACK LH II SAMPLE DILUTING SOLUTION	0020596
AIA-PACK SUBSTRATE SET II	0020968
AIA-PACK WASH CONCENTRATE	0020955
AIA-PACK DILUENT CONCENTRATE	0020956
1-30°C:	
AIA-PACK DETECTOR STANDARDIZATION TEST CUP	0020970
AIA-PACK SAMPLE TREATMENT CUP	0020971

ST AIA-PACK LH II test cups may be stored at 18-25°C for up to 1 day. Calibrators and Sample Diluting Solution should be used within 1 day and 7 days of opening or reconstituting, respectively, provided the vials are kept tightly sealed and refrigerated at 2-8°C. Reconstituted substrate solution is stable for 3 days at 18-25°C or 7 days at 2-8°C. Working diluent and wash solutions are stable for 30 days at 18-25°C. Reagents should not be used if they appear cloudy or discolored.

SPECIMEN COLLECTION AND HANDLING

- Serum or heparinized plasma is required for the assay. EDTA and citrated plasma SHOULD NOT BE USED.
- If using serum, a venous blood sample is collected aseptically without additives. Store at 18-25°C until a clot has formed (usually 15-45 minutes), then centrifuge to obtain the serum specimen for assay.
- If using heparinized plasma, a venous blood sample is collected aseptically with designated additive. Centrifuge and separate plasma from the packed cells as soon as possible.
- Specimen types should not be used interchangeably during serial monitoring of an individual patient. Measured concentrations may vary slightly between sample types in certain patients.
- Samples may be stored at 2-8°C for up to 24 hours prior to analysis. If the analysis cannot be done within 24 hours, the sample should be stored frozen at –20°C or below for up to 60 days.
- Repeated freeze-thaw cycles should be avoided. Turbid serum samples or samples containing particulate matter should be centrifuged prior to testing. Prior to assay, bring frozen samples to 18-25°C slowly and mix gently.
- Diluted serum or heparinized plasma should be used within 24 hours and should not be stored.
- The sample required for analysis is 40 µL.

PROCEDURE

For the AIA Nex·IA / AIA-21, AIA-600 II, AIA-1800, AIA-2000 and AIA-360, please refer to their Operator's Manual for detailed instructions.

I. Reagent Preparation

A) Substrate Solution

Bring all reagents to 18-25°C before preparing the working reagent. Add the entire contents of the AIA-PACK SUBSTRATE RECONSTITUENT II (100 mL) to the lyophilized AIA-PACK SUBSTRATE REAGENT II, mix thoroughly to dissolve the solid material.

B) Wash Solution

Add the entire contents of the AIA-PACK WASH CONCENTRATE (100 mL) to approximately 2.0 L of CAP Class I or NCCLS Type I Reagent Grade water, mix well, and adjust the final volume to 2.5 L.

C) Diluent

Add the entire contents of the AIA-PACK DILUENT CONCENTRATE (100 mL) to approximately 4.0 L of CAP Class I or NCCLS Type I Reagent Grade water, mix well, and adjust the final volume to 5.0 L.

II. Calibration Procedure

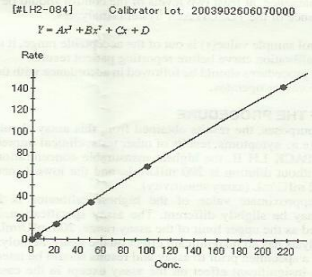
A) Calibration Curve

The calibrators for use with the ST AIA-PACK LH II have been standardized on WHO 2nd IS 80/552.

The calibration curve for ST AIA-PACK LH II is stable for up to 90 days. Calibration stability is monitored by quality control performance and is dependent on proper reagent handling and TOSOH AIA System maintenance according to the manufacturer's instructions.

Recalibration may be necessary more frequently if controls are out of the established range for this assay or when certain service procedures are performed (e.g. temperature adjustment, sampling mechanism changes, maintenance of the wash probe or detector lamp adjustment or change). For further information regarding instrument operation, consult the TOSOH AIA System Operator's Manual.

A sample calibration curve from AIA-1800 follows and shows the algorithm used for calculating results.



INTERFERENCE

Interference is defined, for the purposes of this study, as recovery outside of 10% of the known specimen mean concentration after the following substances are added to human specimens.

- Hemoglobin (up to 410 mg/dL), free bilirubin (up to 17 mg/dL) and conjugated bilirubin (up to 18 mg/dL) do not interfere with the assay.
- Lipemia, as indicated by triglyceride concentration (up to 1,600 mg/dL), does not interfere with the assay.
- Ascorbic acid (up to 20 mg/dL) does not interfere with the assay.
- Protein, as indicated by human albumin concentration (up to 5.0 g/dL), does not interfere with the assay.
- Heparin (up to 100 U/mL) does not interfere with the assay.

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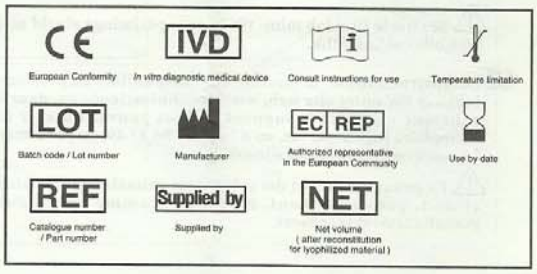
TOSOH CORPORATION
Shiba-koen First Bldg.
3-8-2 Shiba, Minato-ku, Tokyo 105-8623
Japan
Phone :+81-3-5427-5181
Fax :+81-3-5427-5220

EC REP **TOSOH EUROPE N.V.**
Transportstraat 4
B-3980 Tessenderlo, Belgium
Phone :+32-13-66 88 30
Fax :+32-13-66 47 49

Supplied by **TOSOH BIOSCIENCE, INC.**
6000 Shoreline Ct., Suite 101
South San Francisco, CA94080, USA
Phone :+1-650-615-4970
Fax :+1-650-615-0415
Phone :(800)248-6764
Fax :(800)685-7595



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Appendix - 4

ST AIA-PACK PRL

For Quantitative Measurement of prolactin (PRL) in Serum or Heparinized Plasma

NAME AND INTENDED USE

ST AIA-PACK PRL is designed for IN VITRO DIAGNOSTIC USE ONLY for the quantitative measurement of prolactin (PRL) in human serum or heparinized plasma on TOSOH AIA System Analyzers.

