Relationship Between Cervical and Blood Free Radicals Concentrations and Unexplained Infertility In Women

A Thesis

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in Medical Physiology

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2009AD **1430AH**

To

My country

My faithful family

The eyes that observe closely

The hearts that supplicate for success

Ban

بسم الله الرحمن الرحيم

لِلّٰهِ مُلْكُ السَّمٰوٰتِ وَالْاَرْضِ يَخْلُقُ مَا يَشَآءُ يَهَبُ لِمَنْ
يَّشَآءُ اِنَاثًا وَّيَهَبُ لِمَنْ يَّشَآءُ الذُّكُوْرَ ۞ اَوْ يُزَوِّجُهُمْ
ذُكْرَانًا وَّاِنَاثًا وَيَجْعَلُ مَنْ يَّشَآءُ عَقِيْمًا
اِنَّهٗ عَلِيْمٌ قَدِيْرٌ ۞

صدق الله العظيم

سورة الشورى (الآية 49–50)

الخلاصة

يعتبر العقم من أهم المشاكل الصحية في الدول النامية.و يمكن تحديد أسباب العقم في 90% من الحالات بينما يبقى السبب في 10 % من الحالات غير معروف.وهذا العقم غير المفسر ربما تكمن أسبابه في الاضطرابات الفسلجية :هرمونية أو مناعية أو وراثية.

ويبدو في الوقت الحاضر إن أنواع الأوكسجين الفعالة واحدة من الأسباب الرئيسية للعقم الغير مفسر.لذلك صممت هذه الدراسة لتحديد وقياس أنواع الأوكسجين الفعالة والتي تسمى بالجذور الحرة وكذلك قياس مضادات الأكسدة في مصل الدم و الإفرازات المخاطية لعنق الرحم لمعرفة مدى علاقتها بالعقم غير المفسر.

شملت الدراسة (60) امرأة مصابة بالعقم غير المفسر متوسط أعمارهن (29.866±7.195) عاما واللاتي شخصن سريريا و مختبريا . تم فحص النساء من قبل أخصائية نسائية في مستشفى بابل للولادة والأطفال وفي العيادات الخاصة في بابل و للفترة من أيلول 2008 ولغاية أيار 2009. كما تضمنت الدراسة (30) عينة قياسية من النساء المنجبات متوسط أعمارهن (30.133±8.011)عاما ، حيث تم دراسة نسبة ناتج الأكسدة للدهون (المالونداي الدهايد) وكذلك قياس بعض من مضادات الأكسدة (الكلوتاثايون وإنزيم الكاتاليز وفيتامين سي و فيتامين إي) في مصل الدم وفي الإفرازات المخاطية لعنق الرحم ولكلا المجموعتين . بالإضافة إلى بعض العوامل الفسلجية الأخرى (مدة الدورة الشهرية وموعد البلوغ ومعامل كتلة الجسم وفصيلة الدم ونسبة البروتينات الكلية في مصل الدم و نسبة الألبومين في مصل الدم و نسبة الغلوبيولين في مصل الدم).

بينت النتائج إن نسبة المالونداي الدهايد قد ارتفعت معنويا بشكل واضح في مصل الدم و الإفرازات المخاطية لعنق الرحم(3.105 ± 1.441 و 6.729± 3.055مايكرومولاري) للنساء العقيمات عند مقارنتهم بالنساء المنجبات(1.524 ± 0.518 و 3.201±1.028 مايكرومولاري) بالتتابع.

على عكس إنزيم (الكاتاليز) حيث انخفض معنويا بشكل واضح في مصل الدم و الإفرازات المخاطية لعنق الرحم (0.303 ± 0.286 و0.331 ±0.302 ك / مل) للنساء العقيمات عند مقارنتهم بالنساء المنجبات(0.479 ± 0.342 و 0.508 ±0.301 ك/مل).

إما عند دراسة مضادة الأكسدة غير الإنزيمية فقد وجد انخفاض معنوي في نسبة الكلوتاثايون في مصل الدم و الإفرازات المخاطية لعنق الرحم(17.977±8.506 و 51.602 ±27.335 مايكرومولاري) للنساء العقيمات عند مقارنتهم بالنساء المنجبات(27.384 ± 13.547و65.327 ± 18.217 مايكرومولاري).

كذلك نسبة فيتامين إي فقد وجد انخفاض معنوي في مصل الدم و الإفرازات المخاطية لعنق الرحم (5.272± 1.228 و4.644± 1.343 ملغم /لتر) للنساء العقيمات عند مقارنتهم بالنساء المنجبات(7.337 ±3.535 و 0.754± 7.023 ملغم /لتر.

إما نسبة فيتامين سي فقد وجد انخفاض غير معنوي في مصل الدم و الإفرازات المخاطية لعنق الرحم(9.944±1.549 و 14.233± 3.458 ملغم /لتر) للنساء العقيمات عند مقارنتهم بالنساء المنجبات(10.383±3.655 و 16.447 ± 4.042 ملغم /لتر).

العلاقة بين تراكيز الجذور الحرة في الدم وعنق الرحم و العقم المبهم في النساء

رسالة مقدمة إلى
مجلس كلية الطب – جامعة بابل
كجزء من متطلبات نيل درجة ماجستير علوم في
الفسلجة الطبية

من قبل

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1430هـ 2009م

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Supervisors Certification

We , certify that this thesis was prepared under our supervision at College of Medicine at University of Babylon in Partial Fulfillment of the Requirements for the Degree of Master of Science in Medical Physiology

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We ,the examiner committee certify that we have read this thesis entitled (Relationship Between Cervical and Blood Free Radicals Concentrations and Unexplained Infertility In Women) and have examined the student (Ban Jabir Edan)in its contents and that in our opinion it is accepted as a thesis for the Degree of Master of Science in Medical Physiology with excellent estimation.

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Chapter one

Introduction

1.Introduction :

Reproductive failure is a significant public health concern. Infertility, carries significant personal, societal and financial consequences.One of the most important and underappreciated reproductive health problems in developing countries is the high rate of infertility and childlessness (Ruder, *et a.l,*2008).

Infertility is defined as 'the inability to conceive following 12 months of unprotected sexual intercourse, before an investigation is undertaken unless medical history and physical findings dictate earlier evaluation and treatment (ASRM, 2004). infertility is classified as primary in which no previous pregnancy has occurred and secondary in which prior pregnancy has occurred respective of its outcome(Jaiswar ,*et al*., 2006). Causes of infertility can be found in about 90% of cases, about 10 % of patients don't know why they can not conceive this is called unexplained infertility (Siristatidis and Bhattacharya,2007).

Unexplained infertility is a diagnosis of exclusion, when the standard investigation of both the female and male partner has ruled out other infertility diagnoses (Smith, *et al.,* 2003).A couple is considered to have unexplained infertility if the woman ovulated and had a normal and hysterosalpingogram, and the man a normal semen analysis (Edward and Marut, 2008). Critical factors to be considered in evaluating and managing unexplained infertility are the duration of infertility and female age (Siristatidis and Bhattacharya,2007).

In case of unexplained infertility, any form of treatment is speculated.

A period of three years of unexplained infertility is generally accepted as minimum duration before active intervention is considered .

Empirical treatment with clomiphene ,intrauterine insemination are used in treatment of unexplained infertility, if failed , invetro fertilization is considered(Gleicher and Barad,2006).

Because female ovary is the source of oocytes and regulating hormones, free radicals in the gynecologic environment is likely to be an important mediator of conception. Recently there is a growing evidence of possible role of highly reactive products of oxygen, termed free radicals, in infertility(Agarwal and Shyam,2004).

A free radical is any atom (e.g. oxygen, nitrogen) with at least one unpaired electron in the outermost shell (Ruder ,*et al*.,2008). free radicals are neutralized by an elaborate antioxidant defense system .In a healthy body, pro-oxidants and antioxidants maintain a ratio and a shift in this ratio towards pro-oxidants gives rise to oxidative stress(Agarwal,*et al*.,2008).

In this case free radical species which are unstable and highly reactive, will become stable by acquiring electrons from nucleic acids ,lipids, proteins, carbohydrates or any nearby molecule causing a cascade of chain reactions resulting in cellular damage and disease (Van Langendonckt, *et al*, 2002). Because free radicals are unstable, and difficult to measure, traditional indices of oxidative stress include downstream markers of oxidative damage to macromolecules such as lipids, proteins and DNA. Oxidative stress is also indirectly assessed by estimating capacity for antioxidant defense in serum, or other body fluids. Such measures include assessment of enzymatic antioxidant activity and individual assessment of circulating non-enzymatic antioxidant levels(Janicki,2008).

Aims of the study

The aims of the present work is to explore some aspects of unexplained infertility among women. To verify this goal, the following parameters were determined :

1- The malondiadehyde (MDA) concentration in sera and cervical mucus secretions.
2- The activity of antioxidant enzyme, catalase (CAT) in sera and cervical mucus secretions.
3- The level of reduced glutathione (GSH) concentration as non-enzymatic antioxidant in sera and cervical mucus secretions.
4- The concentration of vitamin C as non-enzymatic antioxidant in sera and cervical mucus secretions.
5- Vitamin E concentration as non- enzymatic antioxidant in sera and cervical mucus secretions.
6- The serum proteins concentrations.
7- The relationship between ABO blood group system and unexplained infertility in women.
8- Relationship between oxidative stress markers and some factors (age, body mass index ,smoking ,infertility duration and type of infertility).

Chapter Two

Literatures Review

2.1 Female Reproduction

2.1.1 Anatomy

The female reproductive organs (figure (2.1) consists of the ovaries(as female gonads ,which produce ova and several important sex hormones) , fallopian tubes, uterus, vagina, external genital organs and mammary glands (Rod, *et al* ,1996).

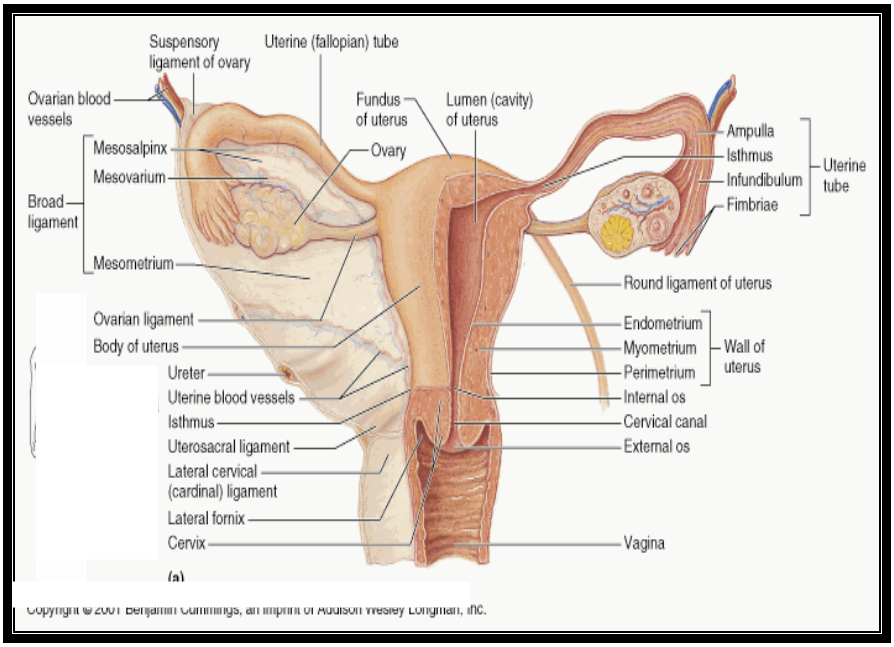


Figure (2.1) Part of female reproductive organs (Rod, *et al* ,1996)

2.1. 2 The ovarian cycle

During fetal development, the ovaries contain over 7 million primordial follicles. However, many undergo atresia before birth. At the time of birth, there are 2 million ova, but 50% of these are atretic. The million that are normal undergo the first part of the first meiotic division at about this time and enter a stage of arrest in prophase in which those that survive persist until adulthood. Atresia continues during

development, and the number of ova in both of the ovaries at the time of puberty is less than 300,000. Only one of these ova per cycle normally reaches maturity; the remainder degenerate(Ganong ,*et al.*,2005).

Just before ovulation, the first meiotic division is completed. One of the daughter cells(the secondary oocyte) receives most of the cytoplasm, while the other(the first polar body), fragments and disappears. The secondary oocyte immediately begins the second meiotic division, but this division stops at metaphase and is completed only when a sperm penetrates the oocyte (Balusik,2003).

2.1. 3 Ovulation

Ovulation in a woman who has a normal 28-day sexual cycle occurs 14 days after the onset of menstruation. Shortly before ovulation, the protruding outer wall of the follicle swells rapidly, and a small area in the center of the follicular capsule, called the *stigma,* protrudes like a nipple then the stigma ruptures widely, allowing a more viscous fluid, to evaginate outward. This fluid carries with it the ovum surrounded by a mass of granulosa cells, called *corona radiate*(Guyton and Hall,2006).

LH is necessary for final follicular growth and ovulation. Without this hormone, the follicle will not progress to the stage of ovulation (Rod, *et al* .,1996). LH causes rapid secretion of follicular steroid hormones that within a few hours, two events occur, necessary for ovulation:

(1) The theca externa begins to release proteolytic enzymes from lysosomes, and these cause dissolution of the follicular capsular wall . (2) Simultaneously, there is rapid growth of new blood vessels into the follicle wall, and prostaglandins (local hormones that cause vasodilation) are secreted into the follicular tissues. These two effects cause plasma transudation into the follicle, which contributes to follicle swelling. Finally, the combination of follicle swelling and simultaneous

degeneration of the stigma causes follicle rupture, with discharge of the ovum(Guyton and Hall,2006) .

2.1. 4 Menstrual cycle

Females begin the menstrual cycle at the time of puberty, around age 13, and experience it monthly until around their late forties to middle fifties, except when interrupted by pregnancy(Shier, *et al.* ,1998). The balance between the hormones of the hypothalamus, pituitary and the ovaries must be in a homeostatic balance, fluctuating together to cause the cyclic changes, the ultimate goal of which is to produce a viable oocyte , ready for fertilization(Rod, et al ,1996). The menstrual cycle is generally accepted to begin on day 1, the first day of Menses, or bleeding. *Menses* usually lasts 4 to 5 days. In this phase, the lining of the uterus, the endometrium, and its blood vessels break down and discharge through the vagina, releasing blood, mucus, and cell debris (Shier, *et al.* ,1998). Also, during this phase, the anterior pituitary gland releases both FSH and LH, which stimulate the ovary and begin follicular growth (Balusik,2003).

The next phase, known as the *proliferative phase*, happens in the time between the end of the Menstrual phase and Ovulation (days 6 – 14). During this time, estrogen from the developing follicle in the ovary stimulates the endometrium of the uterus to grow and thicken. At this phase, the hypothalamus is sending GnRH to the anterior pituitary and at the mid-cycle, around day 14, ovulation occur (Guyton and Hall,2006).

The last phase, the *secretory phase*, involves the release of progesterone and estrogen from the corpus luteum in the ovary. Progesterone causes the endometrium to become more glandular and more vascular, and stimulating the uterine glands to secrete fluids into the endometrium (Shier, *et al*. ,1998). About 10 days after ovulation, the corpus luteum degenerates, and, if fertilization does not happen, the concentrations of

estrogen and progesterone decline markedly. In response to the hormonal decline, the blood vessels to the endometrium constrict, cutting off the nutrient supply. Within a few days (the tissues disintegrate and slough off, starting the Menstrual phase once again (Balusik,2003).

2.1. 5 Hormones and Female Cycles

The female reproductive cycle is hormonally regulated in two phases. The follicle secretes estrogen before ovulation; the corpus luteum secretes both estrogen and progesterone after ovulation. Hormones from the hypothalamus and anterior pituitary control the female reproductive cycle as shows in figure (2.2)(Rod, et al ,1996).

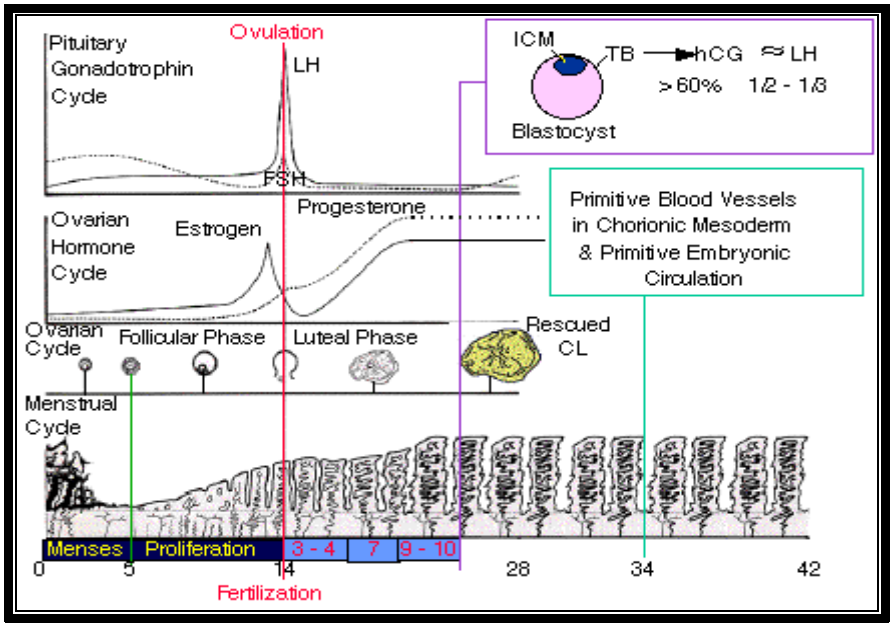


Figure (2.2)Hormonal changes and the female reproductive cycles (Balusik,2003).

During menstruation the uterine lining is broken down and shed as menstrual flow. FSH and LH are secreted on day 1, beginning both the menstrual cycle and the ovarian cycle. Both FSH and LH stimulate the maturation of a single follicle in one of the ovaries and the secretion of estrogen. Rising levels of estrogen in the blood trigger secretion of LH,

which stimulates follicle maturation and ovulation (day 14, or midcycle).

LH stimulates the remaining follicle cells to form the corpus luteum, which produces both estrogen and progesterone(Shier, *et al*. ,1998).Estrogen and progesterone stimulate the development of the endometrium and preparation of the uterine inner lining for implantation of a zygote. If pregnancy does not occur, the drop in FSH and LH cause the corpus luteum to disintegrate. The drop in hormones also causes the sloughing off of the inner lining of the uterus by a series of muscle contractions of the uterus(Balusik,2003).

2.1. 6 Fertilization of the Ovum

After the male ejaculates semen into the vagina during intercourse, a few sperm are transported within 5 to 10 minutes upward from the vagina and through the uterus and fallopian tubes to the *ampullae* of the fallopian tubes where fertilization of the ovum takes place.Before a sperm can enter the ovum, it must first penetrate the *corona radiata* and then bind to and penetrate the *zona pellucida* surrounding the ovum itself(Guyton and Hall,2006).

Capacitation" of the Spermatozoa-making it possible for them to Penetrate the Ovum. This normally requires from 1 to 10 hours. Some changes that are believed to occur are the following:

- The uterine and fallopian tube fluids wash away the various inhibitory factors that suppress sperm activity in the male genital ducts.
- After ejaculation, the sperm deposited in the vagina swim away from the cholesterol vesicles upward into uterine cavity, and they gradually lose much of their other excess cholesterol. In so doing, the membrane at the head of the sperm (the acrosome) becomes much weaker.
- The membrane of the sperm also becomes much more permeable to calcium ions, so that calcium now enters the sperm and changes the

activity of the flagellum.In addition, the calcium ions cause changes in the cellular membrane, making it possible for the acrosome to release its enzymes rapidly and easily(Guyton and Hall,2006).

Also the stored enzymes in the acrosome begin to be released. These enzymes are important in opening pathways between the granulosa cells so that the sperm can reach the ovum(Balusik,2003).

When the sperm reaches the zona pellucida of the ovum, the anterior membrane of the sperm itself binds specifically with receptor proteins in the zona pellucida. Then, rapidly, the entire acrosome dissolves, and all the acrosomal enzymes are released in to ovum(Shier, *et al*. ,1998).Once a sperm has entered the ovum (which is still in the secondary oocyte stage of development), the oocyte divides again to form the *mature ovum* plus a *second polar body* that is expelled. The mature ovum still carries in its nucleus (now called the *female pronucleus*) 23 chromosomes. In the meantime, the fertilizing sperm has also changed. On entering the ovum, its head swells to form a *male pronucleus*, , the 23 unpaired chromosomes of the male pronucleus and the 23 unpaired chromosomes of the female pronucleus align themselves to re-form a complete complement of 46 chromosomes (in the *fertilized ovum)*(Guyton and Hall,2006).

Therefore successful initiation of pregnancy requires the ovulation of a mature oocyte, production of competent sperm, proximity of sperm and oocyte in the reproductive tract, fertilization of the oocyte, transport of the conceptus into the uterus, and implantation of the embryo into a properly prepared, healthy endometrium. A dysfunction in any one of these complex biological steps can cause infertility (Goldman ,*et al*., 2000).

2.2 Infertility

Infertility refers to inability for conceiving following 12 months of unprotected sexual intercourse, before an investigation is undertaken unless medical history and physical findings dictate earlier evaluation and treatment (American Society for Reproductive Medicine (ASRM), 2004). Infertility is either ***primary***, in which no previous pregnancy has occurred, or ***secondary***, in which prior pregnancy has occurred irrespective of its outcome (Jaiswar ,*et al*., 2006).

2.2.1 Prevalence of infertility

The prevalence of female infertility in USA ranges from 7% to 28%, depending on the age of the woman. In developing countries, only a limited number of papers report on the prevalence of infertility in these countries (Boivin ,*et al*., 2007). In Asia and Latin-America, a report compiled by the WHO indicated that the prevalence of infertility in these regions fell within the range 8–12% of couples of reproductive age (WHO, 1991).

In general, an estimated 84% of couples conceive after 1 year of intercourse, and 92% of the couples conceive after 2 years (Yu SL, 2003).

About 40%–50% of the etiology of infertility studied is due to female causes (Duckitt, 2003).A primary diagnosis of male factor infertility is made in 30% of infertile couples. Combined female and male factor infertility is responsible for 20%–30% of cases and , unexplained infertility affects 15% of couples (DeCherney ,*et al*.,2003).

2.2.2 The main causes of female infertility

The main causes of female infertility can be illustrated in Table (2.1) as shown by (Nathan, 2007).In his study, Duckitt, (2003) has shown that about 90% of cases of infertility causes have been uncovered, the other

10% of patient has don't been known the causes ,whereas (10-30) % of infertility has more than one cause. Recently there is a growing evidence of possible role of highly reactive products of oxygen, termed free radicals, in case of infertility (Agarwal ,*et al*.,2004).

Table (2.1) Causes of female infertility

1-Ovulatory factors : 30%
- Central defect:
  Chronic hyperandrogenemic anovulation.
  Hyperprolactenemia.
  Hypothalamic insufficiency.
  Pituitary insufficiency.
- Peripheral defect:
  Gonadal dysgenesis.
  Premature ovarian failure
  Ovarian resistance
- Metabolic diseases:
  Thyroid disease
  Liver disease
  Renal disease
  Androgen excess.

2-Cervical factor: 15%
- congenital factor :
  mullerian duct abnormality.
  Diethyl stelbosterol(DSE) exposure.
- Acquired factor: surgical treatment ,infection

3- Pelvic factor:25%
- Infection:
  appendicitis
  pelvic inflammatory disease
  uterine adhesion.
- Endometriosis.
- Structural abnormalities :
- Diethyl stelbosterol(DSE) exposure.
- Failure of normal fusion of reproductive tract

4- Additional Factors
- Behavioral factors:
  Diet
  Exercise
  Smoking
  Alcohol
  Drugs
- Environmental Factors:

2.2.3 Diagnosis of female infertility

An accurate history of couple's personal and medical details together with a simple light microscopy seminal analysis will identify the majority of infertility problems related to ovulatory disorders and male subfertility (Menkveld,*et al*.,2001).Figure(2.3)shows the diagnostic steps of infertility:

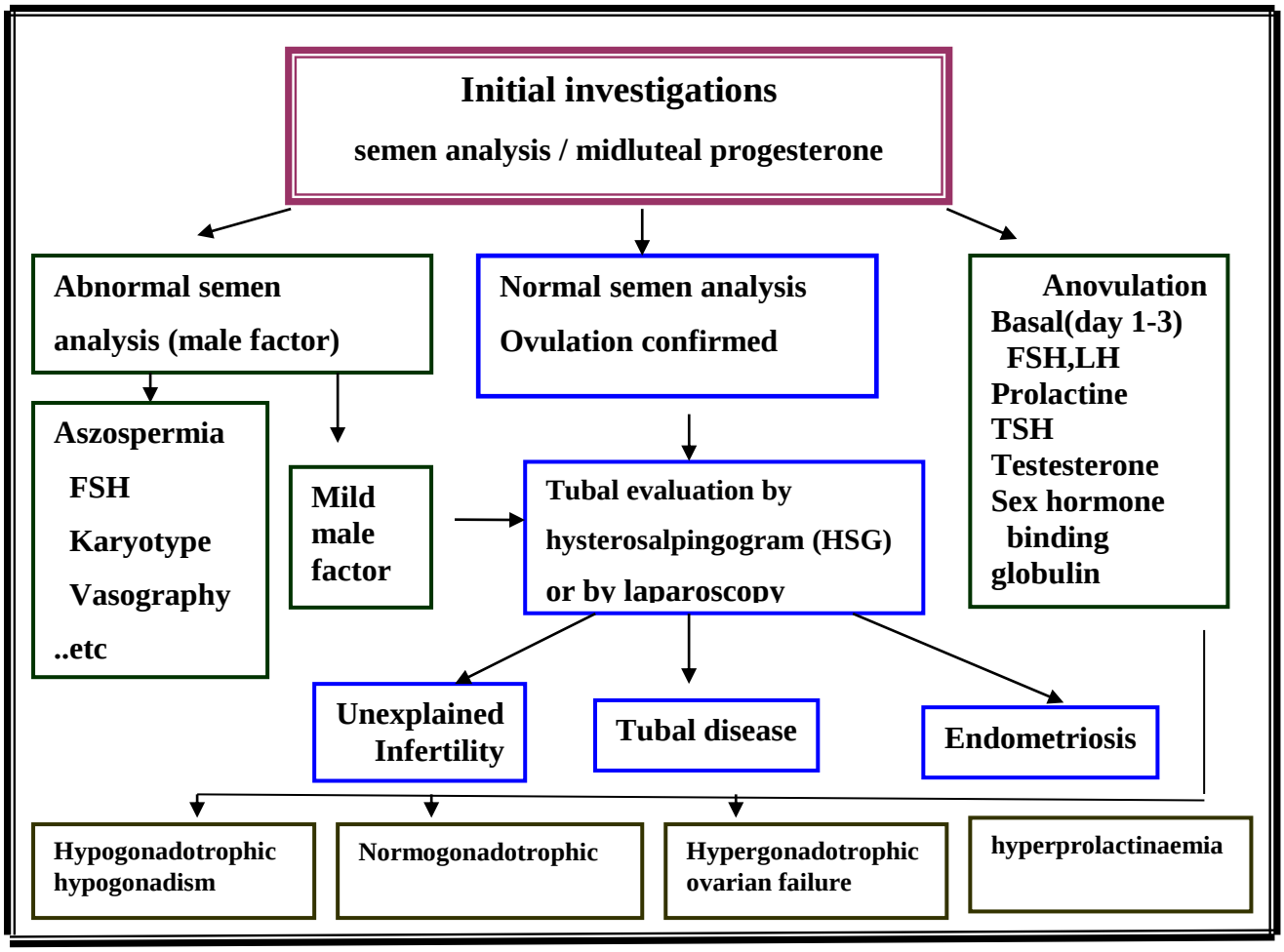


Figure (2.3) Investigations of infertility(Edmonds,2007)

So the initial steps for diagnosis of infertility includes:

- Confirmation of ovulation by History and laboratory tests.
- An assessment of the fallopian tubes and the uterus by the use of an x-ray called Hysterosalpingogram (HSG).
- An assessment of a seminal fluid analysis (SFA).

2.2.4 Mucus secretion and infertility

Under the influence of estrogen, the cervix secretes highly hydrated mucus, specially at midcycle. Mucus consists essentially of two parts, the mucin and the water with its dissolved components **(**Suarez and Pacey, 2005).Cervical mucus has been suggested to influence spermatozoa in a such manner including:

- Removal of spermatozoa from the hostile vaginal environment.
- Exclusion of seminal plasma and its components.
- Selective exclusion of certain sperm cells with morphological and possibly functional abnormalities.
- Retention of spermatozoa for later migration to the upper tract (Perry, *et al*.,1996).

Passage of sperm through the female reproductive tract is regulated to maximize the chance of fertilization and ensure that sperm with normal morphology and active motility will be the ones to succeed **(**Suarez and Pacey ,2005**).** The normal thinning of cervical mucus at ovulation functions to make the passage of sperm easier where as poor mucus at midcycle can be a physical barrier that decreases sperm penetration and associated with a decreased chance for fertility(Speroff ,*et al*., 1999).

Therefore active and highly synchronized interactions between sperm and female tract fluids appear to be critical for the survival and functions of sperm(Kawakami ,*et al*., 2001).

2.2.5 Unexplained Infertility

Guidelines have been established for a standard infertility evaluation by the American Society for Reproductive Medicine . They includes a semen analysis, assessment of ovulation and demonstration of tubal patency by hysterosalpingogram. When the results of a standard infertility evaluation are normal, practitioners assign a diagnosis of unexplained infertility(Wang ,*et al.* ,1997; Valentine, *et al*.,2004; Alexander and Dokras,2008 and Edward, 2008).

It doesn't mean that there is no reason for the infertility, but that the reason is unable to be identified at that time, and its aetiology seems to be heterogeneous(Siristatidis and Bhattacharya,2007). Approximately 10 to 15 percent of couples will receive the diagnosis

of unexplained infertility(Smith ,*et al*., 2003).

In fact there is often something wrong at a more basic level for example, it is possible that there is something wrong at the level of the gametes (ova & sperm); as DNA defect, their interaction with each other, or their interaction with the female reproductive organs as immunological defects Couples with unexplained infertility may have problems with oocyte quality, tubal function, or sperm function that are difficult to diagnose and/or treat (ASRM,2004).

It is also possible to have sperm that appears normal under a microscope, however not perform the function of fertilization adequately. Furthermore, one can have normal sperm but poor quality ova that do not fertilize or fertilize at a lower than expected rate. If normal ova and sperm meet, one can expect a fertilization rate between 60 to 90 %.

However; possible causes of unexplained infertility can be :

1. *Tubal Abnormalities*: It is possible that there may be a subtle defect in the mechanism by which the fimbria "pick up" the ova at ovulation; or the cilia in the tube may not function properly(Evers, 2002).

2. *Genetic factor* (Abnormal ova or sperm): due to a deformed structure or chromosomal abnormalities**(**Golubovsky,2008**).**

3. *Trapped ova:* In some cases the ova is 'trapped' inside the unbroken corpus luteum - called a luteinized unruptured follicle syndrome.

4. *Luteal phase abnormalities.*

5. *Immunological factors:* The immune system can react against the man's sperm and kill them, immobilize them, or make them stick together (Shatavi, *et al*., 2006).

Women can also develop an immune reaction to the coating of their own ova, which can prevent sperm from attaching to them.

6. *Infections.*

7. *Inability of sperm to penetrate ova.*

8. ***Uterine factor:*** Some women have an abnormal endometrium (uterine lining) which does not allow the embryo to implant. This may be associated with inadequate uterine blood flow, or poor estrogen receptors in the endometrial cells.

9. ***Psychological factors.***

Critical factors to be considered in evaluating and managing unexplained infertility are the duration of infertility and age of the female partner. Younger fertile couples have approximately a 20 percent chance of spontaneous conception per month.

In contrast, couples with unexplained infertility, who are infertile for more than three years, have spontaneous conception rates of 1 to 2 percent per month (ASRM,2004).

Because age is an important determinant, further evaluation and therapy should not be deferred in older women (Tummon,*et al*., 1988) .

The average monthly fecundity in normal couples is 30%; the monthly pregnancy rate in couples with unexplained infertility is 1.5–3%. After 3 years of infertility, the prospect of pregnancy decreases by 24% each year(Crosignani, *et al*. ,1993).Approximately 60% of couples with unexplained infertility of less than 3 years duration will become pregnant with 3 years of expectant management (Crosignani, *et al*. , 1992).

In case of unexplained infertility, any form of treatment is speculated. A period of three years of unexplained infertility is generally accepted as minimum duration before active intervention is considered.

Empirical treatment with clomiphene ,intrauterine insemination are used in treatment of unexplained infertility, if failed , in vitro fertilization is considered (Gleicher and Barad, 2006).

The utility of anything other than basic tests of semen quality, ovulation and tubal patency in the diagnosis and management of infertility has yet to be proven (Crosignani, *et al*., 1993).

Recent research on the role of reactive oxygen species (ROS) in human infertility has received a great deal of interest from the scientists and medical practitioners (Saleh , *et a*l., 2002 and Agarwal ,*et al*.,2008).

2.3 Free radicals

A free radical is any atom (e.g. oxygen, nitrogen) with at least one unpaired electron in the outermost shell (Ruder, *et al*,2008).

Free radical atoms are unstable and highly reactive. They become stable by acquiring electrons from nucleic acids, lipids, proteins, carbohydrates or any nearby molecule causing a cascade of chain reactions resulting in cellular damage and disease (Van Langendonckt, *et al*., 2002 and Pierce, *et al*.,2004). The terms free radical and ROS are commonly used in an interchangeable manner, despite the fact that not all ROS are free radicals (Cheeseman and Slater, 1993). For example, hydrogen peroxide (H_2O_2) is considered a ROS but it is not a free radical since it does not contain unpaired electrons. In addition, there is a sub-class of free radicals derived from nitrogen (Tremellen , 2008).

There must be a balance between oxidants and antioxidants, generation of an excess of free radical results in oxidative stress (Fujii , *et al.*,2005).

2.3.1 Types of free radical species:

There are two major types of free radical species: reactive oxygen species (ROS) and reactive nitrogen species (NOS):

2.3.1.1 Reactive oxygen species (ROS)

The Oxygen centered free radicals are class of powerful oxidants in the human body. The most common ROS include: the superoxide anion (O_2^-), the hydroxyl radical ($OH^{\cdot}$), singlet oxygen($^1O_2$), and a number of related species ,such as hydrogen peroxide (H_2O_2),that do not themselves

contain unpaired electrons but are often involved in the generation of free radicals(Christophersen, *et al*.,1991).Some biochemical steps can directly generate the hydrogen peroxide radical as the Figure(2.4) shows:

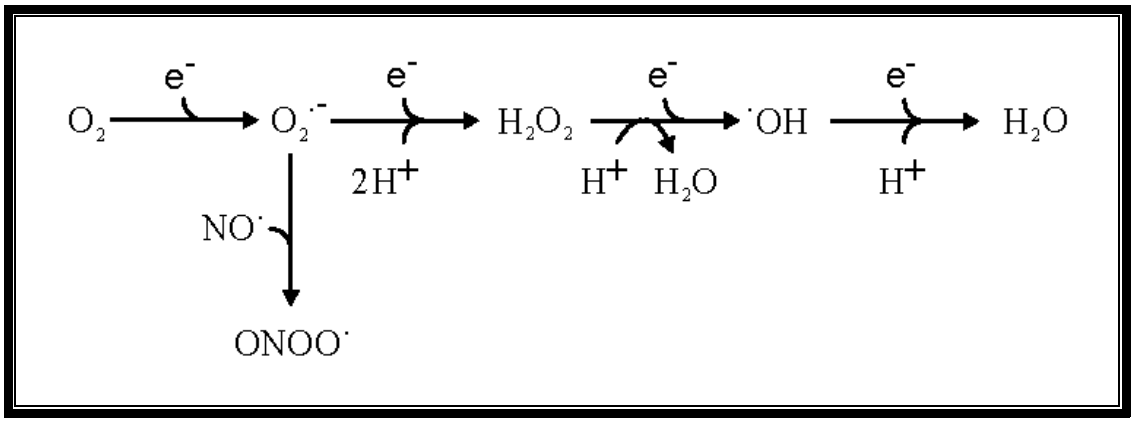


Figure (2.4) :Conversion of free radicals (José ,*et al*. ,1999).

2.3.1.2 Reactive nitrogen species

The two common examples of reactive nitrogen species are nitric oxide (NO) and nitrogen dioxide(NO_2)(Vega, *et al*.,2001).Nitric oxide is a highly reactive free radical results in cells and tissues damage (Dong ,*et al*.,2001).

2.3.2 Sources of free radicals in human body

The main four endogenous normal sources appear to account for most of the oxidants produced by cells; are aerobic respiration in mitochondria, Phagocyte, Peroxisomes and Cytochrome P450 enzymes(Bruce, *et al*., 1993).Exogenous sources may significantly increase the large endogenous oxidant load which include: Iron and copper salts (Lauffer, 1992); smoking and alcohol; normal diets (fried food,caffeen) (Ames, *et al*.,1990 and Gold, *et al*.,1992); radiation, sunlight; pollution and xenobiotics (Drugs ,pesticides , anesthetics ,and industrial solvents) (Bruce ,*et al*.,1993).

2.3.3 Physiological role of free radicals:

Depending on free radicals tissue concentration, they can either exert beneficial physiological effects or pathological damage to cellular components (Sharma and Agarwal , 1996).Free radicals are involved in:

1-Enzyme-catalyzed reactions.

2-Electron transport in mitochondria.

3-Signal transduction and gene expression.

4-Activation of nuclear transcription factors.

5-Oxidative damage to harmful molecules, dead cells, and necrotic tissues.

6-Antimicrobial action of neutrophils and macrophages(Prcker,*et al*.,2000).

2.3.4 Pathological damage of free radicals

Interaction of free radicals with other compounds results in a chain reactions of oxidation and reduction which ultimately can lead to cellular damage as Figure (2.5) shows(Janicki,2008).

Oxidation of DNA molecules, for example, can result in mutation where as oxidation of protein can result in protein cross-linking and loss function **(**Bergamini, *et al*.,2004).

Reactive oxygen species can attack polyunsaturated fatty acids of the cell membrane leading to a chain of chemical reactions called lipid peroxidation and this will lead to decrease structural fluidity of these compounds, thus resulting in loss of integrity of cellular membranes (Janicki,2008).

It is possible to measure the extent of peroxidative damage by estimating the stable end products of lipid peroxidation such as MDA(Agarwal, *et al*., 2004).

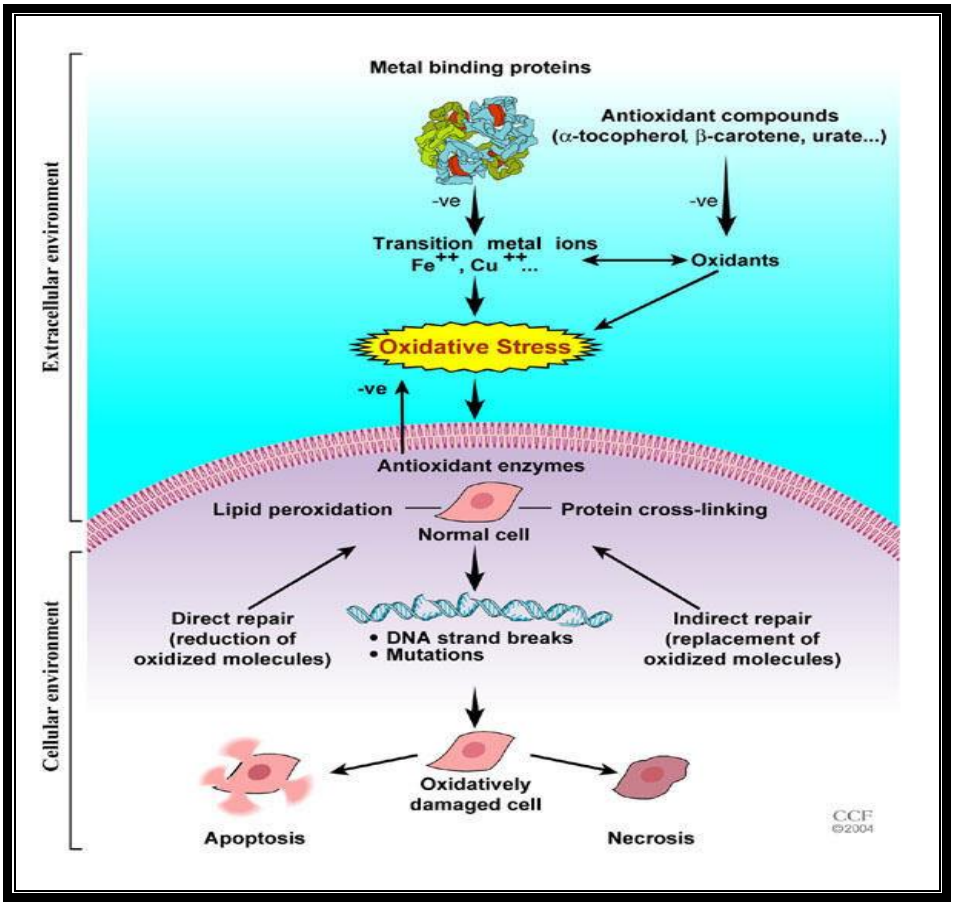


Figure (2. 5) Mechanisms of oxidative stress-induced cell damage(Agarwal ,*et al*.,2005a)

2.4 Antioxidant defense system

Organisms have developed efficient protective mechanisms against excessive accumulation of free radicals called antioxidants(Ebisch ,*et al*.,2007)**.**

Free radicals are neutralized by an elaborate antioxidant defense system. In a healthy body, pro-oxidants and antioxidants maintain a ratio and a shift in this ratio towards pro-oxidants gives rise to oxidative stress. Whenever ROS levels become pathologically elevated, antioxidants begin to work and help minimize the oxidative damage, repair it or prevent it(Zini ,*et al*., 2000).An antioxidant can be defined as any substance that, when present at low concentration compared to those of an oxidizable substance, significantly delays or prevents the oxidation of that substrate(Lunec and Ann ,1990).