SUMMARY AND EXPLANATION OF TEST

Prolactin (PRL) is a protein of 198 amino acids with a molecular weight of approximately 21,000. Prolactin is secreted from the anterior pituitary gland and acts directly on the mammary gland to stimulate milk production *(1,2)*. When secreted in excess, it increases adrenal androgens and blunts the response of gonadotropes and gonadal cells to tropic factors by decreasing gonadoliberin. Thus, chronically elevated prolactin induces amenorrhea and infertility in women *(3-5)* and impotence in men. Prolactin synthesis and secretion is under inhibitory control by the hypothalamus. The hypothalamic prolactin inhibiting factor (PIF) is now established as dopamine *(1,2,6)*.

Measurement of prolactin concentrations in blood is essential in evaluating patients with infertility or suspected hypothalamic-pituitary dysfunction. Increased serum prolactin concentrations are observed in pregnancy and during lactation. Other conditions associated with abnormal prolactin concentrations have been reported *(3,6,7-11)*.

PRINCIPLE OF THE ASSAY

The ST AIA-PACK PRL is a two-site immunoenzymometric assay which is performed entirely in the AIA-PACK test cups. Prolactin present in the test sample is bound with monoclonal antibody immobilized on a magnetic solid phase and enzyme-labeled monoclonal antibody in the AIA-PACK test cups. The magnetic beads are washed to remove unbound enzyme-labeled monoclonal antibody and are then incubated with a fluorogenic substrate, 4-methylumbelliferyl phosphate (4MUP). The amount of enzyme-labeled monoclonal antibody that binds to the beads is directly proportional to the prolactin concentration in the test sample. A standard curve is constructed, and unknown sample concentrations are calculated using this curve.

MATERIAL PROVIDED (ST AIA-PACK PRL, Cat. No. 0025255)

Plastic test cups containing lyophilized twelve magnetic beads coated with anti-prolactin mouse monoclonal antibody and 100 µL of anti-prolactin mouse monoclonal antibody (to human prolactin) conjugated to bovine alkaline phosphatase with sodium azide as a preservative.

MATERIALS REQUIRED BUT NOT PROVIDED

The following materials are not provided but are required to perform prolactin analysis using the ST AIA-PACK PRL (Cat. No. 0025255) on the TOSOH AIA Systems Analyzers. They are available separately from TOSOH.

Materials			Cat. No.
AIA Nex·IA or AIA-21			0018539
AIA Nex·IA or AIA-21 LA			0018540
AIA-1800 ST			0019836
AIA-1800 LA			0019837
AIA-2000 ST			0022100
AIA-2000 LA			0022101
AIA-600 II			0019014
AIA-600 II BCR			0019328
AIA-360			0019945
AIA-PACK SUBSTRATE SET II			0020968
AIA-PACK SUBSTRATE REAGENT II			
AIA-PACK SUBSTRATE RECONSTITUENT II			
AIA-PACK PRL CALIBRATOR SET			0020355
ZERO CALIBRATOR	0	ng/mL	
POSITIVE CALIBRATOR	100	ng/mL (approx.)	
AIA-PACK PRL SAMPLE DILUTING SOLUTION			0020555
AIA-PACK WASH CONCENTRATE			0020955
AIA-PACK DILUENT CONCENTRATE			0020956
SAMPLE CUPS			0018581
AIA-PACK DETECTOR STANDARDIZATION TEST CUP			0020970
AIA-PACK SAMPLE TREATMENT CUP			0020971
Additional Requirements for AIA Nex·IA / AIA-21 only:			
PIPETTE TIPS			0018552
PRELOADED PIPETTE TIPS			0018583
Additional Requirements for AIA-600 II, AIA-1800 and AIA-2000:			
PIPETTE TIPS			0019215
TIP RACK			0019216
PRELOADED PIPETTE TIPS			0022103

Only materials obtained from TOSOH should be used. Materials obtained elsewhere should not be substituted since assay performance is characterized based strictly on TOSOH materials.

WARNINGS AND PRECAUTIONS

1. The ST AIA-PACK PRL is intended for in vitro diagnostic use only.
2. Test cups from different lots or different assays should not be mixed within a tray.
3. The ST AIA-PACK PRL contains sodium azide, which may react with lead or copper plumbing to form potentially explosive metal azides. When disposing of such reagents, always flush with large volumes of water to prevent azide build-up.
4. Human serum is not used in the preparation of this product; however, since human specimens will be used for samples and other quality control products in the lab may be derived from human serum, please use standard laboratory safety procedures in handling all specimens and controls.
5. Do not use beyond the expiration date.
6. The ST AIA-PACK PRL has been designed so that the high dose "hook effect" is not a problem for the vast majority of samples. Samples with prolactin concentrations between 200 and 20,000 ng/mL will read > 200 ng/mL. The "hook effect" phenomenon may occur at prolactin concentrations > 20,000 ng/mL.
7. TOSOH AIA-1200 series or AIA-600 Immunoassay Analyzers can NOT be used to perform the ST AIA-PACK PRL assay.

STORAGE AND STABILITY

All unopened materials are stable until the expiration date on the label when stored at the specified temperature.

Materials	Cat. No.
2-8°C:	
ST AIA-PACK PRL	0025255
AIA-PACK PRL CALIBRATOR SET	0020355
AIA-PACK PRL SAMPLE DILUTING SOLUTION	0020555
AIA-PACK SUBSTRATE SET II	0020968
AIA-PACK WASH CONCENTRATE	0020955
AIA-PACK DILUENT CONCENTRATE	0020956
1-30°C:	
AIA-PACK DETECTOR STANDARDIZATION TEST CUP	0020970
AIA-PACK SAMPLE TREATMENT CUP	0020971

ST AIA-PACK PRL test cups may be stored at 18-25°C for up to 1 day. Calibrators and Sample Diluting Solution should be used within 1 day and 7 days of opening or reconstituting, respectively, provided the vials are kept tightly sealed and refrigerated at 2-8°C. Reconstituted substrate solution is stable for 3 days at 18-25°C or 7 days at 2-8°C. Working diluent and wash solutions are stable for 30 days at 18-25°C. Reagents should not be used if they appear cloudy or discolored.