Under normal conditions, antioxidants convert ROS to H_2O to prevent overproduction of ROS.

The different possible mechanisms by which antioxidants may offer protection against free radical damage include :

- Prevention of formation of free radicals.
- Interception of free radicals by scavenging the reactive metabolites and converting them to less reactive molecules.
- Facilitating the repair of damage caused by free radicals .
- Providing a favourable environment for effective functioning of other antioxidants(Battino ,*et al*.,2002 and Pendyala ,*et al*., 2008).

2.4.1 Types of antioxidants

There are two types of antioxidants in the human body: enzymatic and non-enzymatic antioxidants (Agarwal,*et al*.,2005):

2.4.1.1 Enzymatic antioxidants

Enzymatic antioxidants are also known as natural antioxidants. They are mainly composed of :

- Superoxide dismutase.
- Catalase.
- Glutathione peroxidase .

Antioxidant enzymes may act in a coordinate manner to defend living tissue from oxidant (Quinlan, *et al*.,1994).

2.4.1.1.1 Superoxide dismutase:

Superoxide dismutase is a protein dimer ,destroys the free radical superoxide by converting it to peroxide that can in turn be destroyed by CAT or GPX reactions. Superoxide dismutase converts the highly reactive superoxide radical to the less reactive H2O2(Benov and Fridovich,1998).

2.4.1.1.2 Catalase(CAT):

Catalase is a hemoprotein enzyme of the oxidoreductase class that catalyzes the convertion of hydrogen peroxide to water and oxygen, protecting cells(Boon ,*et al*., 2007). It is found in almost all cells except certain alibgate anaerobic bacteria (Alberts , *et al*., 2002).Deficiency of the enzyme ,an autosomal recessive trait result in acatalasia (Brioukhanov, *et al*.,2006).

2.4.1.1.3 Glutathione peroxidase:

Glutathione peroxidase, is a tetramer protein containing selenium, and uses glutathione as a co-substrate. Glutathione peroxidase is a cytosolic enzyme and also eliminates H_2O_2; but, in comparison to catalase, has a wider range of substrates including lipid peroxides. Glutathione peroxidase primarily functions to detoxify low levels of H_2O_2 in the cell(Jose, *et al* .,1999).

2.4.1.2 Non-enzymatic antioxidants

Non-enzymatic antioxidants are also known as synthetic antioxidants or dietary supplements. The body's complex antioxidant system is influenced by dietary intake of antioxidant vitamins and minerals such as vitamin C, vitamin E, selenium, zinc, glutathione and beta carotene (Agarwal, *et al*.,2005).

2.4.1.2.1 Glutathione

Glutathione is a tripeptide, gamma glutamyl –cysteinyl-glycin, that is widely distributed in animal and plant tissue .It exist in both the reduced thiol form (GSH) 90% and the oxidize disulfied form (GSSG) 10% (Sies,1999).

It function in various redox reactions such as the destruction of peroxide and free radicals , the detoxification of harmful compounds and activity as damage by reduction of methemoglobin and peroxide. Glutathione is also involved in the formation and maintenance of disulfied bonds in protein and in transport of amino acids across cell membrain (Giordano, *et al*. ,2007 and McConnachie, *et al*., 2007).

All cells in the human body are capable of synthesizing glutathione Specially the liver(Chen, *et al* .,2007).

2.4.1.2.1.1 Physiological role of Glutathione

GSH has multiple functions for antioxidant defense for :

1- Glutathione is a major thiol source maintaining essential redox status of the cell.

2- It reduces disulfide linkage of proteins and other molecules maintaining antioxidant enzymes in the reduced state.

3- It is a major nontoxic storage form of cysteine providing a vehicle for transport between organs .

4- It plays an important role in the reduction of ribonucleotides to deoxy-Ribonucleotides (Pollack and Leeuwenburgh,2004).

Without the protection from oxidative injury afforded by glutathione, cells may be damaged or killed (Pereira and Oliveira ,2000).

2.5.1.2.2 Vitamin C (Ascorbic acid)

Vitamin C is a water soluble vitamin found in many fruit and vegetable(Svirbelf and Gyorgyi, 2007). It is a six-carbon keto-lactone ,synthesized from glucose via several intermediates (Davis ,*et al*., 1991). Vitamin C is required for optimal functions of number of enzymes ;deficiency cause scurvy and poor wound repair. It is also considered a chain breaking antioxidant that stops the propagation of the peroxidative process. Vitamin C also helps recycle oxidized vitamin E and glutathione

(Chan ,1993). It is an unstable ,easily oxidized acid and can be destroyed by oxygen, alkaline and high temperature .Humans cannot synthesize vitamin C, so they take it from exogenous supplement or diet found in many fruit and vegetable (Iqbal ,*et al*.,2004).

Ascorbic acid has three main biological functions, each dependent on its role as a reducing agent:

- It is required for biosynthesis of collagen.
- For biosynthesis of steroids and peptide hormones, Indirectly, ascorbic acid plays important regulatory roles throughout the entire body due to its involvement in the synthesis of hormones, hormone-releasing factors, and neurotransmitters (Groff, *et al.*, 1995) and
- For prevention or reduction of the oxidation of biomolecules ,and protects the body from the harmful effects of free radicals and pollutants (Iqbal ,*et al*. ,2004).

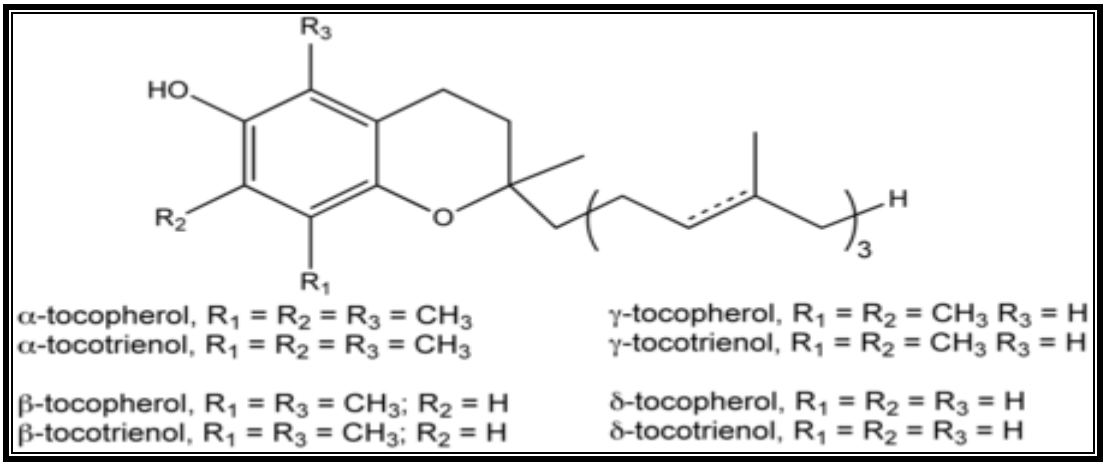
Reactive oxygen species oxidize (take electrons) from ascorbate to be changed to monodehydroascorbate and then dehydroascorbate. The ROS are reduced to water, while the oxidized forms of ascorbate are relatively stable and unreactive, and do not cause cellular damage (Stone, 1972).

The water-soluble properties of vitamin C allow for the quenching of free radicals before they reach the cellular membrane.

Tocopherol and GSH also rely on vitamin C for regeneration back to their active isoforms. The relationship between vitamin C and Glutathione is unique. Vitamin C reduces GSH back to the active form. Once reduced, Glutathione will regenerate vitamin C from its oxidized state. Vitamin C protects the DNA of the cells from the damage caused by free radicals and mutagens. It prevents harmful genetic alterations within cells (Gaby and Singh, 1991).

2.4.1.2.3 Vitamin E

Vitamin E is the collective name for a set of at least eight related tocopherols and tocotrienols compounds with similar biological antioxidants activity , particularly alpha-tocopherol , as shown in Figure (2.6),which are fat-soluble vitamins with antioxidant properties(Herrera and Barbas,2001).



Figure(2.6) Types of Vitamin E (Packer ,*et al*. ,2001)

2.4.1.2.3.1 Physiological Role of Vitamin E

The primary role of vitamin E within the body is to function as an antioxidant. Vitamin E is considered to be the major chain breaking antioxidant in membranes (Chitra and Devi, 2008). Within cells and organelles (e.g. mitochondria) vitamin E is the first line of defense against lipid peroxidation. Moreover, it plays a very important function in lending red blood cells flexibility as they make their way through the arterial network and helps prolong the life of erythrocytes, immune function, and has positive effects in the fertility (Lukaski, 2004). The tocopherol radical is at best useless for further lipid peroxidation termination, and at worst a pro-oxidant. It must be recycled to the reduced form by other antioxidants. Vitamin C regenerates Vitamin E and Vitamin C is ,in turn by regenerated glutathione (Rigotti,2007).

2.5 Oxidative stress in female reproduction

The presence of oxidant and antioxidant systems in various reproductive tissues has evoked great interest in the role of OS in human reproduction (Agarwal ,*et al*.,2004). The role of ROS and antioxidants in relation to female reproductive function has, in contrast, received relatively little attention (Guerin, *et al*., 2001).

Oxidative stress influences the entire reproductive span of women's life and even thereafter (i.e. menopause).

Free radicals appear to have a physiological role in female reproductive system in many different processes such as: oocyte maturation, fertilization, luteal regression, endometrial shedding and progesterone production by the corpus luteum (Sugino ,*et al*., 2004). Protection from ROS is afforded by scavengers present in both male and female reproductive tract fluids, as well as in seminal plasma (Uerin, *et al*.,2001 and Fujii, *et al*.,2005).

2.5.1 Free radicals, antioxidants, and reproductive processes in women

The production of a viable oocyte is modulated by a complex interaction of endocrine, paracrine and autocrine factors, leading to follicular maturation, granulosa cell maturation, ovulation and luteinization (Agarwal, *et al*., 2003). Elevated concentrations of ROS in these environments may have detrimental effects on the spermatozoa, oocytes, sperm oocyte interaction and embryos both in the Fallopian tube and the peritoneal cavity ;therefore oxidative stress modulates a host of reproductive pathologies affecting natural fertility in a woman’s life (Agarwal, *et al.,* 2009).Free radicals plays a role in the physiology of ovarian function (Agarwal ,*et al*.,2006). They may have a regulatory role in oocyte maturation ,folliculogenesis (as shown in Figure (2. 7)),ovarian

steroidogenesis and luteolysis (Agarwal,*et al* ,2005).There is a delicate balance between ROS and antioxidant enzymes in the ovarian tissues (Agarwal ,*et al*.,2005a). Vitamin C deficiency characteristically produces ovarian atrophy and extensive follicular atresia (Agarwal, et al.,2005). Glutathione has been identified as critical for oocyte maturation and formation of the male sperm pronucleus(PN) (Ruder, *et al*.,2008).

Glutathione in mature oocytes is thought to be a highly relevant biochemical marker for the viability of mammalian oocytes (Zuelke, *et al*., 2003; Luberda, 2005).Hence, Follicular ROS initiate apoptosis; whereas follicular Glutathione, in addition to FSH, protect against apoptosis in cultured pre-ovulatory rat follicles (Turton and Luderer, 2006).The secreted Glutathione would protect oocytes against excessively produced ROS that occurs during the ovulation, thus maintaining fertilization potency (Ikeda, *et al*., 2005 and Dalvit, *et al*.,2005).

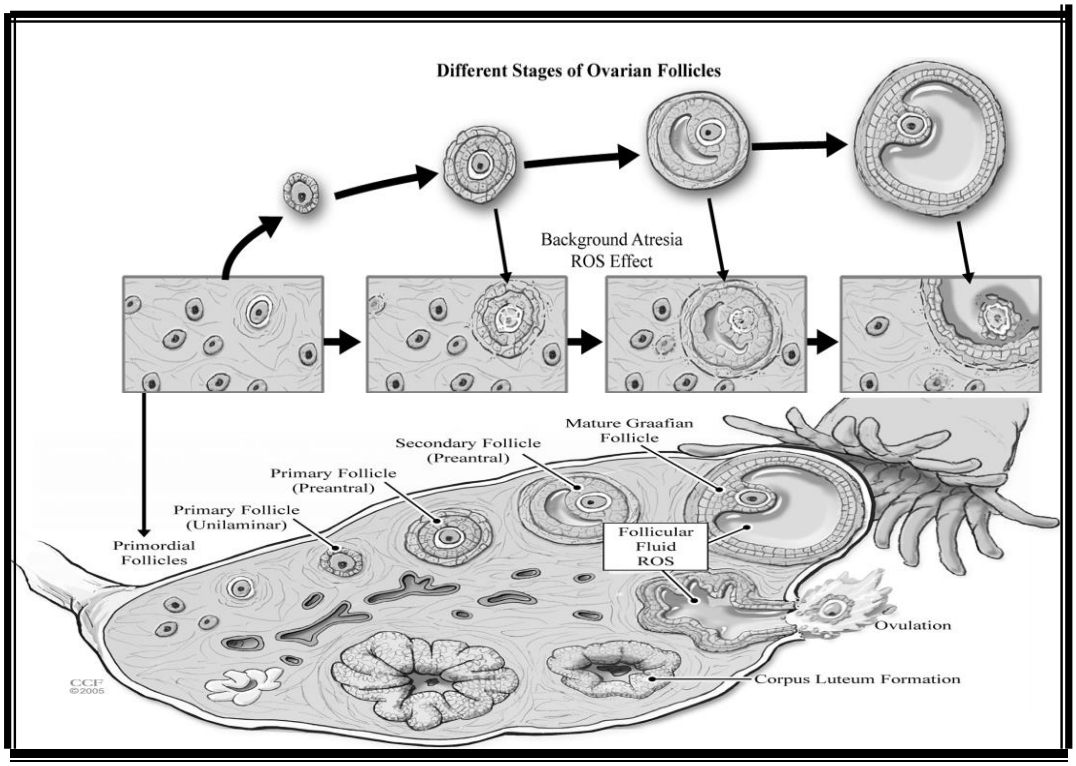


Figure (2. 7) Reactive oxygen species in folliculogenesis in women ovary (Agarwal, *et al*. ,2008).

Goto ,*et al*.(2002) observe that integrity of the antioxidant defenses within the different stages of oocyte development may contribute significantly to the overall quality of the oocytes. One consequence of an excess of ROS in the ovary may be plasma membrane damage of the oocytes. The significance of such damage for female fertility, however, is unknown.

Glutathione peroxidase may also maintain low levels of free radicals inside the follicle and thus play an important role in gametogenesis and fertilization(Agarwal, *et al*.,2005a). Glutathione is considered the major source of redox potential in the oocyte (Fujii,2005).Oocytes are also rich in glutathione reductase (Gardiner, *et al*. 1998 and Paszkowski, *et al*.,1995) .

Reactive oxygen species are produced during luteal regression (Behrman, *et al*., 2001). Because the corpus luteum produces much of the progesterone ; ROS are produced as a byproduct (Behrman ,*et al.*, 2001). The detoxification of the produced ROS by GSH in conjunction with antioxidative enzymes would be particularly important for the corpus luteum and surrounding cells (Tatemoto, *et al*.,2000).

Reactive oxygen species may act as important mediators in hormone signaling, ovarian steroidogenesis and germ cell function (Agarwal ,*et al*.,2006).

Oxidative stress may affect theca-interstitial cells by inducing their proliferation and growth. Higher doses of Oxidative stress inhibited the proliferation of the theca-interstitial cells while antioxidants stimulate the release of gonadotrophins from the adenohypophysis (Duleba ,*et al*.,2004).

Cells involved in steroidogenesis such as theca cells, granulosa lutein cells, and hilus cells show stronger oxidative enzyme activity (Scully and Cohen, 1964). Over-exposure of the ovary to H_2O_2 causes the

LH receptor to uncouple from adenyle cyclase, thereby impairing protein synthesis and cholesterol utilization by mitochondria (Behrman, *et al*., 2001).

Data suggest that vitamin C has defined functions in hormone secretion, gamete protection, and gonadal tissue remodeling (Franceschi, 1992).Steroidogenesis appears to be ascorbate-dependent(Tsuji, *et al*., 1989) and the reduced concentrations of antioxidants often coincide with poor fertilization success rates (Paszkowski, *et al*.,2002).

Reactive oxygen species therefore play a role in the formation of the corpus luteum and steroidogenesis. Imbalance in redox leading to luteal regression that results in lack of luteal support to pregnancy (Agarwal and Allamaneni ,2004).

Endogenous NO system exists in the fallopian tubes(Agarwal, *et al*., 2005). NO has a relaxing effect on smooth muscles and it has similar effects on tubular contractility. Deficiency of NO may lead to tubal motility dysfunction, resulting in retention of the ovum, delayed sperm transport and infertility. Increased NO levels in the fallopian tubes are cytotoxic to the invading microbes and also may be toxic to spermatozoa(Agarwal,*et al*., 2005a).

In male ,ROS cause infertility by two principal mechanisms:

- Reactive oxygen species damage the sperm membrane which, in turn, reduces the sperm's motility and ability to fuse with the oocyte.
- Damage sperm DNA, compromising the paternal genomic contribution to the embryo (Tremellen,2008).

The percentage of sperm with DNA damage is negatively correlated with the fertilization rate (Sun , *et al*.,1997). Oocytes can repair DNA damage to some extent, but when the damage is severe, embryo death and miscarriages can occur.

The inability of sperm to fuse with an oocyte appears to be due to the effects of ROS on the sperm membrane. As a result of lipid peroxidation process, spermatozoa are unable to initiate the necessary biochemical reactions associated with acrosome reaction, zona pellucida binding and oocyte penetration (Griveau and Lannou, 1997).

In addition, ROS brings about changes in the endometrium that prepare it for implantation (Agarwal1, *et al*., 2006). Nitric oxide functions as an important vasodilator, neurotransmitter, regulator of implantation (Guerin, *et al*.,2001) and may also contribute as an anti-platelet agent during implantation (Cameron and Campbell, 1998) .

2.6 Oxidative Stress and Female Infertility

The role of Oxidative Stress in female reproductive diseases and infertility is under intense investigations (Guerin, *et al*., 2001;Bedaiwy, *et al*., 2002 and Paszkowski, *et al*, 2002).Whenever there is imbalance in the levels of ROS and antioxidants, damage can occur to oocytes and embryos through various pathological mechanisms (Agarwal ,*et al*. ,2005).

Oxidative Stress has been reported to have an important role in the pathogenesis of female infertility (Agarwal and Allamaneni, 2004).

Some studies have suggested that ROS are involved in various causative factors of infertility, i.e. peritoneal factor, tubal factor, endometriosis and unexplained infertility. The scientific basis of unexplained infertility remains a challenge, and OS may have a role in its pathophysiology (Agarwal, *et al*.,2005 a).

Free radicals can affect the female fertility potential in number of ways which can contribute negatively to a number of reproductive processes including folliculogenesis, oocyte maturation, sperm DNA

damage, necrozoospermia, asthenospermia, endometriosis (Guerin, *et al*., 2001 and Behrman , *et al*., 2001). High levels of ROS play an important role in the etiology of male and female infertility .It has been proposed that the imbalance between antioxidants and ROS, favoring the latter, is responsible for increased OS levels that induce infertility(Savita,*et al*.,2009). Fissore ,*et al*.(2002) have found that OS is associated with maternal aging and postovulatory aging of the ova. The Role of oxidative stress in fertility is shown in Figure (2.8) :

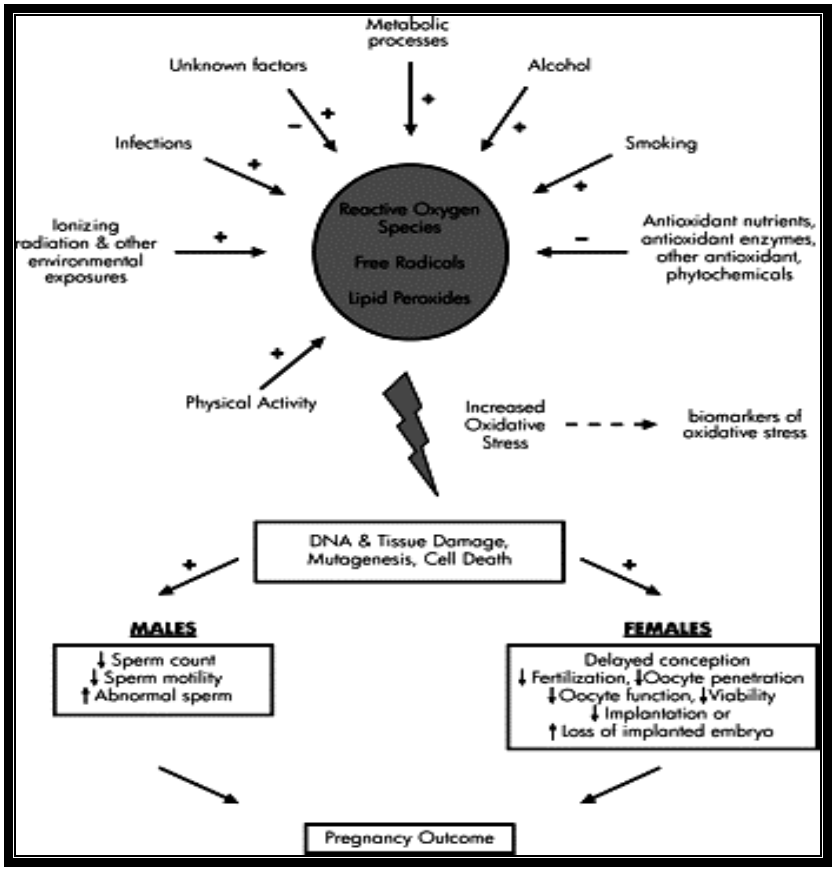


Figure (2.8) Role of oxidative stress in fertility (Ruber, *et al*.,2008)

2.6.1 Unexplained infertility

Unexplained infertility can result from increased ROS production in the peritoneal cavity. Samples of peritoneal fluid from women with unexplained infertility often are characterized by increased ROS levels;

furthermore, antioxidant levels in patients with unexplained infertility are significantly lower than those of healthy controls (Polak, *et al*.,2001). Elevated OS and ROS levels as well as decrease antioxidant concentrations in peritoneal fluid and serum have also been demonstrated in unexplained infertility, tubal infertility, and endometriosis patients (Agarwal, *et al*.,2008).