SPECIMEN COLLECTION AND HANDLING

- Serum or heparinized plasma is required for the assay. EDTA and citrated plasma SHOULD NOT BE USED.
- Since physical or emotional stress may elevate blood levels of prolactin, patients should be under resting basal conditions prior to sampling. Also, prolactin levels rise rapidly with sleep, so samples ideally should not be taken until 1-2 hours after awakening. Prolactin, like other pituitary hormones, is secreted episodically; it may be advantageous to pool equal volumes of two or more sera from samples drawn at 6-18 minute intervals *(12)*.
- If using serum, a venous blood sample is collected aseptically without additives. Store at 18-25°C until a clot has formed (usually 15-45 minutes), then centrifuge to obtain the serum specimen for assay.
- If using heparinized plasma, a venous blood sample is collected aseptically with designated additive. Centrifuge and separate plasma from the packed cells as soon as possible.
- Specimen types should not be used interchangeably during serial monitoring of an individual patient. Measured concentrations may vary slightly between sample types in certain patients.
- Samples may be stored at 2-8°C for up to 24 hours prior to analysis. If the analysis cannot be done within 24 hours, the sample should be stored frozen at –20°C or below for up to 60 days.
- Repeated freeze-thaw cycles should be avoided. Turbid serum samples or samples containing particulate matter should be centrifuged prior to testing. Prior to assay, bring frozen samples to 18-25°C slowly and mix gently.
- The sample required for analysis is 30 µL.

PROCEDURE

For the AIA Nex·IA / AIA-21, AIA-600 II, AIA-1800, AIA-2000 and AIA-360, please refer to their Operator's Manual for detailed instructions.

I. Reagent Preparation

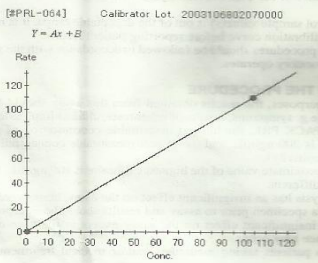
A) Substrate Solution
Bring all reagents to 18-25°C before preparing the working reagent. Add the entire contents of the AIA-PACK SUBSTRATE RECONSTITUENT II (100 mL) to the lyophilized AIA-PACK SUBSTRATE REAGENT II, mix thoroughly to dissolve the solid material.

B) Wash Solution
Add the entire contents of the AIA-PACK WASH CONCENTRATE (100 mL) to approximately 2.0 L of CAP Class I or NCCLS Type I Reagent Grade water, mix well, and adjust the final volume to 2.5 L.

C) Diluent
Add the entire contents of the AIA-PACK DILUENT CONCENTRATE (100 mL) to approximately 4.0 L of CAP Class I or NCCLS Type I Reagent Grade water, mix well, and adjust the final volume to 5.0 L.

II. Calibration Procedure

A) Calibration Curve
The calibrators for use with the ST AIA-PACK PRL have been standardized on WHO 2nd IS 83/562 (1986).
The calibration curve for ST AIA-PACK PRL is stable for up to 90 days. Calibration stability is monitored by quality control performance and is dependent on proper reagent handling and TOSOH AIA System maintenance according to the manufacturer's instructions.
Recalibration may be necessary more frequently if controls are out of the established range for this assay or when certain service procedures are performed (e.g. temperature adjustment, sampling mechanism changes, maintenance of the wash probe or detector lamp adjustment or change). For further information regarding instrument operation, consult the TOSOH AIA System Operator's Manual.
A sample calibration curve from AIA-1800 follows and shows the algorithm used for calculating results.



B) Calibration Procedure
1. Refer to the appropriate TOSOH AIA System Operator's Manual for the procedural instructions.
2. Verify that both the calibrator lot and concentration numbers have been correctly entered into the software.
3. ZERO CALIBRATOR for ST AIA-PACK PRL is provided ready for use. TOSOH recommends that all calibrators be run in triplicate.
4. POSITIVE CALIBRATOR for ST AIA-PACK PRL is lyophilized. It should be reconstituted with 1.0 mL of CAP Class I or NCCLS Type I Reagent Grade water. TOSOH recommends that all calibrators be run in triplicate.

C) Calibration Acceptability Criteria
1. The mean rate for the ZERO CALIBRATOR should be < 3.0 nmol/(L·s).
2. Since there is a direct relationship between concentration and rate, the rate should increase as the concentration increases.
3. The replicate values should be within a 10% range.

D) Calibration Review and Acceptance
1. Review the calibration curve carefully, using the criteria listed above.
2. Edit the calibration if necessary, then accept the calibration.

For further information regarding calibration, consult the TOSOH AIA System Operator's Manual.

III. Quality Control Procedure

A) Commercially Available Controls

Commercially available controls should be run at least once per day. It is recommended that at least two levels of controls, normal and abnormal, be used. Laboratory policy for this particular assay designates the following:

Control Material: _____
Frequency: _____

Lot number of control material, acceptable limits, and corrective action to be taken if controls do not meet laboratory criteria will be found in a separate quality control document maintained by the laboratory.

B) Quality Control Procedure

1. Assay quality control specimens as instructed in the specific Operator's Manual for your analyzer. In addition, refer to the TOSOH AIA System Operator's Manual for detailed instructions on defining and editing the files.
2. Quality control material to be run with this assay is defined by individual laboratory policy.

IV. Specimen Processing

A) Preparation

Following specific instructions in the Operator's Manual for the analyzer, place samples on the instrument appropriately. Barcoded primary tubes as well as sample cups can be run on the AIA Nex·IA / AIA-21, AIA-600 II, AIA-1800, AIA-2000 and AIA-360.

B) Assay Procedure

1. Ensure a sufficient quantity of ST AIA-PACK PRL test cups for the number of samples to be run.
2. Load patient samples as instructed in the Operator's Manual and proceed with analysis. Note: The AIA Nex·IA / AIA-21, AIA-600 II, AIA-1800 and AIA-2000 will require AIA-PACK SAMPLE TREATMENT CUPs if onboard dilutions are utilized.

PROCEDURAL NOTES

1. Lyophilized substrate must be completely dissolved.
2. Ligand assays performed by the TOSOH AIA System Analyzers require that the laboratory use water designated by the College of American Pathologists as Class I or by NCCLS as Type I. Water should be tested at least once per month and should be free of particulate matter including bacteria. The pH of the water should also be routinely tested. For further information, consult the NCCLS document "Preparation and Testing of Reagent Water in the Clinical Laboratory," NCCLS Document C3-A3, Volume 11 No. 13, originally approved as a guideline by NCCLS in October 1997.
3. If a specimen prolactin concentration is found to be greater than the upper limit of the assay range, 200 ng/mL, the specimen should be diluted with the AIA-PACK PRL SAMPLE DILUTING SOLUTION and reassayed according to the Assay Procedure. The recommended dilution for specimens containing greater than 200 ng/mL is 1:10 or 1:100. It is desirable to dilute the specimen so that the diluted specimen reads between 5 and 200 ng/mL. The dilution factor should be entered into the software. For further information on the dilution of specimens, refer to the TOSOH AIA System Operator's Manual.
4. The TOSOH AIA System Analyzers can store two different calibration curves for each analyte at one time. Therefore, up to two different lots of ST AIA-PACK PRL test cups can be used during the same run.
5. If the assay specifications for this test are not ready in the system software, the specifications must be entered under test code **041**.

CALCULATION OF RESULTS

The TOSOH AIA System Analyzers perform all sample and reagent handling operations automatically. The TOSOH AIA System Analyzers read the rate of fluorescence produced by the reaction and automatically convert the rate to prolactin concentration in ng/mL.

For samples requiring dilution, the AIA Nex·IA / AIA-21, AIA-600 II, AIA-1800 and AIA-2000 will automatically perform dilutions and calculate results if the dilution factors are entered into the software. Dilution factors may be entered into the Test File, or pre-defined dilution factors may be selected in Specimen Processing.

EVALUATION OF RESULTS

Quality Control

In order to monitor and evaluate the precision of the analytical performance, it is recommended that commercially available control samples should be assayed according to the local regulations.

The minimum recommendations for the frequency of running internal control material are:

After calibration, three levels of the internal control are run in order to accept the calibration curve.

The three levels of controls are repeated when certain service procedures are performed (e.g. temperature adjustment, sampling mechanism changes, maintenance of the wash probe or detector lamp adjustment or change).

After daily maintenance, at least two levels of the control should be run in order to verify the overall performance of the TOSOH AIA System Analyzers.

If one or more control sample value(s) is out of the acceptable range, it is necessary to investigate the validity of the calibration curve before reporting patient results.

Standard laboratory procedures should be followed in accordance with the strict regulatory agency under which the laboratory operates.

LIMITATIONS OF THE PROCEDURE

- For diagnostic purposes, the results obtained from this assay should be used in conjunction with other data (e.g. symptoms, results of other tests, clinical impressions, therapy, etc.).
- Using ST AIA-PACK PRL, the highest measurable concentration of prolactin in specimens without dilution is 200 ng/mL, and the lowest measurable concentration in specimens is 1.0 ng/mL (assay sensitivity).
- Although the approximate value of the highest calibrator is 100 ng/mL, the exact concentration may be slightly different.
- Although hemolysis has an insignificant effect on the assay, hemolyzed samples may indicate mistreatment of a specimen prior to assay and results should be interpreted with caution.
- Lipemia has an insignificant effect on the assay except in the case of gross lipemia where spatial interference may occur.
- Specimens from patients taking medicines and/or medical treatment may show erroneous results.
- Specimens from patients who have received preparations of mouse monoclonal antibodies for diagnosis or therapy may contain human anti-mouse antibodies (HAMA). Such specimens may show falsely elevated values when tested for prolactin.
- Samples containing "macroprolactin" may cause falsely elevated results *(13,16)*. "Macroprolactin" is thought to be a complex of prolactin with human immunoglobulin G (hIgG) along with possible aggregates of prolactin *(14)*.
- For a more complete understanding of the limitations of this procedure, please refer to the SPECIMEN COLLECTION AND HANDLING, WARNINGS AND PRECAUTIONS, STORAGE AND STABILITY, and PROCEDURAL NOTES sections in this insert sheet.

EXPECTED VALUES

Each laboratory should determine a reference interval which corresponds to the characteristics of the population being tested. As with all diagnostic procedures, clinical results must be interpreted with regard to concomitant medications administered to the patient *(15)*.

Reference Ranges

The interval given here was determined in 158 serum samples from apparently healthy Asian individuals.

Male (n = 134)	3.6 - 16.3 ng/mL
Female (n = 24)	4.1 - 28.9 ng/mL

Conversion Factors

Prolactin concentrations in this application are in units of ng/mL. Conversion to SI units of μg/L and mIU/L may be made using the following equations:

μg prolactin /L = ng/mL x 1.0
mIU prolactin /L = ng/mL x 27.0

PERFORMANCE CHARACTERISTICS

ACCURACY

a. Recovery: Three serum pools were spiked with three different levels of prolactin and assayed before and after spiking.

Sample	Initial Value (ng/mL)	Prolactin Added (ng/mL)	Expected Value (ng/mL)	Measured Value (ng/mL)	Percent Recovery (%)
Serum A1	6.11	40.9	47.0	43.5	92.5
	6.11	81.8	87.9	84.4	96.0
	6.11	163.6	169.8	169.1	99.6
Serum B1	7.46	40.9	48.4	43.7	90.4
	7.46	81.8	89.3	83.7	93.8
	7.46	163.6	171.1	170.8	99.8
Serum C1	3.01	40.9	43.9	40.4	91.9
	3.01	81.8	84.8	80.2	94.5
	3.01	163.6	166.7	167.0	100.2

b. Dilution: Three serum samples containing high concentrations of prolactin were serially diluted with AIA-PACK PRL SAMPLE DILUTING SOLUTION and assayed.

Sample	Dilution Factor	Expected Value (ng/mL)	Measured Value (ng/mL)	Percent Recovery (%)
Serum A2	none		186.2	
	7.5/10	139.7	131.7	94.3
	5.0/10	93.1	83.8	90.0
	2.5/10	46.6	40.4	86.8
	1.0/10	18.6	16.1	86.6
Serum B2	none		181.5	
	7.5/10	136.1	126.0	92.6
	5.0/10	90.7	82.5	90.9
	2.5/10	45.4	40.0	88.1
	1.0/10	18.1	15.4	84.6
Serum C2	none		156.4	
	7.5/10	117.3	111.6	95.1
	5.0/10	78.2	72.0	92.0
	2.5/10	39.1	34.0	87.0
	1.0/10	15.6	13.3	85.3

PRECISION

a. Within run precision was determined using three controls in a total of 20 runs. Within each run, one set of duplicates per control was assayed. The mean of each duplicate was used to obtain the pooled standard deviation (SD), which was then used to calculate the coefficient of variation (CV).

Sample	Mean (ng/mL)	Pooled SD (ng/mL)	CV (%)
Serum A3	4.32	0.0898	2.1
Serum B3	20.6	0.299	1.5
Serum C3	100.7	1.77	1.8

b. Total precision was determined by the duplicate assay of three controls in 20 separate runs. The means of each run were used to calculate the pooled standard deviation (SD) and coefficient of variation (CV).