In human, Paszkowski ,*et al*. (1999) find decreased levels of glutathioine peroxidase in follicular fluid of women with unexplained infertility .

Bedaiwy , *et al*.(2002), find that ROS levels in the peritoneal fluid are significantly higher in the patients with idiopathic infertility compared with the fertile women.

Women with unexplained infertility have reduced concentrations of antioxidants and increased ROS-induced lipid peroxidation damage resulting in infertility (Agarwal, *et al*.,2005). Dong *et al*. (2001) have shown significantly high levels of nitric oxide in peritoneal fluid of patients with unexplained infertility and endometriosis and Pyari , *et al*.(2006) have shown significantly lowest levels of catalase enzyme in cases of unexplained infertility.

Agarwal ,*et al*. (2006) find that reduced levels of antioxidants and increased ROS-induced lipid peroxidation damage may have a role in pathophysiology of unexplained infertility.

Explicating the role of OS in unexplained infertility is important to design strategies to ameliorate the OS related adverse effects(Agarwal, *et al*.,2009).

2.7 The effect of some factors on free radicals levels

2.7.1 Body weight

The effects of body weight and weight change on OS have only recently been investigated. More studies on this topic are needed, since

both inadequate and excessive energy intakes have been associated with reduced fertility among women (Allaire, 2006).

Research has focused on the effects of energy intake on hormonal patterns and menstrual cycles , ovulatory dysfunction and later age at menarche have been associated with both low and high body mass index (BMI, calculated as kg/m^2) , energy intake and high levels of physical activity (De Souza and Williams, 2004).

2.7.2 Age

It has been suggested that the age-related decline in fertility is modulated by OS (de Bruin, *et al*., 2002). Moreover, there is an age related decline in the number and quality of follicles in females (Tarin , 1996 and Dumollard ,*et al*. ,2007).

Several studies have investigated if there is an age-associated increase in the generation of oxidants by mitochondria (Agarwal, *et al*.,2005a). Free radical activity of human follicular fluid increases with age (Wiener-Megnazi , *et al*., 2004) as does apoptosis(programmed cell death) of human granulosa and cumulus cells (Sadraie ,*et al*., 2000 ; Moffatt ,*et al*., 2002 and Takahashi, *et al*. 2003). Carbone ,*et al*.(2003) find that a reduction in the expression of glutathione and CAT activity is demonstrated in older women compared with young controls. Yeh, *et al*.(2005) show alterations in antioxidant defense with age. It is hypothesized that diminished antioxidant status may induce apoptosis during luteal regression and lead to decreased progesterone synthesis.

2.7.3 Smoking

Smoking is known to decrease fertility in women (Hakim, *et al*., 1998), likely through an increase in OS. A history of smoking is associated with high levels of oxidative stress (Saleh and Agarwal,2002).

The oxides of nitrogen (NO) in cigarette smoke damage macromolecules and deplete antioxidant. This is likely to contribute significantly to the pathology of smoking (Agarwal, *et al*. ,2005).

Dietary intakes of smokers; however, are different from non-smokers, confounding this relationship (Agarwal ,*et al*.,2005a). It is found that intrafollicular exposure to cigarette smoking metabolites was associated with a significant increase in follicular lipid peroxidation and decrease in the local antioxidative potential(Dumollard,*et al*.,2007).Smoking significantly reduced glutathione peroxidase concentration in the follicular fluid (Paszkowski , *et al*., 2002). Consequently, OS imbalance may be responsible for impaired folliculogenesis in female smokers (Paszkowski, *et al*.,2002 and Agarwal ,*et al*.,2006).

2.8 Overcoming OS in female infertility

Oxidative stress can be overcome by reducing generation of ROS or increasing the amounts of antioxidants available(Agarwal ,*et al*,2005).So prior to the treatment of female infertility, ROS levels should be assessed. By estimating ROS levels, it may be possible to identify the causes of infertility, especially in cases of idiopathic infertility (Agarwal, *et al*.,2008). It is important to identify the source of increased ROS generation(Agarwal, *et al*.,2003). Patients with history of smoking should be advised to stop smoking. In addition, Any exposure to drugs, toxic substances and radiation should be checked and patients should be advised to stop exposure to them.

Infections of the reproductive tract should be treated with appropriate antibiotics (Agarwal ,*et al*. ,2004).Initially, specific therapeutic options directed against the etiological cause of raised ROS should be tried(Agarwal ,*et al*. ,2004). After treating the primary cause, patients can be advised to take antioxidant supplementation.Antioxidants can be

started directly when a specific aetiology cannot be identified (unexplained infertility) (Agarwal ,*et al*. ,2008).Considerable interest has been generated in the use of antioxidants to overcome the adverse and pathological results of OS. Some studies that used nutritional supplements and antioxidants, like vitamin C supplementation to protect against ROS and OS. However, there is a lack of consensus on the type and dosage of antioxidants to be used (Agarwal ,*et al*.,2005). In vivo antioxidants may be helpful in smoker infertile women (Paszkowski ,*et al*.,2002). A study , impact of a nutritional supplement, containing vitamin E, iron, zinc and selenium, is examined. The patients receiving the supplement experienced a significant increase in ovulation rates and pregnancy rates compared with the placebo group (Westphal ,*et al*., 2004). Indirect evidence of the importance of OS and its control with antioxidant intake is provided by studies that have shown that preconceptional multivitamin supplementation may enhance fertility, perhaps by increasing menstrual cycle regularity (Czeizel, *et al*., 1994 and Dudas, *et al*., 1995) or via prevention of ovulatory disorders (Chavarro ,*et al*., 2007).In general, when supplemental vitamins C and E are given to older mice, the age-associated reduction in ovulation is partially prevented (Tarin, *et al*., 2002).There is sufficient evidence to hypothesize that diet, particularly its constituent antioxidants, and OS may influence the timing and maintenance of a viable pregnancy(Ruder, *et al.* ,2008).

Chapter Three

Materials and Methods

3.1 Materials

3.1.1 Patients and control groups

3.1.1.1 Control group

This group consists of thirty apparently healthy fertile women, with a mean age (30.133±8.011years), and range from 18 to 44 years were investigated to serve as a controlling group. None of them had clinical or laboratory evidence of diseases that would affect the parameters to be measured.

3.1.1.2 Patients group

Couples resident in Babylon with unexplained infertility of more than 12 months duration were identified from the Fertility Clinic database in Babylon maternity hospital and private clinic. The patients were seen between August 2008 and May 2009.

The following criteria have been used to establish the diagnosis of unexplained infertility: mid-luteal serum progesterone concentration >20 nmol/l, bilateral tubal patency demonstrated by laparoscopy or hysterosalpingogram and normal semen parameters (WHO,1999).

Sixty women had been studied, with a mean of age (29.866 ±7.195 years), and from 18 to 44 years ; 26 with primary unexplained infertility and 34 have secondary unexplained infertility. Those having male factor of infertility or female factors or any other an associated condition which could alter the level of free radicals like, hypertension, diabetes mellitus, heart disease, malignancy, and antioxidant therapy, had been excluded from the study. Each subject was involved to

detailed clinical history and physical examination. The infertile group had undergone baseline investigations of infertility.

3.1.2 Instruments and Equipments

Table 3.1 The apparatus used in this study:

Instrumental	Supplied Company
Spectrophotometer, Cecil 10ll (UV-visible)	Spectronic 303,Milton Roy(USA)
Spectrophotometer	Apel (Japan)
Vortex mixer	Janke and Kunkel (Germany)
Water Bath	Schutzart Din 40050-IP memert GMBH,Schwabach 20FRG(Germany)
Incubator	Fisher Scientific (USA)
Centrifuge	Sigma (Germany)
Glass slide	Sigma (Germany)
Casco's speculum	Sigma (Germany)
Micropipette 20,100 – 1000, 20 - 200 ,5-50.	Slamed(Germany)
Electronic balance	Sartorius(Germany)
Distillator	Bibby science(England)
Glass stirring	Standard (Germany)
Centrifuge tube	Mes(1 gm) Fisonsm(England)
Plane tube	Afma-Dispo-(Jordan)

3.1.3 Chemicals

Table 3.2 The Chemicals used in this study:

Chemicals	Origin
Ethylenediaminetetraacetic acid dihydrate (EDTA)·$2H_2O$	Gainlang chemical (England)
Methanol	Fluka (Switzerland)
5,5-Dithio bis(2-nitrobenzoic acid) (DTNB)	Fluka (Switzerland)
Metaphosphoric acid (m-HPO_3)	Fluka(Switzerland)
Sulfuric acid (H_2SO_4)	Fluka(Switzerland)
Thiourea	Fluka(Switzerland)
Copper sulfate	Fluka(Switzerland)
Potassium dihydrogen orthophosphate ($KH2PO_4$. $2H_2O$)	Chem-supply (south Australia)
H_2O_2 30%	BDH (England)
Thiobarbutric acid (TBA)	BDH(England)
Disodium hydrogen orthophosphate($Na_2HPO_4.2H_2O$)	BDH (England)
Tris hydroxymethylene Aminomethane	BDH(England)
Hydrochloric acid (HCL).	BDH (England)
N acetyl L cystiene	BDH (England)
Ascorbic acid standards	Biochemical
Glutathione Standard (GSH)	Merch (Germany)
Trichloroacetic acid(TCA)	Thomas Baker (India)
Total protein Kit	Biomegreb (Tunis)
Serum Albumin Kit	Biomegreb (Tunis)
Sodium hydroxide (NaOH)	Merch (Germany)
ferric chloride ($Fecl_3$)	BDH (England)
α -α dipyridyle	Merch (Germany)
Heptane	Merch (Germany)
Ethanol	Fluka(Switzerland)
Anti A, Anti B, Anti D	Sigma (Germany)
pH-paper	HANNA (Portugal)

3.2 Methods

3.2.1 Collection of blood and cervical secretion

3.2.1.1 Collection of blood and serum preparation

About 5 ml of venous blood for specific test of markers was collected by vein puncture using 5ml disposable syringes. Some blood drops are put on slide for blood group typing .The reminder blood was put in plain tubes and allowed to clot, then separated by centrifugation 5-10 minutes at 1000xg - 2000xg (Bioshop ,*et al*.,2000). The obtained sera was put then in another disposable tubes and labeled. The samples were transferred to the biochemical laboratory for analysis of MDA as a marker of free radicals, CAT as scavenging enzymes and vitamin C , vitamin E and GSH as non-enzymatic scavengers as well as for analysis of serum proteins level.

3.2.1.2 Collection of cervical secretion

About 0.5 ml of cervical secretion was taken by syringe from high cervix using cusco speculum, labeled and storage at – 20 C◦.
For biochemical tests, the mucus must be liquefied by mucolytic agent of N-acetyl L- cycteine at concentration 0.2 mg/ml which prepared by weight 0.2 mg of N-acetyl L- cycteine and complete to one milliliter with DW (White,2007 and Tirouvanziam, 2006).

3.2.2 Hematological test

3.2.2.1 Determination of ABO and Rh (D) blood groups

3.2.2.1.1 ABO blood groups

There are four main blood groups: A,B,AB and O according to the presence or absence of two antigens ;A and B (Dacie and Lewis,2005) as follows:

blood groups	Antigens in red blood cells	Antibodies in serum
A	A	Anti-B
B	B	Anti-A
AB	A+B	none
O	Neither A or B	Anti-A + Anti

3.2.2.1.1.1 Principle

Determination of ABO blood groups of red cells depends on testing them with known anti-A and anti-B sera. The presence or absence of agglutination indicating the group of each cell sample(Dacie and Lewis,2005).

3.2.2.1.1.2 Reagents

1-anti-A sera.

2-anti-B sera.

3.2.2.1.2 Rh (D) blood groups

Besides the A and B agglutinoges , human red cells carry many other antigen .The only one which needs specific consideration was that of Rh system.

3.2.2.1.2.1 Principle

Determination of Rh blood groups of red cells depends on testing them with known anti-D sera. The presence or absence of agglutination indicating the presence or absence of Rh antigen in each cell sample.

3.2.2.1.2.2 Reagents

anti-D sera

3.2.2.1.3 Procedure

The slide method includes the following steps:

- The microscope slide is divided into three areas: A,B,D.

- One drop of anti-A serum, anti-B serum and anti-D serum was placed in area A,B and D division respectively.
- One drop of blood was added into each division (A,B and D) next to the drop of test serum and mixed them with clean match stick.
- After 2 minutes, the area was observed for the evidence of agglutination of the red cells (read microscopically).

The blood group was found using the following table:

blood groups	Anti-A	Anti-B
A	+	-
B	-	+
AB	+	+
O	-	-

Rh was found using the following table:

Rh types	Anti-D
Rh+	+
Rh-	-

(+) indicate agglutination.

(-) indicate no agglutination.

3.2.3 Biochemical tests

3.2.3.1 Measurement of serum protein

3.2.3.1.1 Measurement of total serum protein

Many methods have been developed to measure the total protein content of biological fluids, such as, ultraviolet absorption, dye binding and electrophoresis (Burtis and Ashwood ,1999).

In the present study, total serum protein was measured by the use of commercially available kits (Biomegreb (Tunis)), based on biuret method (Bishop,et al.,2000; Varley ,et al.,1991).

3.2.3.1.1.1 Principle

Peptide bonds of protein react with cupric ion (Cu^{2+}) in alkaline solution to form a coloured product whose absorbance was measured spectrophotometrically at 540 nm (Bishop, *et al*.,2000).

3.2.3.1.1.2 Reagents

Reagent1

Alkaline reagent :potassium –sodium tartrate 12 mmol \l

Sodium hydroxide 0.6 mmol \l

Potassium iodide 30 mmol \l

Reagent2

Colouring Reagent cooprrsulphate 0.6 mol \l

Reagent3

Standard bovine albumin 5g\dl

3.2.3.1.1.3 Procedure

Three set of tubes were prepared as follows:

Reagent	Blank reagent	Standard	sample
Sample	-	-	20 µl
R3:standard	-	20 µl	-
Working reagent (R1 &R2)	1 ml	1 ml	1 ml

Tubes were mixed and incubated for 5 minutes at 20-25 C°, and the absorbency of standard, blank and sample was read by

spectrophotometer at absorbency (A) 546 nm (the colour of reaction was stable for 30 minutes).

3.2.3.1.1.4 Calculation

The total serum protein level was obtained from the following equation:

$$\text{Total protein} = \frac{A_{\textbf{sample}} - A_{\textbf{blank}}}{A_{\textbf{standard}} - A_{\textbf{blank}}} \times n$$

g\dl : n = 5 ; g\l : n=50

3.2.3.1.2 Measurement of serum albumin

The determination of albumin in serum or plasma was usually based on the binding behavior of the protein with anionic dyes such as, bromocresol green (BCG), in a manual or automated procedure. Electrophoretic and immunochemical methods were also available for the analysis of albumin (Burtis and Ashwood ,1999).

In the present study, serum albumin was measured by the use of commercially available kits (Biomegreb (Tunis)), based on bromocresol green method (Doumas ,*et al* .,1971).

3.2.3.1.2.1 Principle

The measurement of serum albumin was based on its quantitative binding to the indicator 3,3`, 5,5`-tetrabromo-m-cresol sulphonphthalin (bromocresol green). The absorption of bromocresol green -albumin complex was determined spectrophotometrically at 628nm. The binding of bromocresol green to albumin was not wholly specific; some dye binds also to α_1-and α_2-glubulins, although more slowly than with albumin (Burtis and Ashwood, 1999 and Varley ,*et al*. , 1991).

3.2.3.1.2.2 Reagents

Reagent1 : bromocresol green 0.14 g\l

Reagent2 : Succinate Buffer 75 mmol\l

Brij 7mol\l

Reagent3 : bovine albumin 50 g\l

724 Mm ol\l

3.2.3.1.2.3 Procedure

Three set of tubes are prepared as follows:

Reagent	Blank reagent	Standard	sample
sample	-	-	10 µL
R3:standard	-	10µL	-
Working reagent (R1 &R2)	2 ml	2 ml	2 ml

Tubes were mixed. The absorbency of standard, blank and sample was read by spectrophotometer at absorbency (A) 628 nm after 5 minutes (the colour of reaction is stable for 30 minutes).

3.2.3.1.2.4 Calculation

The serum albumin level is obtained from the following equation:

$$\text{serum albumin level} = \frac{A_{\textbf{sample}} - A_{\textbf{blank}}}{A_{\textbf{standard}} - A_{\textbf{blank}}} \times n$$

g\dl :n = 5 ; g\l : n=50

3.2.3.1.3 Measurement of serum globulin

Serum globulin was measured by subtraction of serum albumin from total serum protein (Bishop ,*et al*.,2000) as follows:

serum globulin = total serum protein – serum albumin

3.2.3.2 Determination of Malondialdehyde level in sera and cervical secretions

3.2.3.2.1 Principle

Measurement of MDA, a secondary product of lipid peroxidation, was based on the calorimetric reaction with thiobarbituric acid (TBA) to form pink color product ,which could be measured by spectrophotometer(Lunec ,1990). :

3.2.3.2.2 Preparation of reagents

1. 17.5% trichloroacetic acid (TCA).
   17.5 gm of trichloroacetic acid were dissolved in 100 ml DW.
2. 0.6% thiobarbituric acid (TBA).
   0.6gm thiobarbituric acid were dissolved in 100 ml DW.
3. 70% trichloroacetic acid (TCA).
   70 gm of trichloroacetic acid were dissolved in 100 ml DW**.**

3.2.3.2.3 Procedure

The level of MDA was determined by a modified procedure described by Burtis and Ashwood (1999) in which two groups of tubes were prepared as follows :

reagent	sample	blank
Serum or cervical secretion	150 µl	-
TCA (17.5)	1ml	1ml
TBA(0.6%)	1ml	1ml

All tubes were mixed by vortex . Then they were put in boiling water bath for 15 minutes and allowed to cool in room temperature.

After that, 1 ml of TCA 70% was added for each tube, and the tubes were let to stand at room temperature for 20 minutes .All tubes were

centrifuged at 3000 x g for 15 minutes and the supernatant was taken out to measure the absorbance of sample at 532nm by spectrophotometer.

3.2.3.2.4 Calculation

The concentration of MDA was obtained from following equation:

$$\text{The concentration of MDA} = \frac{\text{Absorbance at 532 nm}}{\mathbf{E_0 \times L}} \times \text{D}$$

Where:

L : light bath (1 cm)

E_0 : extinction coefficient = 1.56×10^5 M/cm

$$\text{D : dilution factor} = \frac{\text{1 ml vol. Used in ref.}}{0.15} = \mathbf{6.7}$$

3.2.3.3 Measurement of Catalase Activity in sera and cervical secretions

As mention , CAT was a heme enzyme that contains four ferriprotoporphyrin groups molecules and was present in peroxisomes. It catalyzes the divalent reduction of H_2O_2 (at high concentration) to water. Its activity could be induced by oxidative stresses (Cohen, *et al*.,1970**).**

3.2.3.3.1 Principle

Catalase activity was determined by the decrease in absorbance due to H_2O_2 conception (Abi,1974).

3.2.3.3.2 Preparation of reagent

1- Phosphate buffer solution (50mM, pH 7.0) which was prepared from:

a- $KH_2PO_4.2H_2O$(50mM) 6.81g/l.

b- $Na_2HPO_4.2H_2O$ 6.9 g/l.

Then 390 ml of solution (a) was mixed with 610 ml of solution (b) and adjust the pH to 7.0 with 1M HCl and store in sterile containers at 4°C.

2- Hydrogen peroxide

Dilute 0.34ml of 30% H_2O_2 with phosphate buffer to 100 ml prepare fresh prior to analysis.