Sample	Mean (ng/mL)	Pooled SD (ng/mL)	CV (%)
Serum A3	4.32	0.126	2.9
Serum B3	20.6	0.580	2.8
Serum C3	100.7	2.72	2.7

CORRELATION

The correlation between serum (x) and heparinized plasma (y) on ST AIA-PACK PRL was carried out using 197 patient specimens.

Slope	0.997
y-Intercept	0.048
Correlation Coefficient	0.999
Range of Samples	2.2-192.5
Number of Samples	197

SPECIFICITY

The following substances were tested for cross-reactivity. The cross-reactivity (%) is the percentage of the compound which will be identified as prolactin. If these compounds are present in the specimen at the same concentration as prolactin, the final result will be increased by these percentages.

Compound	Cross-reactivity (%)
PRL	100
LH	0.03
FSH	< 0.01
TSH	< 0.01
hPL	< 0.01
hGH	0.04

SENSITIVITY

The minimal detectable concentration (MDC) of prolactin is estimated to be 1.0 ng/mL. The MDC is defined as the concentration of prolactin which corresponds to the rate of fluorescence that is two standard deviations from the mean rate of fluorescence of 20 replicate determinations of a ZERO CALIBRATOR.

INTERFERENCE

Interference is defined, for the purposes of this study, as recovery outside of 10% of the known mean concentration of the specimen after the following substances are added to human specimens.

- Hemoglobin (up to 390 mg/dL), free bilirubin (up to 17 mg/dL) and conjugated bilirubin (up to 18 mg/dL) do not interfere with the assay.
- Lipemia, as indicated by triglyceride concentration (up to 1,600 mg/dL), does not interfere with the assay.
- Ascorbic acid (up to 20 mg/dL) does not interfere with the assay.
- Protein, as indicated by human albumin concentration (up to 5.0 g/dL), does not interfere with the assay.
- Heparin (up to 100 U/mL) does not interfere with the assay.

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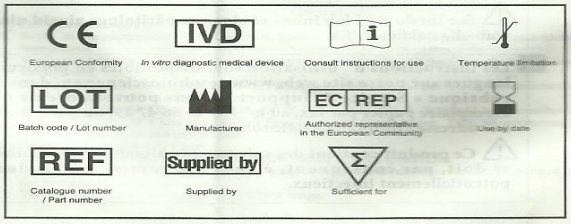
TOSOH CORPORATION
Shiba-koen First Bldg.
3-8-2 Shiba, Minato-ku, Tokyo 105-8623
Japan
Phone :+81-3-5427-5181
Fax :+81-3-5427-5220

EC REP

TOSOH EUROPE N.V.
Transportstraat 4
B-3980 Tessenderlo, Belgium
Phone :+32-13-66 88 30
Fax :+32-13-66 47 49

Supplied by

TOSOH BIOSCIENCE, INC.
6000 Shoreline Ct., Suite 101
South San Francisco, CA94080, USA
Phone :+1-650-615-4970
Fax :+1-650-615-0415
Phone :(800)248-6764
Fax :(800)685-7595



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Rev. 03/10

Appendix - 5

ST AIA-PACK Testosterone

For Quantitative Measurement of testosterone in Serum or Heparinized Plasma

NAME AND INTENDED USE

ST AIA-PACK Testosterone is designed for IN VITRO DIAGNOSTIC USE ONLY for the quantitative measurement of testosterone in human serum or heparinized plasma on TOSOH AIA System Analyzers. Measurement of testosterone is used to aid in the diagnosis and management of conditions involving excess or deficiency of this androgen.

SUMMARY AND EXPLANATION OF TEST

Testosterone is one of the major male sex hormones produced by the interstitial cells of Leydig in the testes. The measurement of the total testosterone in serum can provide information to evaluate adrenal and testicular functions. It is useful for the diagnosis of the hypergonadism *(1)* and hyperpituitarism *(2)* in men, and hirsutism *(3)*, menstrual disorders *(4)*, and polycystic ovarian syndrome *(5)* in women. It is also useful for the characterization and follow-up of some cancers such as testicular *(6)*, breast, ovarian, and adrenal tumors *(7-9)*.

PRINCIPLE OF THE ASSAY

ST AIA-PACK Testosterone is a competitive enzyme immunoassay which is performed entirely in the ST AIA-PACK Testosterone test cups. Testosterone present in the test sample competes with enzyme-labeled testosterone for a limited number of binding sites on the testosterone specific monoclonal antibody immobilized on a magnetic solid phase. The magnetic beads are washed to remove unbound enzyme-labeled testosterone and are then incubated with a fluorogenic substrate, 4-methylumbelliferyl phosphate (4MUP). The amount of enzyme-labeled testosterone that binds to the beads is inversely proportional to the testosterone concentration in the test sample. A standard curve is constructed, and unknown sample concentrations are calculated using this curve.

MATERIAL PROVIDED (ST AIA-PACK Testosterone, Cat. No. 0025204)

Plastic test cups containing lyophilized twelve magnetic beads coated with mouse anti-testosterone monoclonal antibody and 45 µL of testosterone conjugated to bovine alkaline phosphatase with sodium azide as a preservative.

MATERIALS REQUIRED BUT NOT PROVIDED

The following materials are not provided but are required to perform testosterone analysis using the ST AIA-PACK Testosterone (Cat. No. 0025204) on the TOSOH AIA System Analyzers. They are available separately from TOSOH.

Materials			Cat. No.
AIA Nex-IA or AIA-21			0018539
AIA Nex-IA or AIA-21 LA			0018540
AIA-1800 ST			0019836
AIA-1800 LA			0019837
AIA-2000 ST			0022100
AIA-2000 LA			0022101
AIA-600 II			0019014
AIA-600 II BCR			0019328
AIA-360			0019945
AIA-PACK SUBSTRATE SET II			0020968
AIA-PACK SUBSTRATE REAGENT II			
AIA-PACK SUBSTRATE RECONSTITUENT II			
ST AIA-PACK Testosterone CALIBRATOR SET			0025304
ST AIA-PACK Testosterone CALIBRATOR (1)	0	ng/dL	
ST AIA-PACK Testosterone CALIBRATOR (2)	30	ng/dL (approx.)	
ST AIA-PACK Testosterone CALIBRATOR (3)	100	ng/dL (approx.)	
ST AIA-PACK Testosterone CALIBRATOR (4)	350	ng/dL (approx.)	
ST AIA-PACK Testosterone CALIBRATOR (5)	900	ng/dL (approx.)	
ST AIA-PACK Testosterone CALIBRATOR (6)	2,200	ng/dL (approx.)	
ST AIA-PACK Testosterone SAMPLE DILUTING SOLUTION			0025504
AIA-PACK WASH CONCENTRATE			0020955
AIA-PACK DILUENT CONCENTRATE			0020956
SAMPLE CUPS			0018581
AIA-PACK DETECTOR STANDARDIZATION TEST CUP			0020970
AIA-PACK SAMPLE TREATMENT CUP			0020971
Additional Requirements for AIA Nex-IA / AIA-21 only:			
PIPETTE TIPS			0018552
PRELOADED PIPETTE TIPS			0018583
Additional Requirements for AIA-600 II, AIA-1800 and AIA-2000:			
PIPETTE TIPS			0019215
TIP RACK			0019216
PRELOADED PIPETTE TIPS			0022103

Only materials obtained from TOSOH should be used. Materials obtained elsewhere should not be substituted since assay performance is characterized based strictly on TOSOH materials.