3.2.3.3.3 Procedure

Fifty µl of serum was diluted with 5 ml of Phosphate buffer solution and pH 7.0 immediately before assay .

Two groups of tubes were prepared as follows:

Reagents	Sample	Blank
Phosphate buffer solution pH 7.0	-	1ml
Diluting serum	2ml	2ml
H_2O_2	1ml	-

The reaction was started by adding H_2O_2 . All tubes were then mixed immediately and initial absorbance after 15 seconds (t_1) and final absorbance after 30 seconds (t_2) were read at 240 nm by using spectrophotometer .

3.2.3.3.4 Calculation

The enzyme activity in unit (U) was expressed as the rate constant first order reaction (K) is used according to the following equation:

$$K = \frac{V_t}{V_s} \times \frac{2.3}{\Delta T} \times \log \frac{A_1}{A_2} \times 60$$

Where:

A_1= A_{240} at t=15s

A_2=A_{240} at 30s

V_t= was total assay volume.

V_s= was a sample volume in the assay mixture.

$\triangle T$= 15 sec.

3.2.3.4 Determination of glutathione level in sera and cervical secretions

Determination of GSH depends on the action of sulfhydryl groups(Boyer ,2000).

3.2.3.4.1 Principle

Sulfhydryl group of GSH could reduce disulfide chromogen of 5,5´-Dithiobis 2.nitrobenzoic acid (DTNB) and change it to an intensely yellow compound which could measure its absorbance directly by spectrophotometer at 412 nm and it was directly proportional to the GSH concentration (Burtis and Ashwood,1999).

3.2.3.4.2 Preparation of reagents

1. Trichloroacetic acid (TCA) 50% in which 50gm of TCA was dissolved in a final volume of 100 ml of DW. This acts as Precipitating solution.
2. Ethylenediamine tetracetic acid – di sodium (EDTA Na_2) (0.4 M) in which 148.9 gm of EDTA was dissolved in a final volume of 1 liter DW.
3. Tris-EDTA buffer (0.4M) pH 8.9.

   48.458 gm of Tris were dissolved in 800 ml of DW. Then 100ml of (0.4M) EDTA solution were added and brought to a final

volume of 1 litter with DW. The pH was adjusted by pH paper to 8.9 by addition of 1M HCl. (this solution was stable for at least 10 days)

4. DTNB reagent (0.01M)

   0.099 gm of DTNB was dissolved in absolute methanol, and brought to a final volume of 25 ml, (this reagent was stable for at least 13 weeks at 4C° .)

5. GSH standards

   Stock standard solution (0.001M) was prepared by dissolving 0.0307gm of GSH in a final volume of 100 ml of (0.4M) EDTA solution ,then dilutions were made in EDTA solution to 5,10,15,20, 30 and 50 µM (working standard solution prepared daily).

3.2.3.4.3 procedure

Glutathione was determined by using a modified procedure utilizing Elaman's reagent (DTNB) (Burtis and Ashwood,1999) which was summarized as follows:

Three set of tubes were prepared as follows :

Reagents	Sample µL	Blank µL	Standard µL
Serum or cervical secretion	200	-	-
Standard	-	-	100
DDW	800	1000	900
TCA	100	100	100

Then all tubes were mixed in vortex mixture intermittently for 10 -15 minutes, and centrifuged for 15 minutes at 3000 xg then pipettes into test tubes .The reagents were added for each tube as follows:

Reagents	Sample µL	Reagent Blank µL	Standard µL
Supernatant	400	400	400
Tris–EDTA buffer	800	800	800
DTNB reagent	20	20	20

Tubes were mixed in vortex mixture ,and the absorbency of standards and sample was read by spectrophotometer at absorbency (A) 412 nm within 1 minute of the addition of DTNB.

3.2.3.4.4 Calculation of serum glutathione concentration

The concentration of serum GSH was obtained from calibration curve in µM.

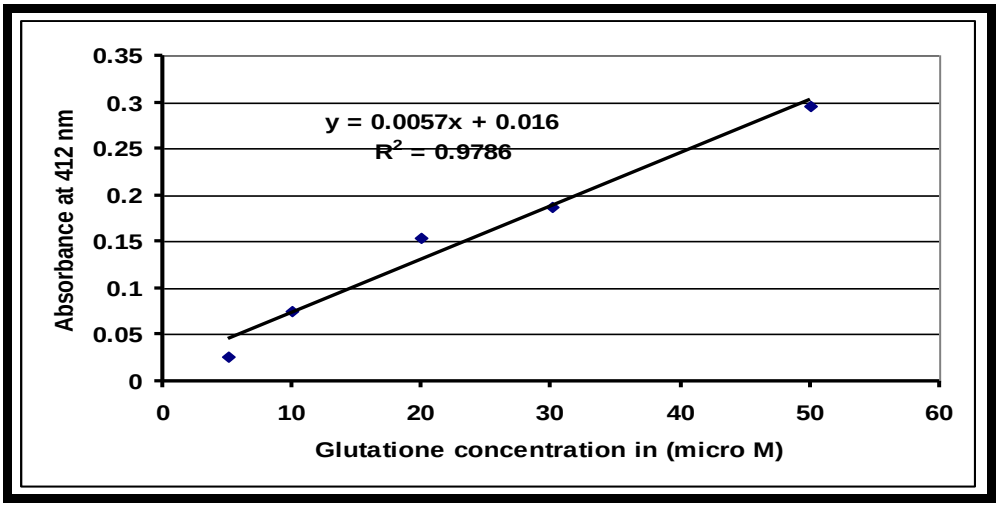


Figure (3.1) Standard curve for reduced glutathione (GSH)

3.2.3.5 Determination of total vitamin C in sera and cervical secretions

Numerous analytical methods were available for assessment of vitamin C nutritional status. In most methods, protein was precipitated with metaphosphoric or perchloric acid before analysis (Boyer ,2000).

3.2.3.5.1 Principle

According to 2,4-dinitrophenylhydrazine (DNPH) methods, vitamin C was oxidized by Cu^{+2} to dehydroascorbic acid and diketogulonic acid (Rifal, *et al*.,1999; Boyer,2000). When treated with 2,4-DNPH, the hydrazones derivatives were produced , which, in the presence of sulfuric acid, form an orange – red complex that absorbs at 520 nm .

3.2.3.5.2 Preparation of reagents

1. Metaphosphoric acid (m-HPO_3) (0.75 M)
   30 gm of m-HPO_3 were dissolved in a final volume of 500 ml of DW (Stable for 1 week).
2. Sulfuric acid (H_2SO_4) (4.5 M)
   Carefully 250 ml of concentrated H_2SO_4 was added to 500 ml of cold DW .When the solution cooled to room temperature , DW. is added to 1 litter, with mixing (stable for 2 years) .
3. Sulfuric acid (H_2SO_4) (12M)
   Carefully 650 ml of concentrated H_2SO_4 were added to 300 ml of cold DW. and brought to a final volume of 1 litter (stable for 2 years).
4. 2,4- DNPH reagent (0.01 M)
   10 gm of 2,4-DNPH were dissolved in 400 ml of (4.5 M) H_2SO_4 and bought to a final volume of 500 ml with (4.5 M) H_2SO_4, then refrigerated overnight, and filtered (stable for at least 1week at refrigerated temperature).
5. Thiourea (0.66 M)
   5 gm of thiourea were dissolved in a final volume of 100 ml of DW. (stable for 1 month at 4C°) .
6. Copper sulfate (0.027 M)

0.6 gm of anhydrous copper sulfate was dissolved in a final volume of 100 ml of DW (Stable for 1 year at room temperature).

7. 2,4- DNPH- thiourea- copper sulfate reagent(DTCS reagent)

   5 ml of the thiourea, 5 ml of the copper sulfate, and 100 ml of the 2,4-DNPH reagent were combined (Store at 4 C° for a one week).

8. Ascorbic acid standards

Stock standard solution (2.8 mM) was prepared by dissolving 50 mg ofin m-HPO_3 to 2.5, 5,10,15 and 20 mg/L (0.014, 0.028,0.055,0.083 and 0.11mM) respectively. These were the working standards (All working standards should be prepared daily).

3.2.3.5.3 Procedure

The procedure for the determination of vitamin C in serum and cervical secretion by 2,4-DNPH methodwas summarized as follows:

Duplicates of each standards and sample test tubes were prepared then pipettes into test tubes.

Reagent	Sample (µL)	Reagent	Blank (µ L)	Standard (µL)
M-HPO_3	800	-	-	-
Serum or cervical secretion	200	-	-	-

Tubes were mixed in vortex mixture, then centrifuged at 2500xg for 10 minutes.

Reagent	Sample (µL)	Reagent Blank (µ L)	Standard (µL)
Supernatant	600	-	-
Standard	-	-	600
m-HPO_3	-	600	-
DTCS regent	200	200	200

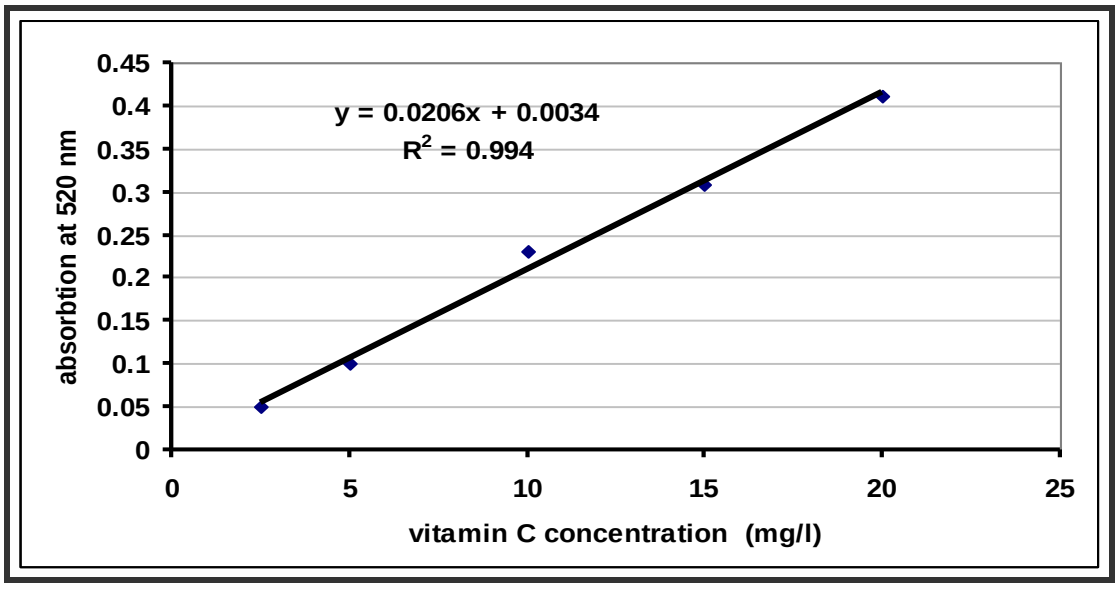
Tubes were capped and mixed in vortex mixture, then incubated in a water bath at 37C° for 3 hours.The tubes were removed from the water both and chilled for 10 minutes in an ice bath, with slow mixing.

Reagent	Sample (µL)	Reagent Blank (µ L)	Standard (µL)
Cold H_2SO_4	1000	1000	1000

Tubes were mixed in vortex mixture and returned immediately to the ice bath. The spectrophotometer was adjusted with blank to read zero absorbency (A) at 520 nm, and the absorbance of standards and sample was read.

3.2.3.5.4 Calculation of total vitamin C

The concentration of the samples were obtained from the calibration curve (Figure 2.5) and multiplied by 5 (to correct for dilution of the samples by m- HPO_3) to give the concentration of vitamin C in mg per litter :



Figure(3.2) Standard curve for ascorbic acid.

3.2.3.6 Determination of Vitamin E in sera and cervical secretions

3.2.3.6.1 Principle

After the proteins in the cervical secretion or serum are precipitated by an equal volume of absolute ethanol, the whole mixture was subjected to extraction by an equal volume of n-heptane .

The α -α dipynidyl was added to an aliquot of the upper layer to estimate the principal interfering substance, B-carotene, at 460 nm. At this time, the ferric chloride (FeCl3) reagent was added to the system to produce the colour which was measured at 510 nm (Hashim and Schttringer**,**1966).

3.2.3.6.2 Preparation of reagents

1. α -α dipyridyle solution :

0.12 gm of α -α dipyridyle were dissolved in a final volume of 100 ml of absolute ethanol.

2. ferric chloride ($FeCl_3$) :

1 gm of ferric chloride ($FeCl_3$) were dissolved in a final volume of 100 ml of DW (put it in brown bottle).

3.2.3.6.3 Procedure

Vitamin E was determined by using a Hashim and Schuttringe procedure (Hashim and Schuttringe,1966) which was summarized as follows:

One tube was prepared as follows :

Reagents	Sample μL
Serum or cervical secretion	500
Absolute ethanol	500
Heptane	1000

The tube was mixed after that in vortex mixture intermittently for 10 minutes, and centrifuged for 5 minutes at 1000 xg. Then the outer most

heptane layer (Supernatant) was drawn by pipette into test tube. Two set of tubes were then prepared as follows:

Reagents	Sample μL	Blank μL
Supernatant	800	_
Heptane	_	800
α -α dipyridyle	600	600

Tubes were mixed and the absorbency of standards and sample was read by spectrophotometer at absorbency (A) 460 nm.

Then the contents of each tube was returned back to its tube and ferric chloride ($FeCl_3$) 0.2 ml was added to each tube. After 45 minutes, the absorbency of standards and sample was read by spectrophotometer at absorbency (A) 510 nm.

3.2.3.6.4 Calculation of Vitamin E level

The concentration of Vitamin E level was obtained from the following equation:

$$\text{Vitamin E in mg/dl} = \frac{\text{Log T}_{510} - 1.36 - (0.32 \times \text{Log T}_{460})}{-0.46082}$$

3.3 Statistical Analysis

SPSS program was used in this study.All values were expressed as mean ± standard deviation (SD). Independent t-test was used to estimate differences between groups. The differences were considered significant when the probability (P) was less than 0.05($P< 0.05$)(Daniel,1999).

Chapter Four

The Results

4.1 History and physical examination

4.1.1 Patient inhabited areas

The percentage of patients with unexplained infertility lived in urban areas more than rural area as shown in Figure (4.1):

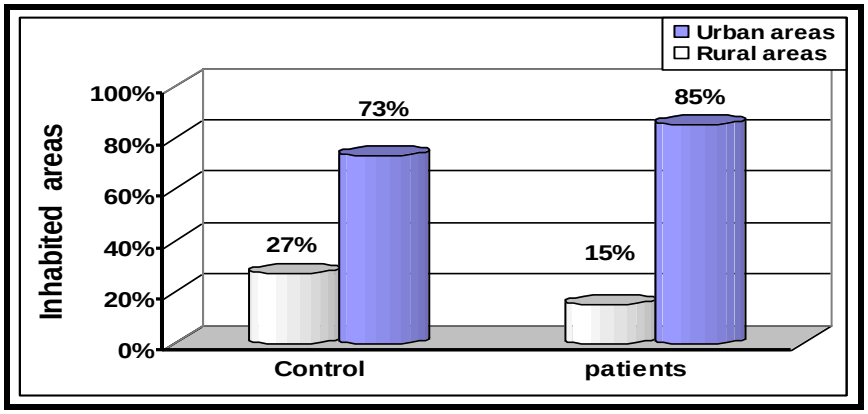


Figure (4.1) The percentage of inhabited areas in patient with unexplained infertility and fertile control(p>0.05).

4.1.2 Menstrual phase duration

The menstrual phase duration shows insignificant difference (p> 0.05) between women with unexplained infertility and fertile group as shown in Figure (4.2):

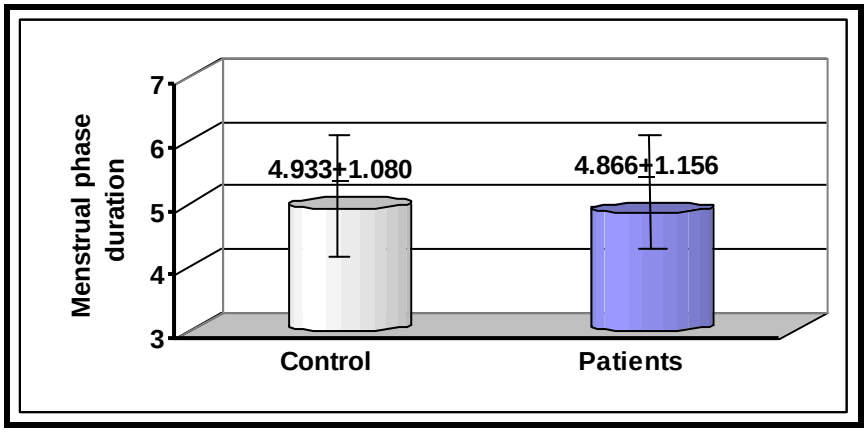


Figure (4 .2) The menstrual phase duration (days) in women with unexplained infertility and fertile control(p>0.05).

4.1.3 Onset of menarche

The onset of menarche shows insignificant differences(p> 0.05) between women with unexplained infertility and fertile group as shown in Figure (4.3):

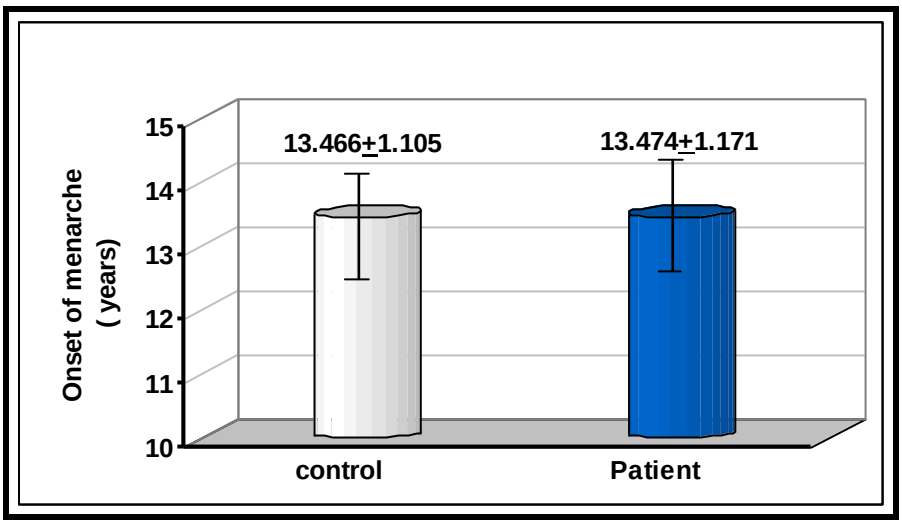


Figure (4. 3) The onset of menarche (years) in women with unexplained infertility and fertile control(p>0.05).

4.1.4 Body mass index(BMI)

The Body mass index shows insignificant differences(p> 0.05) between women with unexplained infertility and fertile group as shown in figure (4.4):

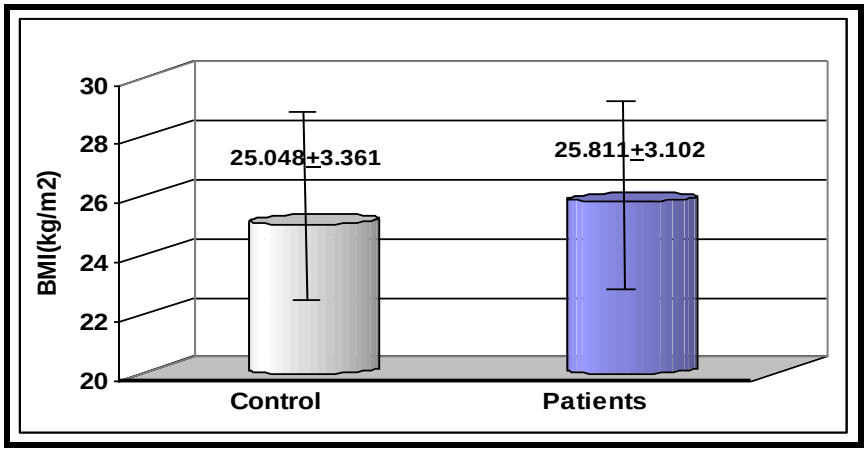


Figure (4.4) The BMI (kg/m^2) in women with unexplained infertility and fertile control(p>0.05).

4.2 Hematological test

4.2.1 ABO blood group system

The result of ABO erythrocyte surface antigen and their distribution among unexplained infertile women show that blood group A is present to be more distributed (40%)than other types of ABO blood group system when compared with fertile group who have mostly blood group B(36%) as shown in Figure(4.5):

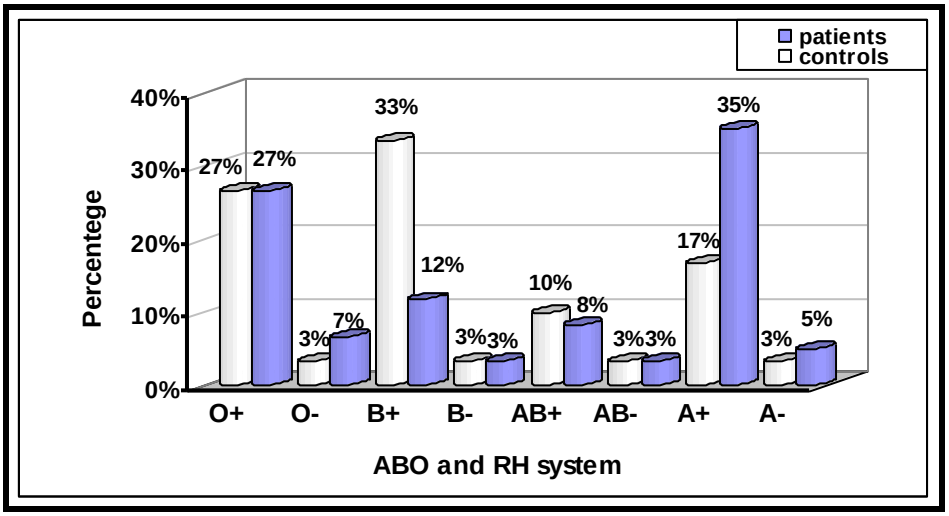


Figure (4. 5) The ABO blood group system percentage in women with unexplained infertility and fertile control.

4.3 Biochemical tests

4.3.1 Measurement of serum proteins

The total serum protein ,serum albumin and serum globulin shows insignificant differences($p > 0.05$) between women with unexplained infertility and fertile group as shown in Figure (4.6):

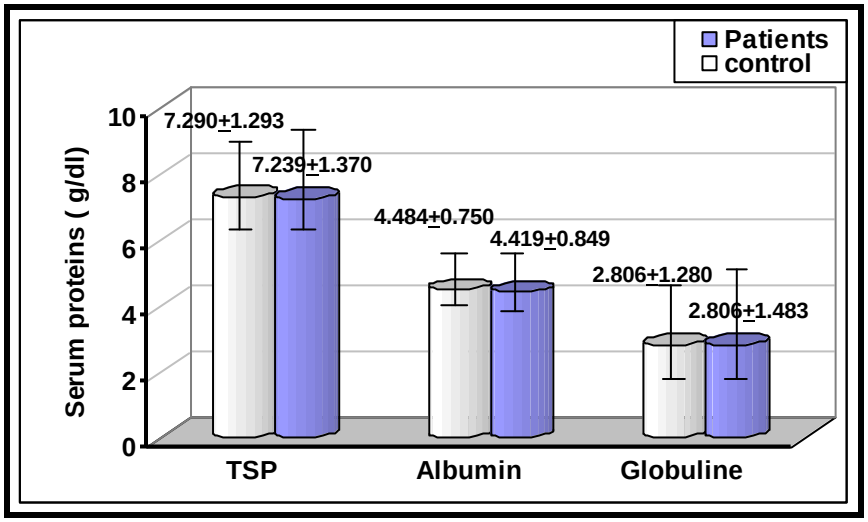


Figure (4.6) The total serum protein ,serum albumin and serum globulin (mean ±S.D g/dl) in women with unexplained infertility and fertile control (p>0.05)

4.3.2 Measurement of malondialdehyde levels

The total MDA levels in sera and cervical secretions of infertile women with unexplained infertility shows a significant increase($p<0.001$) when compared with fertile control as shown in Table (4.1):

Table (4.1)The total malondialdehyde levels in sera and cervical secretions in µM in infertile women with unexplained infertility and fertile Controls

Sample	Control Mean ±SD µM	Patients Mean ±SD µM	P value
Serum MDA	1.524 ± 0.518	3.105 ± 1.441	P<0.001
C.mucus MDA	3.201 ± 1.028	6.729 ± 3.055	P<0.001

4.3.2.1 Malondialdehyde levels and types of infertility

Malondialdehyde levels in sera and cervical secretions of infertile women with unexplained infertility show insignificant differences($p> 0.05$) between primary and secondary infertilityas shown in Figure (4.7):

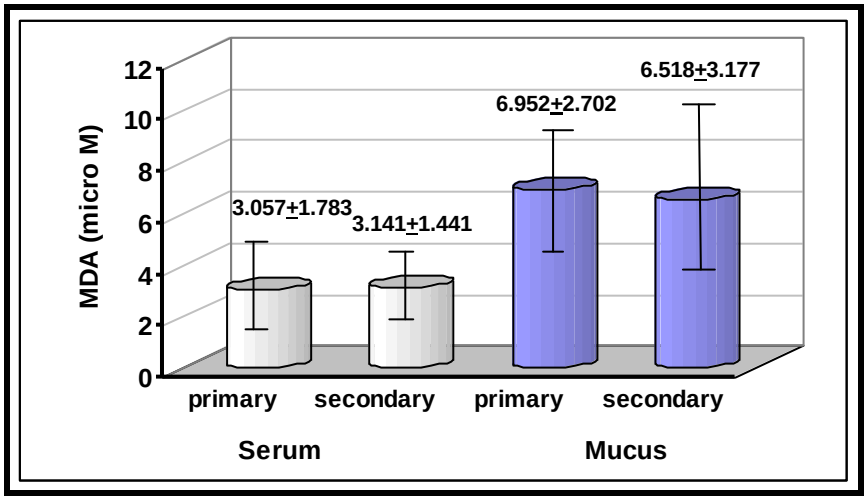


Figure (4.7)The total malondialdehyde **levels in sera and cervical secretions(mean ±S.D** μM**) in primary and secondary infertile women with unexplained infertility(p>0.05).**

4.3.2.2 Malondialdehyde levels and smoking

The total MDA levels in sera and cervical secretions show insignificant differences (p> 0.05) between passive and not smoker in infertile women with unexplained infertility and fertile control as shown in Figure(4.8)and (4.9):

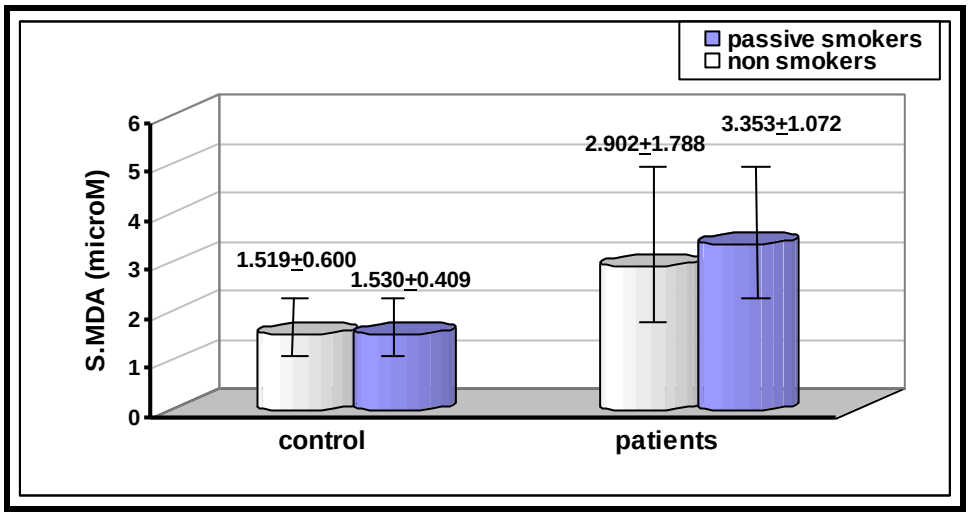


Figure (4.8) The total malondialdehyde levels in sera(mean ±S.D μM**) in passive and non smoker of infertile women with unexplained infertility and fertile control(p>0.05).**

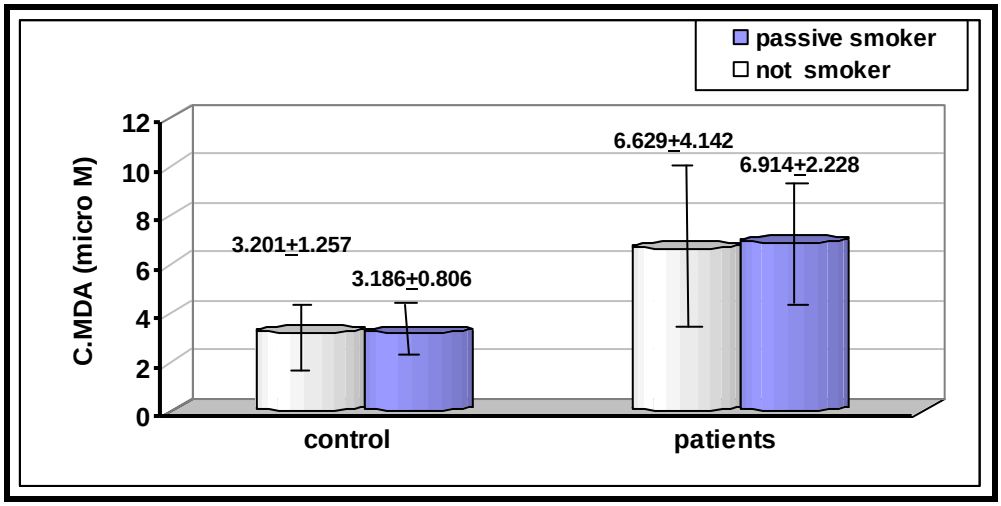


Figure (4 .9) The total malondialdehyde levels in cervical secretions (mean ±S.D μM) in passive and nonsmoker of infertile women with unexplained infertility and fertile control(p>0.05)

4.3.2.3 The Relationship between age and Malondialdehyde

The total MDA levels in sera and cervical secretions of infertile women with unexplained infertility shows a significant positive correlation with age increment when compared with fertile control ($p<0.05$) as shown in Figure (4.10) and (4.11):

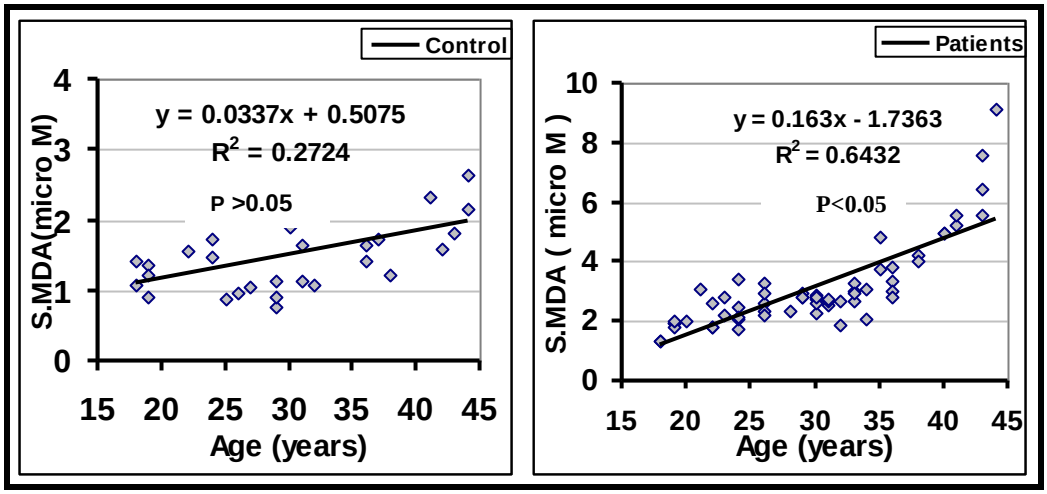


Figure (4.10) Relationship between age in years and serum malondialdehyde levels(μ M)in patients with unexplained infertility and fertile control

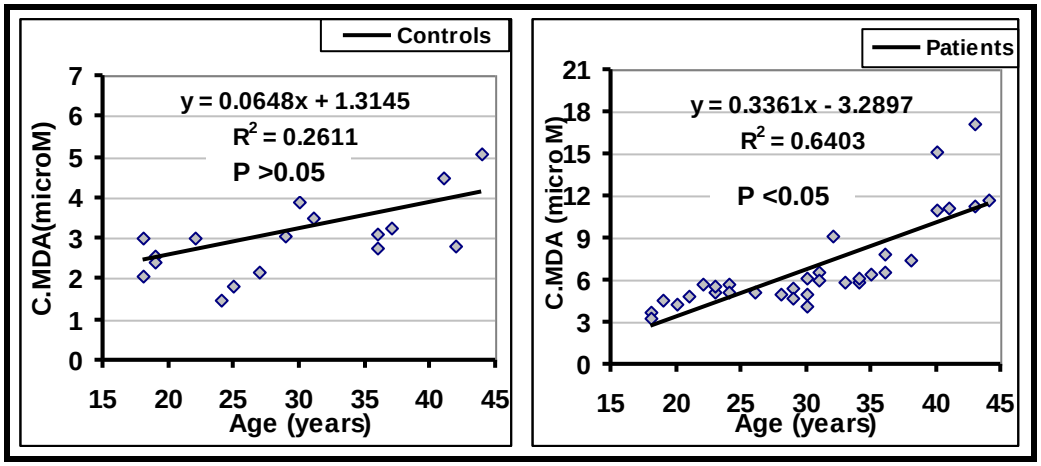


Figure (4 .11)The correlation between age in years and malondialdehyde levels in cervical secretions (µ M)in patients with unexplained infertility and fertile control.

4.3.2.4The Relationship between body mass index and malondialdehyde level

The total malondialdehyde levels in sera and cervical secretions of infertile women with unexplained infertility shows insignificant positive correlation with body mass index when compared with fertile control (p>0.05)as shown in Figure (4.12) and (4.13):

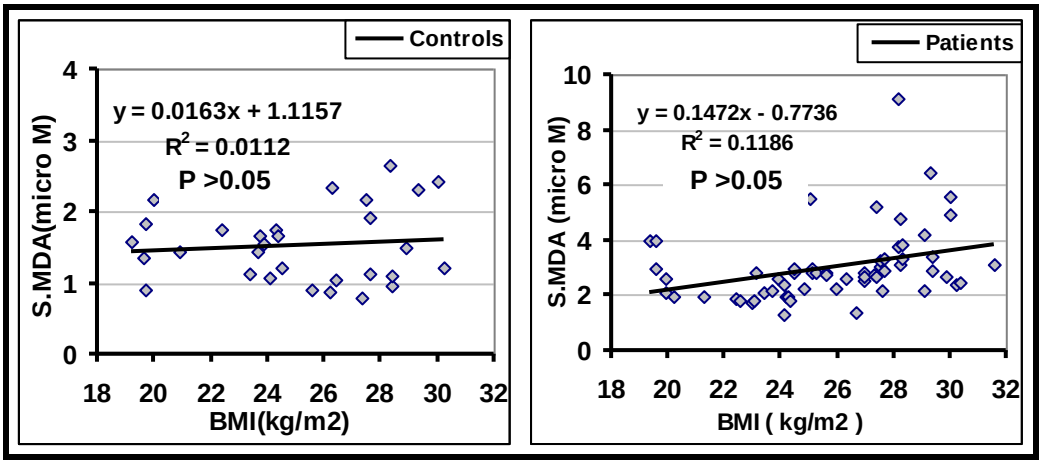


Figure (4. 12) Relationship between BMI (kg/m^2)and serum malondialdehyde levels(µ M)in patients with unexplained infertility and fertile control.

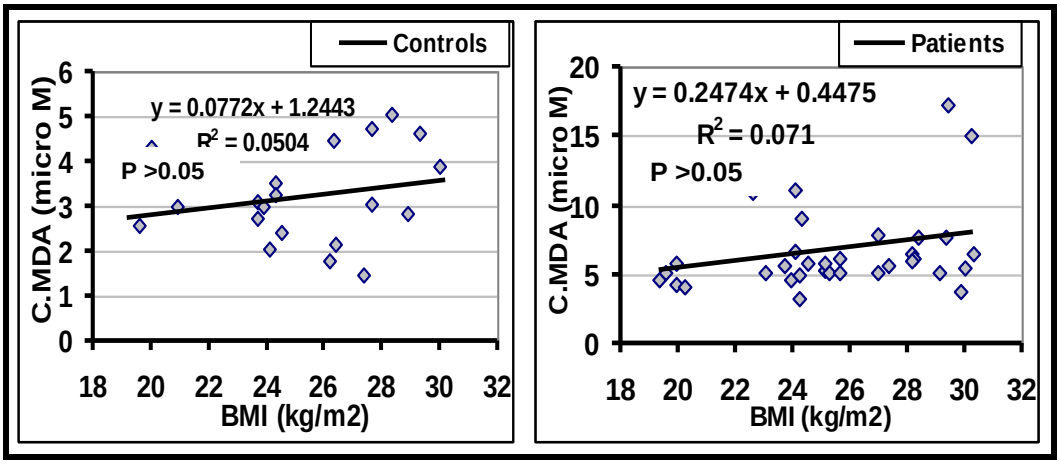


Figure (4. 13) Relationship between BMI (kg/m^2) and malondialdehyde levels in cervical secretions (µ M)in patients with unexplained infertility and fertile control.

4.3.2.5 The Relationship between duration of infertility and malondialdehyde level

The total malondialdehyde levels in sera and cervical secretions of infertile women with unexplained infertility shows insignificant positive correlation (p>0.05) with duration of infertility as shown in Figure (4.14):

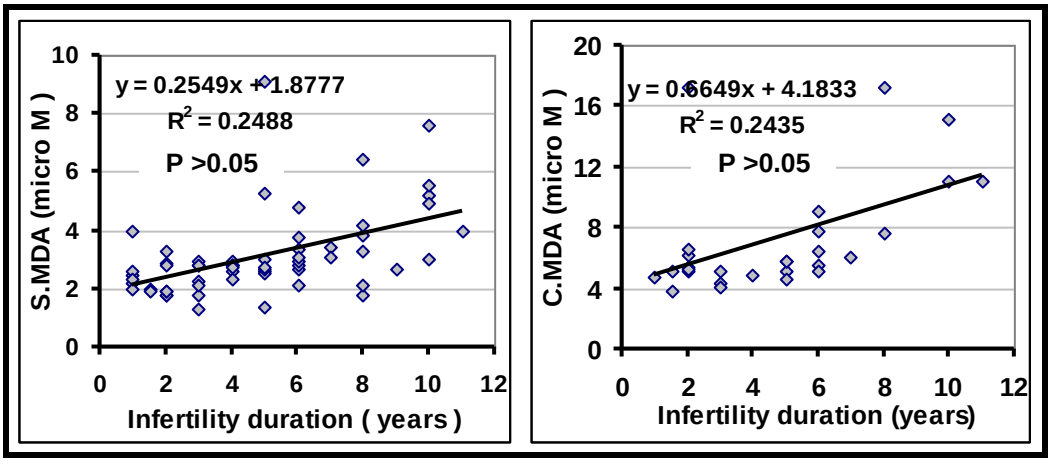


Figure (4.14)Relationship between duration of infertility in years and malondialdehyde levels in sera and cervical secretions(µM) in patients with unexplained infertility.

4.3.3 Measurement of catalase enzyme activity

The catalase levels in sera and cervical secretions of infertile women with unexplained infertility shows a significant decrease ($p< 0.05$) when compared with fertile control as shown in Table (3.2):

Table (4.2)Catalase levels in sera and cervical secretions in K/ml of infertile women with unexplained infertility and Control

Sample	Control Mean ±SD **K/ml**	Patients Mean ±SD **K/ml**	P value
Serum catalase	**0.479 ± 0.342**	**0.303 ± 0.286**	P<0.05
C.mucus catalase	**0.508 ± 0. 301**	**0.331 ± 0.302**	P<0.05

4.3.3.1 Catalase levels and types of infertility

The catalase levels in sera and cervical secretions of infertile women with unexplained infertility shows insignificant differences between primary and secondary infertility ($p>0.05$) as shown in Figure (4.15):

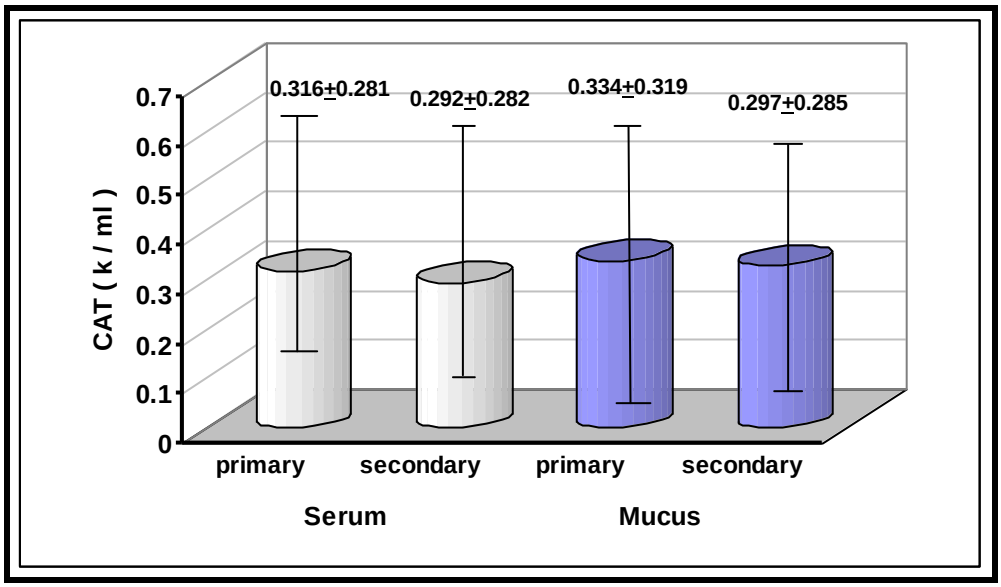


Figure (4.15) The catalase levels(mean ±SD k/ml) in sera and cervical secretions in primary and secondary infertile women with unexplained infertility(p>0.05).