WARNINGS AND PRECAUTIONS

1. The ST AIA-PACK Testosterone is intended for in vitro diagnostic use only.
2. Test cups from different lots or different assays should not be mixed within a tray.
3. The ST AIA-PACK Testosterone contains sodium azide, which may react with lead or copper plumbing to form potentially explosive metal azides. When disposing of such reagents, always flush with large volumes of water to prevent azide build-up.
4. Human serum is not used in the preparation of this product; however, since human specimens will be used for samples and other quality control products in the lab may be derived from human serum, please use standard laboratory safety procedures in handling all specimens and controls.
5. Do not use beyond the expiration date.
6. TOSOH AIA-1200 series or AIA-600 Immunoassay Analyzers can NOT be used to perform the ST AIA-PACK Testosterone assay.

STORAGE AND STABILITY

All unopened materials are stable until the expiration date on the label when stored at the specified temperature.

Materials	Cat. No.
2-8°C:	
ST AIA-PACK Testosterone	0025204
ST AIA-PACK Testosterone CALIBRATOR SET	0025304
ST AIA-PACK Testosterone SAMPLE DILUTING SOLUTION	0025504
AIA-PACK SUBSTRATE SET II	0020968
AIA-PACK WASH CONCENTRATE	0020955
AIA-PACK DILUENT CONCENTRATE	0020956
1-30°C:	
AIA-PACK DETECTOR STANDARDIZATION TEST CUP	0020970
AIA-PACK SAMPLE TREATMENT CUP	0020971

ST AIA-PACK Testosterone test cups may be stored at 18-25°C for up to 1 day. ST AIA-PACK Testosterone CALIBRATOR SET and ST AIA-PACK Testosterone SAMPLE DILUTING SOLUTION should be used within 1 day and 30 days of opening, respectively, provided the vials are kept tightly sealed and refrigerated at 2-8°C. Reconstituted substrate solution is stable for 3 days at 18-25°C or 7 days at 2-8°C. Working diluent and wash solutions are stable for 30 days at 18-25°C. Reagents should not be used if they appear cloudy or discolored.

SPECIMEN COLLECTION AND HANDLING

1. Serum or heparinized plasma is required for the assay. EDTA and citrated plasma SHOULD NOT BE USED.
2. When using serum, a venous blood sample is collected aseptically without additives. Store at 18-25°C until a clot has formed (usually 15-45 minutes), then centrifuge to obtain the serum specimen for assay.
3. When using heparinized plasma, a venous blood sample is collected aseptically with designated additive. Centrifuge and separate plasma from the packed cells as soon as possible.
4. Specimen types should not be used interchangeably during serial monitoring of an individual patient. Measured concentrations may vary slightly between sample types in certain patients.
5. Samples may be stored at 2-8°C for up to 24 hours prior to analysis. If the analysis cannot be done within 24 hours, the sample should be stored frozen at –20°C or below for up to 60 days.
6. Repeated freeze-thaw cycles should be avoided. Turbid serum samples or samples containing particulate matter should be centrifuged prior to testing. Prior to assay, slowly bring frozen samples to 18-25°C and mix gently.
7. The sample required for analysis is 85 µL.

PROCEDURE

For the AIA Nex-IA / AIA-21, AIA-600 II, AIA-1800, AIA-2000 and AIA-360, please refer to their Operator's Manual for detailed instructions.

I. Reagent Preparation

A) Substrate Solution

Bring all reagents to 18-25°C before preparing the working reagent. Add the entire contents of the AIA-PACK SUBSTRATE RECONSTITUENT II (100 mL) to the lyophilized AIA-PACK SUBSTRATE REAGENT II and mix thoroughly to dissolve the solid material.

B) Wash Solution

Add the entire contents of the AIA-PACK WASH CONCENTRATE (100 mL) to approximately 2.0 L of CAP Class I or NCCLS Type I Reagent Grade water, mix well, and adjust the final volume to 2.5 L.

C) Diluent

Add the entire contents of the AIA-PACK DILUENT CONCENTRATE (100 mL) to approximately 4.0 L of CAP Class I or NCCLS Type I Reagent Grade water, mix well, and adjust the final volume to 5.0 L.

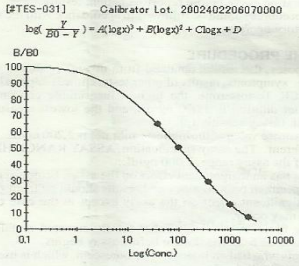
II. Calibration Procedure

A) Calibration Curve

The calibrators for use with the ST AIA-PACK Testosterone were compared to the USP reference material. The recovery of this reference material over the assay range is 64-133% depending on concentration.

The calibration curve for ST AIA-PACK Testosterone is stable for up to 90 days. Calibration stability is monitored by quality control performance and is dependent on proper reagent handling and TOSOH AIA System maintenance according to the manufacturer's instructions. Recalibration may be necessary more frequently if controls are out of the established range for this assay or when certain service procedures are performed (e.g. temperature adjustment, sampling mechanism changes, maintenance of the wash probe or detector lamp adjustment or change). For further information regarding instrument operation, consult the TOSOH AIA System Operator's Manual.

A sample calibration curve from AIA-1800 follows and shows the algorithm used for calculating results.



B) Calibration Procedure

1. Refer to the appropriate TOSOH AIA System Operator's Manual for the procedural instructions.
2. Verify that both the calibrator lot and concentration numbers have been correctly entered into the software.
3. The ST AIA-PACK Testosterone CALIBRATOR SET is provided ready for use. TOSOH recommends that all calibrators be run in triplicate.

C) Calibration Acceptability Criteria

1. Since there is an inverse relationship between concentration and rate, the rate should decrease as the concentration increases.
2. The replicate values should be within a 10% range.