4.3.3.2 Catalase levels and smoking

The Catalase levels in sera and cervical secretions show insignificant differences($p > 0.05$) between passive and non smoker in infertile women with unexplained infertility and fertile control as shown in Figure (4.16) and (4.17):

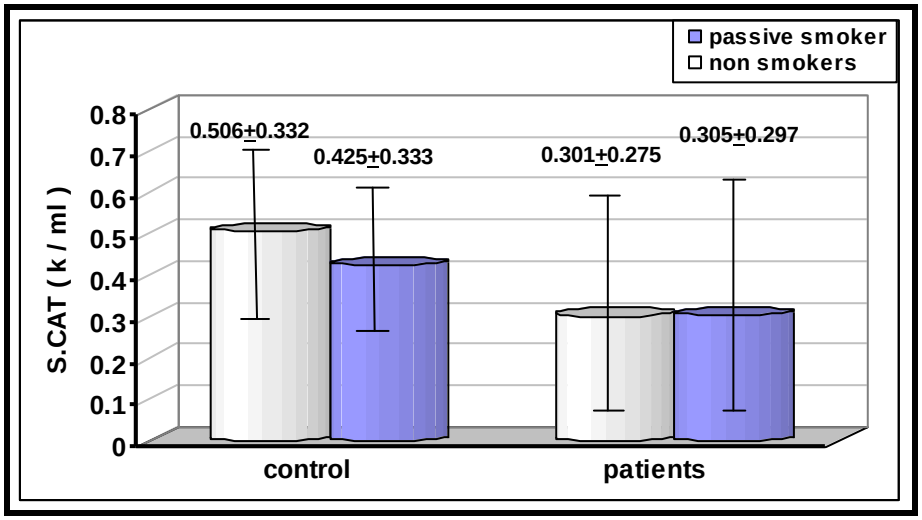


Figure (4.16) The serum catalase levels(mean ±SD k/ml) in passive and non smoker in infertile women with unexplained infertility and fertile control($p > 0.05$).

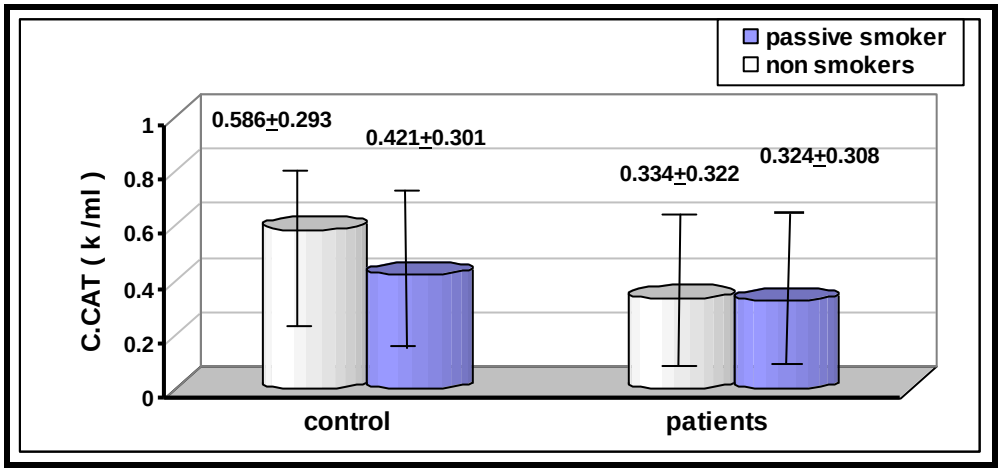


Figure (4.17) The catalase levels(mean ±SD k/ml) in cervical secretions in passive and non smoker in infertile women with unexplained infertility and fertile control ($p > 0.05$).

4.3.3.3 The Relationship between age and catalase enzyme

The catalase level in sera and cervical secretions of infertile women with unexplained infertility shows a insignificant negative Correlation ($p>0.05$) with age increment when compared with fertile controls as shown in Figure (4.18) and (4.19):

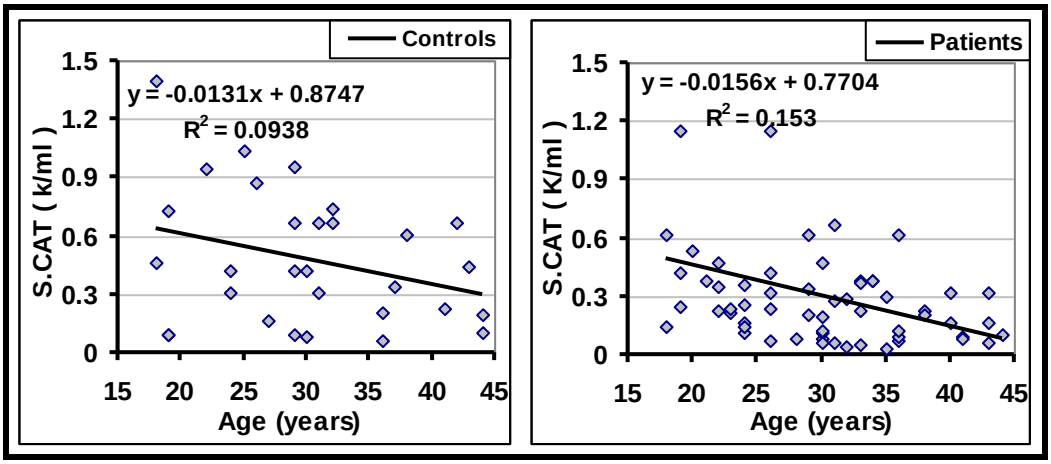


Figure (4 .18) Relationship between age(years) and serum CAT levels (k/ml) in patients with unexplained infertility and fertile control.

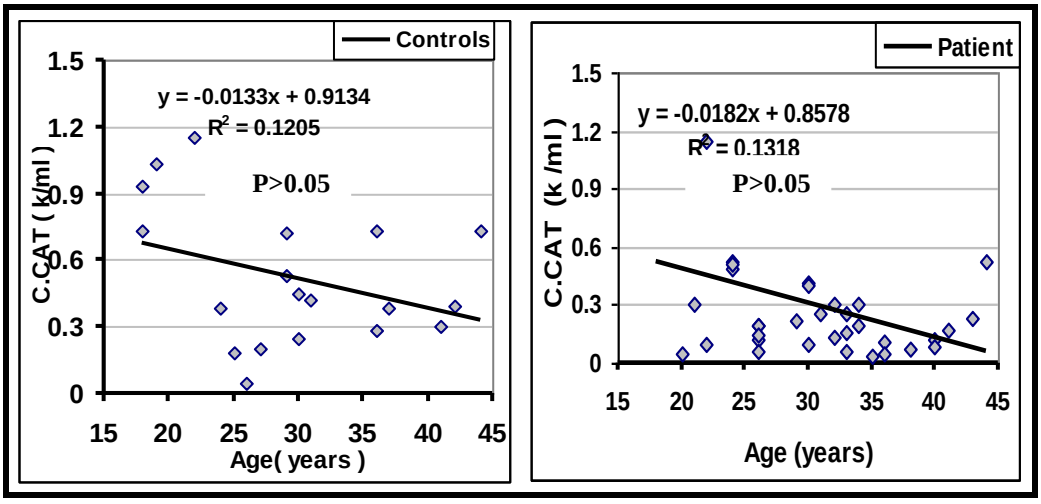


Figure (4.19) Relationship between age (years) and catalase levels in cervical secretions (k/ml) in patients with unexplained infertility and fertile control.

4.3.3.4 The Relationship between body mass index and catalase enzyme

The catalase levels in sera and cervical secretions of infertile women with unexplained infertility show insignificant negative correlation ($p>0.05$) with body mass index when compared with fertile control as shown in Figure (4.20) and (4.21):

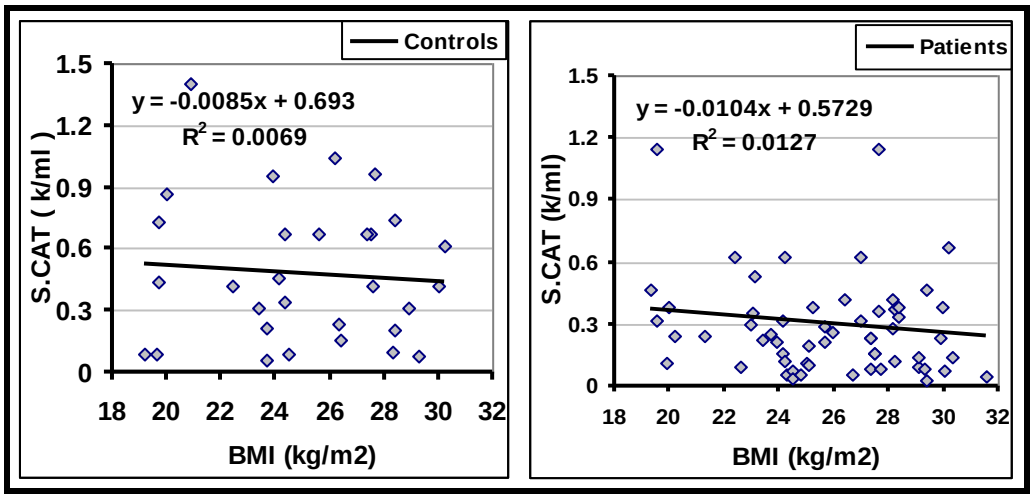


Figure (4 .20) Relationship between BMI (kg/m^2) and serum catalase levels (k/ml) in patients with unexplained infertility and fertile control.

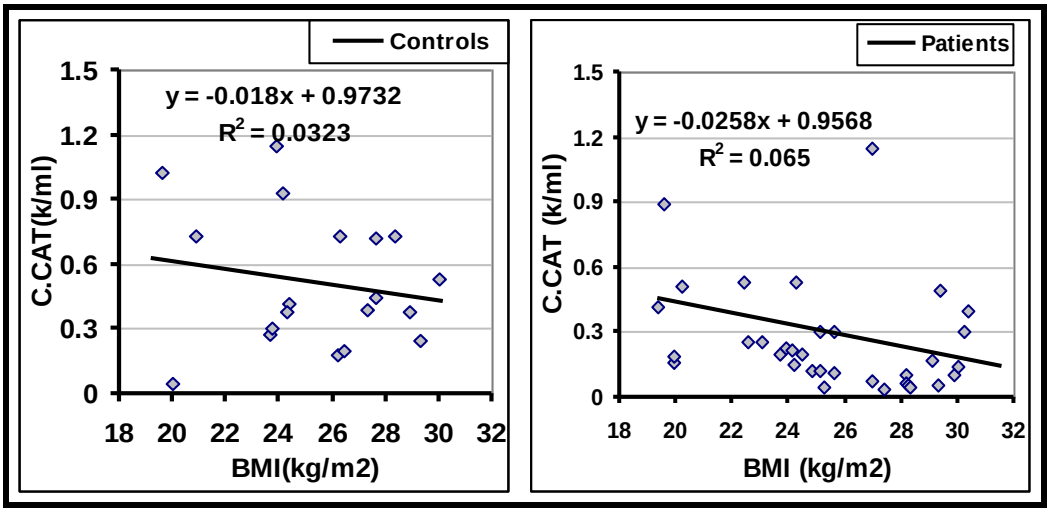


Figure (4. 21)The correlation between (kg/m^2) and catalase levels (k/ml)in cervical secretion in patients with unexplained infertility and fertile control.

4.3.3.5 The Relationship between duration of infertility and catalase enzyme levels

The catalase levels in sera and cervical secretions of infertile women with unexplained infertility show insignificant negative correlation (p> 0.05) with duration of infertility as shown in Figure (4.22):

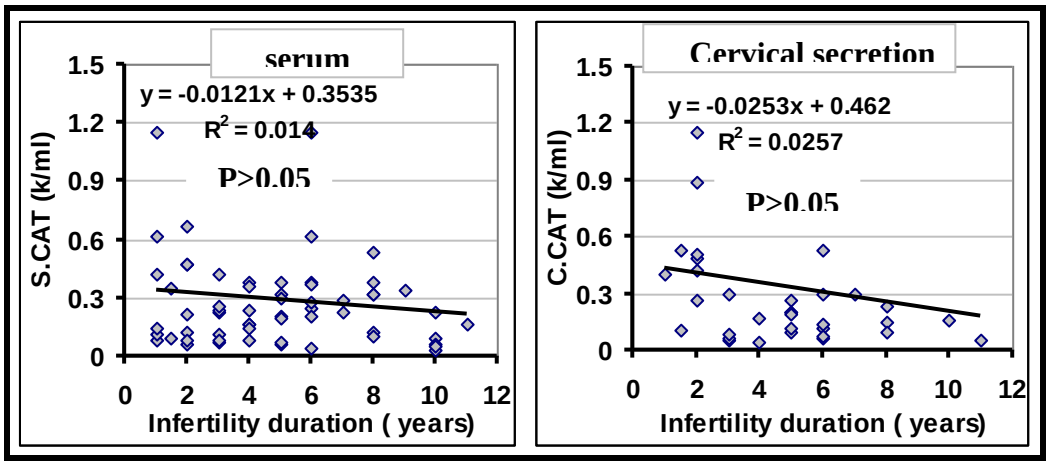


Figure (4. 22) Relationship between duration of infertility(years) and catalase levels (k/ml) in patients with unexplained infertility and fertile control.

4.3.4 Measurement of glutathione levels

The glutathione levels in sera and cervical secretions of infertile women with unexplained infertility show a significant decrease (p< 0.001,P<0.05) respectively when compared with fertile controls as shown in Table (3.3):

Table (4.3) Glutathione levels in sera and cervical secretions in µM of infertile women with unexplained infertility and fertile control

Sample	Control Mean ±SD µM	Patients Mean ±SD µM	P value
Serum glutathione	27.384 ±13.547	17.977 ± 8.506	P<0.05
C.mucus glutathione	65.327± 18.217	51.602 ± 27.335	P<0.001

4.3.4.1 Glutathione levels and types of infertility

Glutathione levels in sera and cervical secretions of infertile women with unexplained infertility show insignificant differences (p> 0.05) between primary and secondary infertility as shown in Figure(4.23):

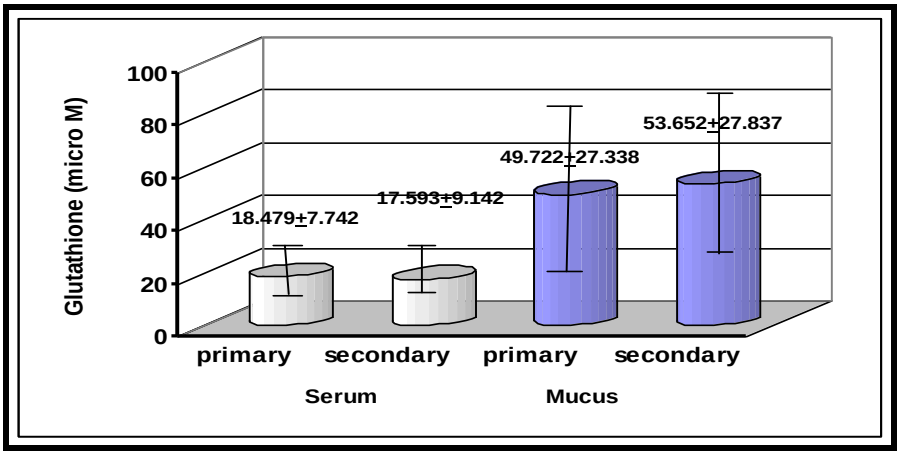


Figure (4.23)The Glutathione levels(mean ±S.D µM) in sera and cervical secretions in primary and secondary infertile women with unexplained infertility (p>0.05).

4.3.4.2 Glutathione levels and smoking

Glutathione levels in sera and cervical secretions show insignificant differences (p> 0.05)between passive and non smoker in infertile women with unexplained infertility and fertile control as shown in Figure(4.24)and(4.25):

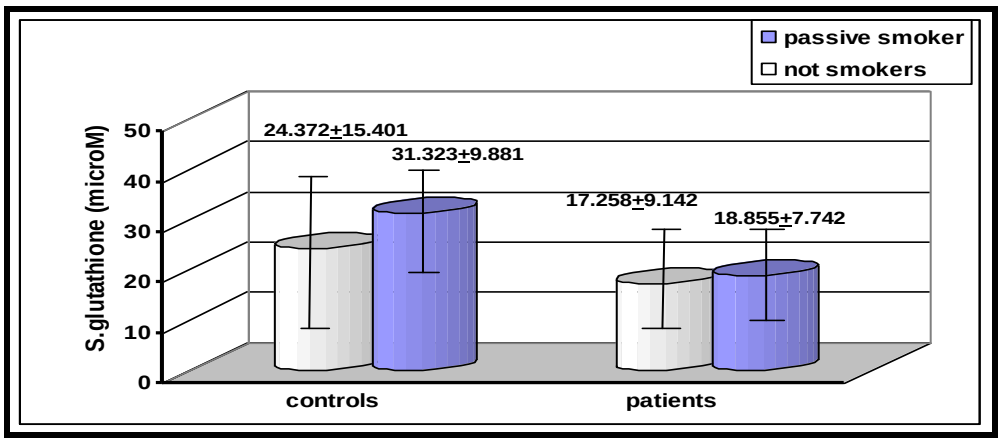


Figure (4. 24)The serum glutathione levels (mean ±S.D µM) in passive and non smoker in infertile women with unexplained infertility and fertile control(p>0.05).

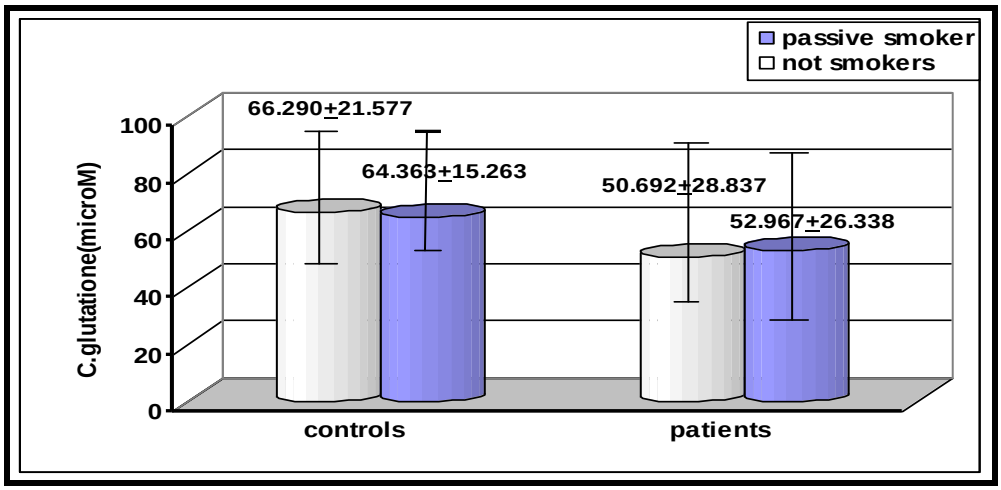


Figure (4 .25) The GSH levels in cervical secretions (mean ±S.D µM) in passive and non smoker in infertile women with unexplained infertility and fertile control(p>0.05).

4.3.4.3 The Relationship between age and glutathione levels

The glutathione levels in sera and cervical secretions of infertile women with unexplained infertility show a significant negative correlation with age increment when compared with fertile control ($p < 0.05$) as shown in Figure (4.26) and (4.27):

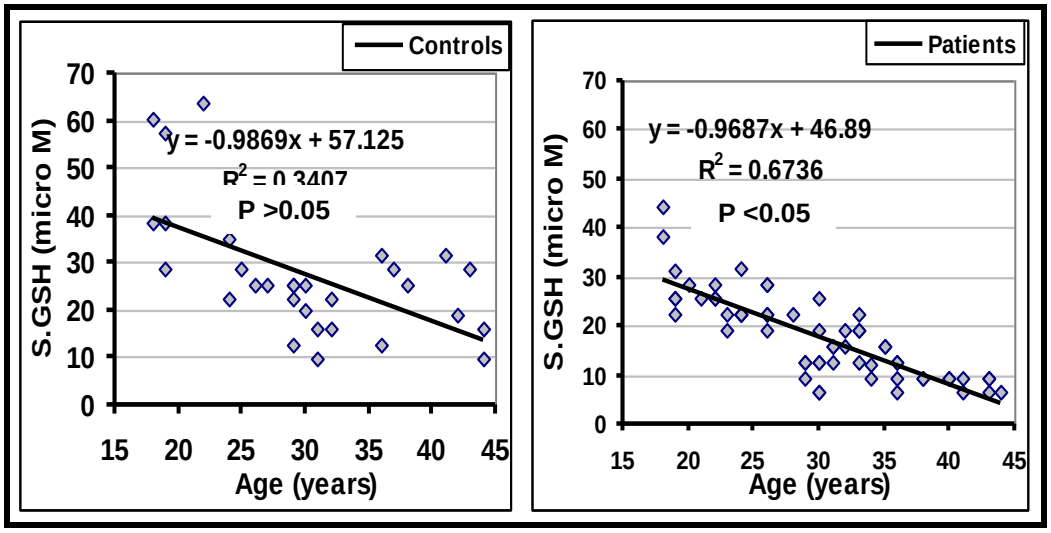


Figure (4. 26) Relationship between age (years) and serum glutathione levels (mean ±S.D µM) in patients with unexplained infertility and fertile control.