D) Calibration Review and Acceptance

1. Review the calibration curve carefully, using the criteria listed above.
2. Edit the calibration if necessary, then accept the calibration.

For further information regarding calibration, consult the TOSOH AIA System Operator's Manual.

III. Quality Control Procedure

A) Commercially Available Controls

Commercially available controls should be run at least once per day. It is recommended that at least two levels of controls, normal and abnormal, be used. Laboratory policy for this particular assay designates the following:

Control Material: _____

Frequency: _____

Lot number of control material, acceptable limits, and corrective action to be taken if controls do not meet laboratory criteria will be found in a separate quality control document maintained by the laboratory.

B) Quality Control Procedure

1. Assay quality control specimens as instructed in the specific Operator's Manual for your analyzer. In addition, refer to the TOSOH AIA System Operator's Manual for detailed instructions on defining and editing the files.
2. Quality control material to be run with this assay is defined by individual laboratory policy.

IV. Specimen Processing

A) Preparation

Following the specific instructions in the Operator's Manual for the analyzer, place samples on the instrument appropriately. Barcoded primary tubes as well as sample cups can be run on the AIA Nex-IA / AIA-21, AIA-600 II, AIA-1800, AIA-2000 and AIA-360.

B) Assay Procedure

1. Ensure a sufficient quantity of ST AIA-PACK Testosterone test cups for the number of samples to be run.
2. Load patient samples as instructed in the Operator's Manual and proceed with analysis. Note: The AIA Nex-IA / AIA-21, AIA-600 II, AIA-1800 and AIA-2000 will require AIA-PACK SAMPLE TREATMENT CUPs if onboard dilutions are utilized.

PROCEDURAL NOTES

1. Lyophilized substrate must be completely dissolved.
2. Ligand assays performed by the TOSOH AIA System analyzers require that the laboratory use water designated by the College of American Pathologists as Class 1 or by NCCLS as Type I. Water should be tested at least once per month and should be free of particulate matter including bacteria. The pH of the water should also be routinely tested. For further information, consult the NCCLS document "Preparation and Testing of Reagent Water in the Clinical Laboratory," NCCLS Document C3-A3, Volume 11 No. 13, originally approved as a guideline by NCCLS in October 1997.
3. If a specimen testosterone concentration is found to be greater than the upper limit of the assay range, 2,000 ng/dL, the specimen should be diluted with the ST AIA-PACK Testosterone SAMPLE DILUTING SOLUTION and reassayed according to the Assay Procedure. The recommended dilution for specimens containing greater than 2,000 ng/dL is 1:3. It is desirable to dilute the specimen so that the diluted specimen reads between 200 and 2,000 ng/dL. The dilution factor should be entered into the software. For further information on the dilution of specimens, refer to the TOSOH AIA System Operator's Manual.
4. The TOSOH AIA System Analyzers can store two different calibration curves for each analyte at one time. Therefore, up to two different lots of ST AIA-PACK Testosterone test cups can be used during the same run.
5. If the assay specifications for this test are not ready in the system software, the specifications must be entered under test code **024**.

CALCULATION OF RESULTS

The TOSOH AIA System Analyzers perform all sample and reagent handling operations automatically. The TOSOH AIA System Analyzers read the rate of fluorescence produced by the reaction and automatically convert the rate to testosterone concentration in ng/dL.

For samples requiring dilution, the AIA Nex-IA / AIA-21, AIA-600 II, AIA-1800 and AIA-2000 will automatically perform dilutions and calculate results if the dilution factors are entered into the software. Dilution factors may be entered into the Test File, or pre-defined dilution factors may be selected in Specimen Processing.

EVALUATION OF RESULTS

Quality Control

In order to monitor and evaluate the precision of the analytical performance, it is recommended that commercially available control samples should be assayed according to the local regulations.

The minimum recommendations for the frequency of running internal control material are:

After calibration, three levels of the internal control are run in order to accept the calibration curve.

The three levels of controls are repeated when certain service procedures are performed (e.g. temperature adjustment, sampling mechanism changes, maintenance of the wash probe or detector lamp adjustment or change).

After daily maintenance, at least two levels of the control should be run in order to verify the overall performance of the TOSOH AIA System Analyzers.

If one or more control sample value(s) is out of the acceptable range, it is necessary to investigate the validity of the calibration curve before reporting patient results.

Standard laboratory procedures should be followed in accordance with the strict regulatory agency under which the laboratory operates.

LIMITATIONS OF THE PROCEDURE

1. For diagnostic purposes, the results obtained from this assay should be used in conjunction with other data (e.g. symptoms, results of other tests, clinical impressions, therapy, etc.).
2. Using ST AIA-PACK Testosterone, the highest measurable concentration of testosterone in specimens without dilution is 2,000 ng/dL, and the lowest measurable concentration in specimens is 7 ng/dL (assay sensitivity).
3. Although the approximate value of the highest calibrator is 2,200 ng/dL, the exact concentration may be slightly different. The assay specification, ASSAY RANGE HIGH, should be defined as the upper limit of the assay range, 2,000 ng/dL.
4. Although hemolysis has an insignificant effect on the assay, hemolyzed samples may indicate mistreatment of a specimen prior to assay and results should be interpreted with caution.
5. Lipemia has an insignificant effect on the assay except in the case of gross lipemia where spatial interference may occur.
6. Samples containing fibrin may exhibit either falsely elevated or falsely decreased results. Fibrin must be eliminated from the sample before assay begins.
7. Samples from patients who had an injection of fluorescein, which is used in fluorescein fundus angiography, may cause falsely elevated results.
8. Specimens from patients taking medicines and/or medical treatment may show erroneous results.
9. Specimens from patients who have received preparations of mouse monoclonal antibodies for diagnosis or therapy may contain human anti-mouse antibodies (HAMA). Such specimens may show falsely elevated values when tested for testosterone.
10. For a more complete understanding of the limitations of this procedure, please refer to the SPECIMEN COLLECTION AND HANDLING, WARNINGS AND PRECAUTIONS, STORAGE AND STABILITY, and PROCEDURAL NOTES sections in this insert sheet.

EXPECTED VALUES

Each laboratory should determine a reference interval which corresponds to the characteristics of the population being tested. As with all diagnostic procedures, clinical results must be interpreted with regard to concomitant medications administered to the patient *(10)*.

Reference Ranges

The interval given here was determined in serum samples from 288 (Male: 150, Female: 138) apparently healthy Asian individuals.

	Number	Ranges (ng/dL)	Average (ng/dL)
Male	150	262 - 870	514
Female	138	9 - 56	28

Conversion Factors

Testosterone concentrations in this application are in units of ng/dL. Conversion to SI units of nmol/L may be done using the following equation:

nmol testosterone / L = ng testosterone / dL x 0.03467

PERFORMANCE CHARACTERISTICS

ACCURACY

a. Recovery: Three serum pools were spiked with three different levels of testosterone and assayed before and after spiking.

Sample	Initial Value (ng/dL)	Testosterone Added (ng/dL)	Expected Value (ng/dL)	Measured Value (ng/dL)	Percent Recovery (%)
Serum A	15.1	249.1	264.2	282.9	107.1
	15.1	124.6	139.7	127.5	91.3
	15.1	62.3	77.4	70.9	91.6
Serum B	11.6	228.5	240.1	272.3	113.4
	11.6	114.3	125.9	126.0	100.1
	11.6	57.1	68.7	64.5	93.9
Serum C	12.9	256.0	268.9	272.3	101.3
	12.9	128.0	140.9	130.8	92.8
	12.9	64.0	76.9	65.7	85.4

b. Dilution: Three serum samples containing high concentrations of testosterone were serially diluted with the ST AIA-PACK Testosterone SAMPLE DILUTING SOLUTION and assayed.

Sample	Dilution Factor	Expected Value (ng/dL)	Measured Value (ng/dL)	Percent Recovery (%)
Serum A	none		2096	
	7.5/10	1571	1633	103.9
	5.0/10	1048	1101	105.1
	2.5/10	524	549	104.8
	1.0/10	210	182	86.7
Serum B	none		1989	
	7.5/10	1492	1574	105.5
	5.0/10	995	1100	110.6
	2.5/10	497	550	110.6
	1.0/10	199	182	91.5
Serum C	none		2048	
	7.5/10	1536	1523	99.1
	5.0/10	1024	1122	109.6
	2.5/10	512	581	113.5
	1.0/10	205	193	94.1

PRECISION

a. The intra-assay coefficient of variation (within run precision) was evaluated in three control samples by 10 replicate determinations.

Reg.Lot	Sample	Number of Replicates	Mean (ng/dL)	Standard Deviation (ng/dL)	Coefficient of Variation (%)
	Sample A	10	52.0	1.8	3.5
BZ12472	Sample B	10	800.0	19.6	2.4
	Sample C	10	1633.0	45.9	2.8
	Sample A	10	53.0	2.8	5.3
BZ12473	Sample B	10	798.0	15.4	1.9
	Sample C	10	1558.0	28.2	1.8
	Sample A	10	61.4	3.2	5.2
C212474	Sample B	10	796.0	25.9	3.3
	Sample C	10	1503.0	46.0	3.1

b. The inter-assay coefficient of variation (between run precision) was evaluated at three different concentrations by analyzing control samples in 20 separate runs.

Sample	Number of Replicates	Mean (ng/dL)	Standard Deviation (ng/dL)	Coefficient of Variation (%)
Sample A	20	88.6	5.3	6.0
Sample B	20	627.2	16.8	2.7
Sample C	20	1402.5	34.8	2.5

CORRELATION

The correlation between serum (x) and heparinized plasma (y) on ST AIA-PACK Testosterone was carried out using 239 patient specimens.

Slope	1.000
y-Intercept	2.830
Correlation Coefficient	0.998
Range of Samples	7.9-1988.7
Number of Samples	239

SPECIFICITY

The following substances were tested for cross-reactivity. Cross-reactivity (%) is the percentage of the compound which will be identified as testosterone. If these compounds are present in the specimen at the same concentration as testosterone, the final result will be increased by these percentages.

Substance	Concentration added (ng/dL)	Cross-reactivity (%)
Androstenedione	200,000	1.72
Aldosterone	160,000	N.D.
Androsterone	2,000,000	0.03
Corticosterone	100,000	0.01
Cortisol	160,000	N.D.
Cortisone	160,000	N.D.
Danazol	4,000	N.D.
11-deoxycortisol	20,000	N.D.
Dexamethasone	160,000	N.D.
DHEA	5,000	0.01
DHEA-sulfate	20,000	N.D.
5α-dihydrotestosterone	1,000	0.08
Estradiol	200,000	N.D.
Estrone	10,000	N.D.
Ethisterone	1,000	0.02
Fluoxymesterone	100,000	N.D.
19-hydroxyandrostenedione	5,000	0.04
Methyltestosterone	600	0.14
Norethindrone	500	N.D.
Prednisone	16,000	N.D.
Progesterone	20,000	0.02
Norethynodrel	1,000	0.03
Spironolactone	20,000	0.01
Triamcinolone	1,000	N.D.

(N.D.: not detectable)

SENSITIVITY

The minimal detectable concentration (MDC) of testosterone is estimated to be 7 ng/dL. The MDC is defined as the concentration of testosterone which corresponds to the rate of fluorescence that is two standard deviations from the mean rate of fluorescence of 20 replicate determinations of the ST AIA-PACK Testosterone CALIBRATOR (1).

INTERFERENCE

Interference is defined, for the purposes of this study, with recovery outside of 10% of the known concentration of the specimen after the following substances are added to human specimens. For these studies, interfering substances are added to normal human serum.

1. Hemoglobin (up to 410 mg/dL), free bilirubin (up to 17 mg/dL) and conjugated bilirubin (up to 18 mg/dL) do not interfere with the assay.
2. Lipemia, as indicated by triglyceride concentration (up to 500 mg/dL), does not interfere with the assay.
3. Ascorbic acid (up to 20 mg/dL) does not interfere with the assay.
4. Protein, as indicated by human albumin concentration (up to 0.5 g/dL), does not interfere with the assay.
5. Heparin (up to 100 U/mL) does not interfere with the assay.

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TOSOH CORPORATION
Shiba-koen First Bldg.
3-8-2 Shiba, Minato-ku, Tokyo 105-8623
Japan
Phone :+81-3-5427-5181
Fax :+81-3-5427-5220

EC REP

TOSOH EUROPE N.V.
Transportstraat 4
B-3980 Tessenderlo, Belgium
Phone :+32-13-66 88 30
Fax :+32-13-66 47 49

Supplied by

TOSOH BIOSCIENCE, INC.
6000 Shoreline Ct., Suite 101
South San Francisco, CA94080, USA
Phone :+1-650-615-4970
Fax :+1-650-615-0415
Phone :(800)248-6764
Fax :(800)685-7595