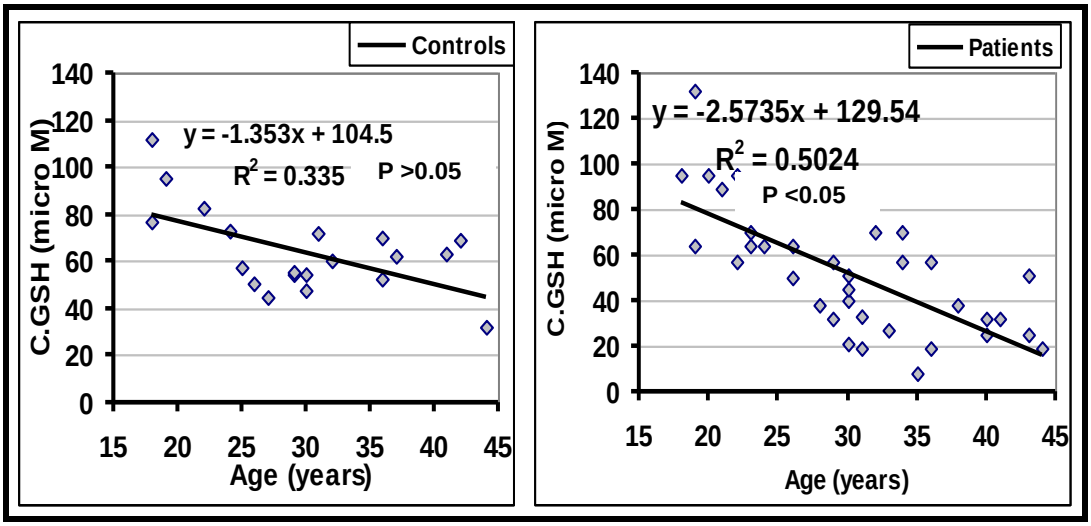


Figure (4 .27) Relationship between age (years) and GSH levels in cervical secretions (mean ±S.D µM)in patients with unexplained infertility and fertile control.

4.3.4.4 The Relationship between body mass index and glutathione levels

The glutathione levels in sera and cervical secretions of infertile women with unexplained infertility show insignificant negative correlation (p> 0.05) with body mass index when compared with fertile controls as shown in Figure (4.28) and (4.29):

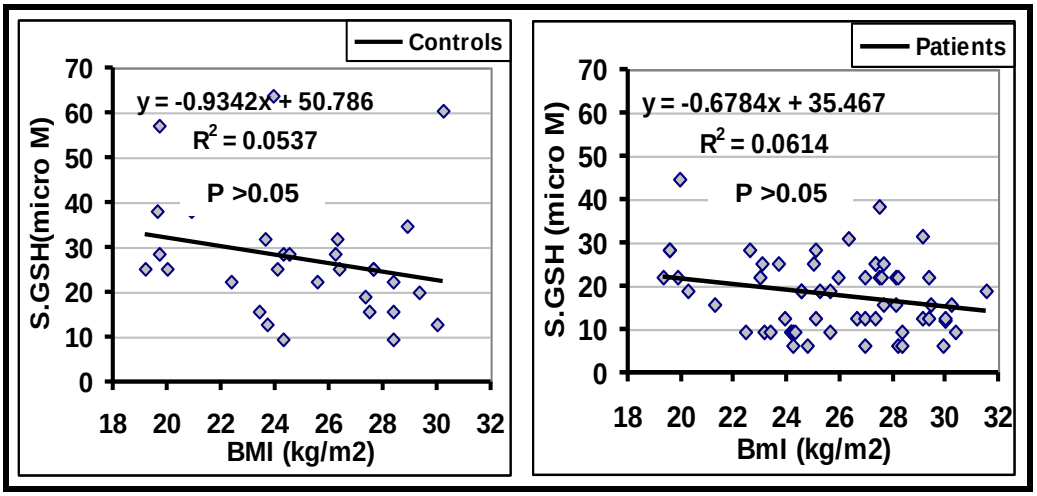


Figure (4. 28) Relationship between BMI (kg/m^2) and serum GSH levels (mean ±S.D µM) in patients with unexplained infertility and fertile control.

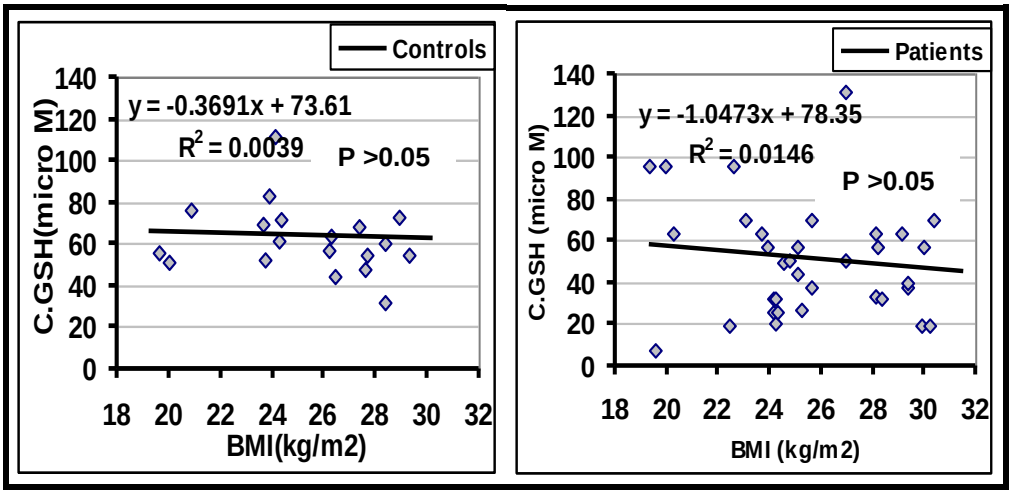


Figure (4.29)The correlation between BMI (kg/m ^{2}) and GSH levels in cervical secretion (mean ±S.D µM) in patients with unexplained infertility and fertile control.

4.3.4.5 The Relationship between duration of infertility and glutathione levels

The glutathione levels in sera and cervical secretions of unexplained infertile women show significant negative correlation with the increasing infertility duration ($p<0.05$)as shown in Figure (3.30):

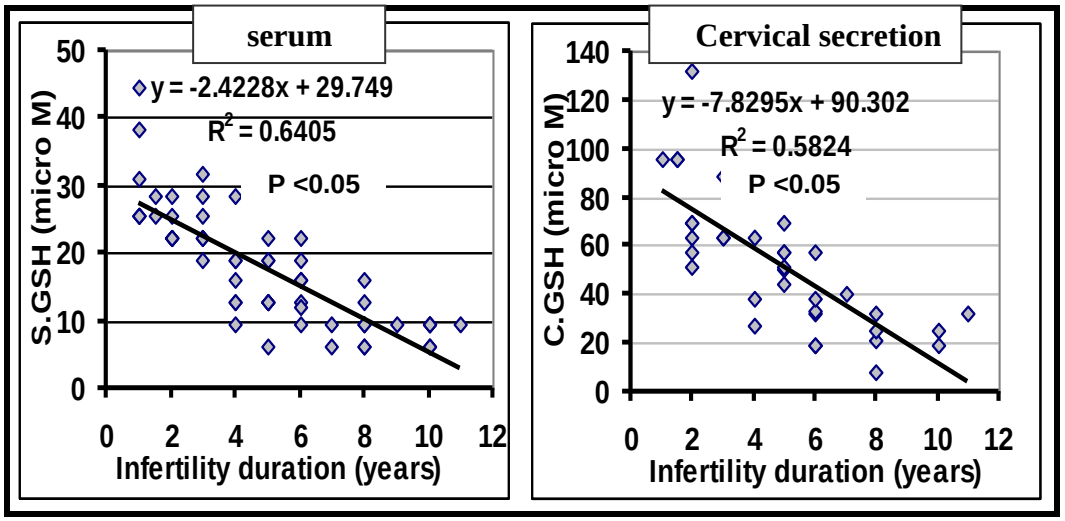


Figure (4 .30) Relationship between duration of infertility (years) and glutathione levels in serum and cervical secretion (mean ±S.D µM) in patients with unexplained infertility.

4.3.5 Measurement of vitamin C activity

Vitamin C levels in sera and cervical secretions of infertile women with unexplained infertility show insignificant decrease (p>0.05) when compared with fertile control as shown in Table (4.4):

Table (4.4) Vitamin C levels(mg/l) in sera and cervical secretions of infertile women with unexplained infertility and Control.

Sample	Control Mean ±SD mg/l	Patients Mean ±SD mg/l	P value
Serum Vitamin C	10.383 ± 3.655	9.944 ± 1.549	**P>0.05**
C.mucus Vitamin C	16.447 ± 4.042	14.233 ± 3.458	**P>0.05**

4.3.5.1 Vitamin C levels and types of infertility

Vitamin C levels in sera and cervical secretions of infertile women with unexplained infertility show insignificant differences(p>0.05) between primary and secondary infertility as shown in Figure (4.31):

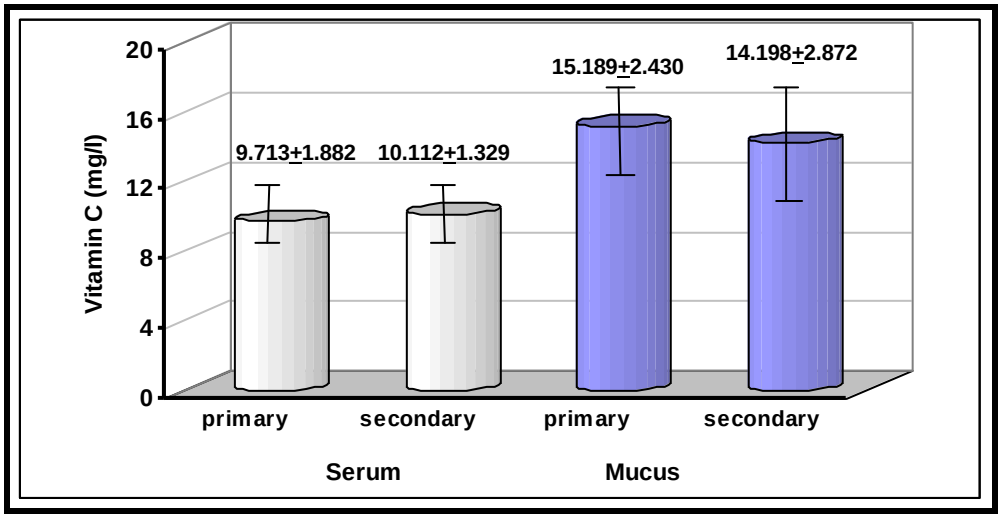


Figure (4 .31) Vitamin C levels (mean ±S.D mg/l) in sera and cervical secretions in primary and secondary infertile women with unexplained infertility(p>0.05).

4.3.5.2 Vitamin C levels and smoking

Vitamin C levels in sera and cervical secretions show insignificant differences($p > 0.05$) between passive and non smoker in infertile women with unexplained infertility and fertile control as shown in Figure (4.32) and (4.33):

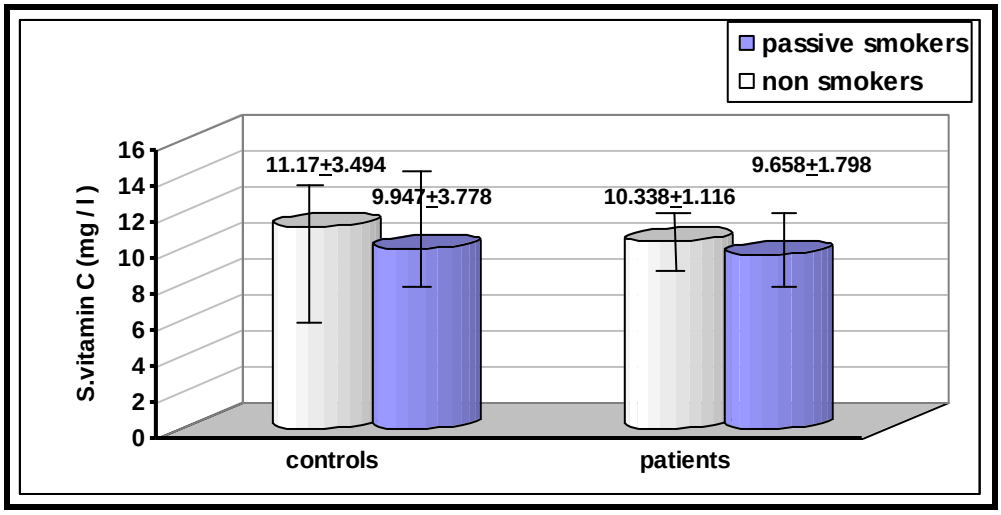


Figure (4 .32) The serum vitamin C levels (mean ±S.D mg/l)in passive and non smoker in infertile women with unexplained infertility and fertile control(p>0.05) .

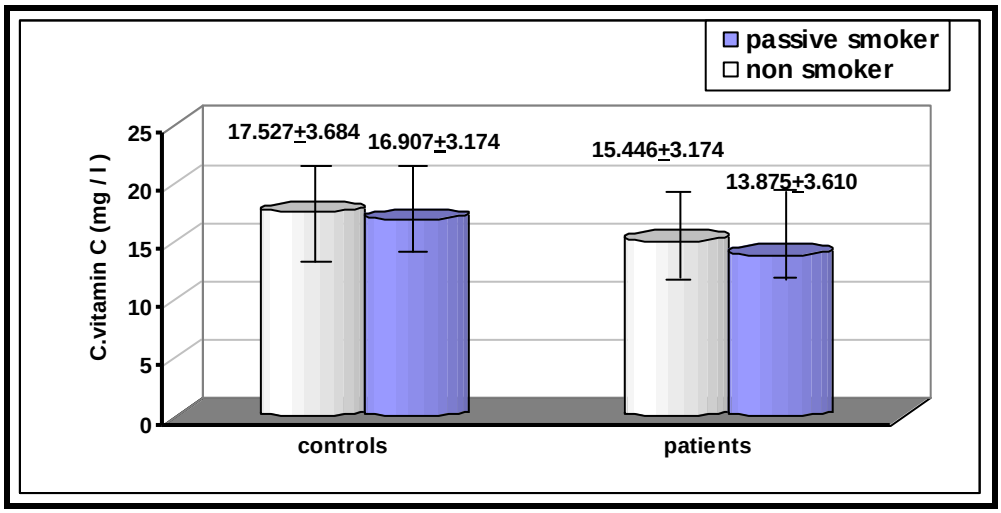


Figure (4.33) Vitamin C level in cervical secretions (mean ±S.D mg/l)in passive and non smoker in infertile women with unexplained infertility and fertile control(p>0.05) .

4.3.5.3 The Relationship between age and vitamin C

Vitamin C levels in sera and cervical secretions of infertile women with unexplained infertility show a significant negative correlation with age increment when compared with fertile controls ($p<0.05$) as shown in Figure (3.34) and (3.35):

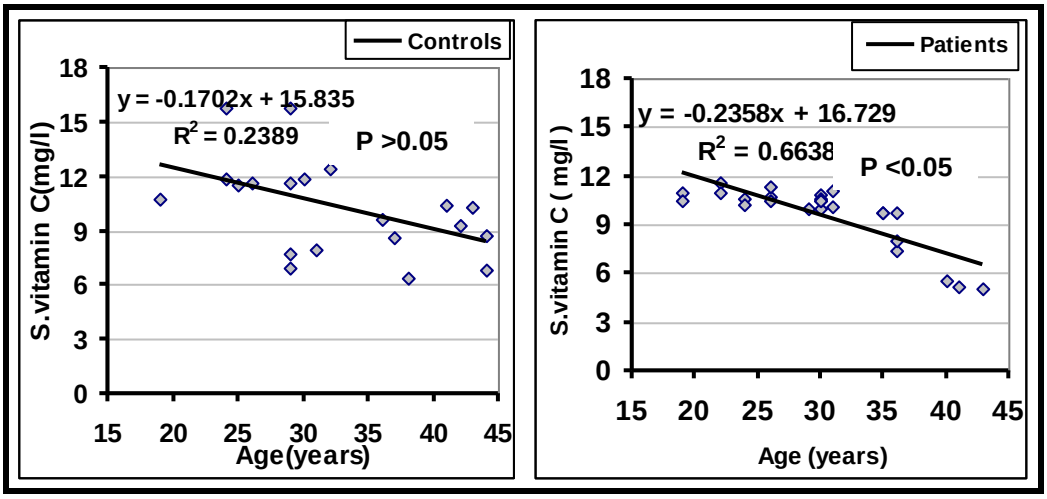


Figure (4.34) Relationship between age (years) and serum vitamin C levels (mean ±S.D mg/l) in infertile women with unexplained infertility and fertile control.

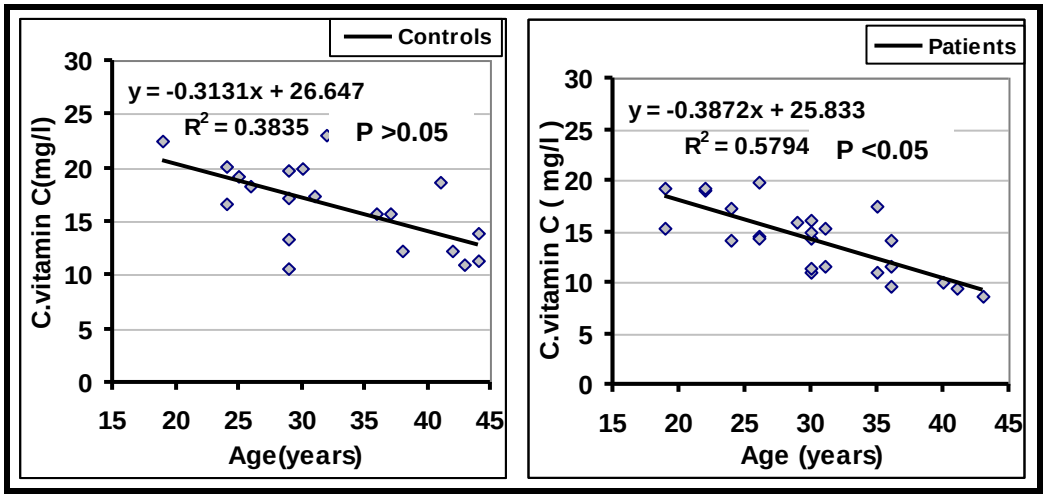


Figure (4. 35) Relationship between age (years) and vitamin C levels in cervical secretions (mean ±S.D mg/l) in infertile women with unexplained infertility and fertile control.

4.3.5.4 The Relationship between body mass index and vitamin C

Vitamin C levels in sera and cervical secretions of infertile women with unexplained infertility show insignificant negative correlation($p>0.05$) with BMI increment when compared with fertile control as shown in Figure (4.36) and (4.37):

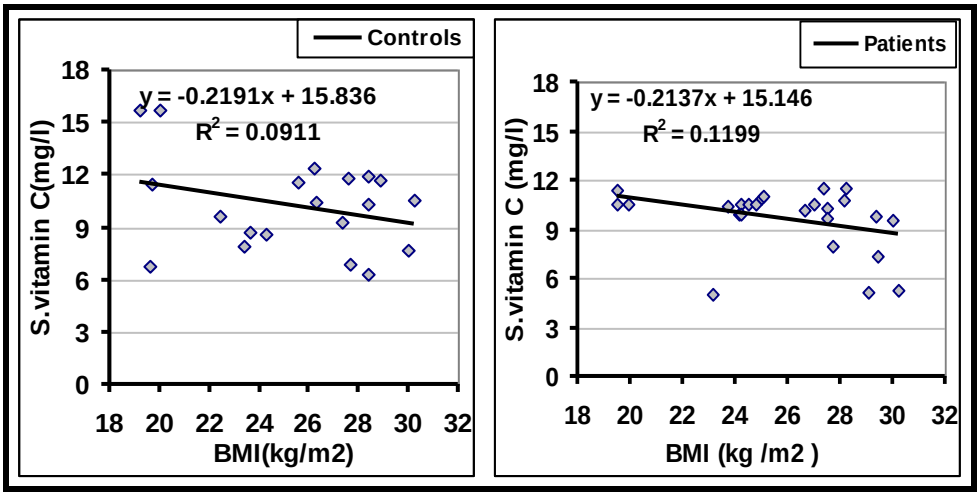


Figure (4.36)Relationship between BMI (kg/m^2) and serum vitamin C levels (mean ±S.D mg/l) in infertile women with unexplained infertility and fertile control.

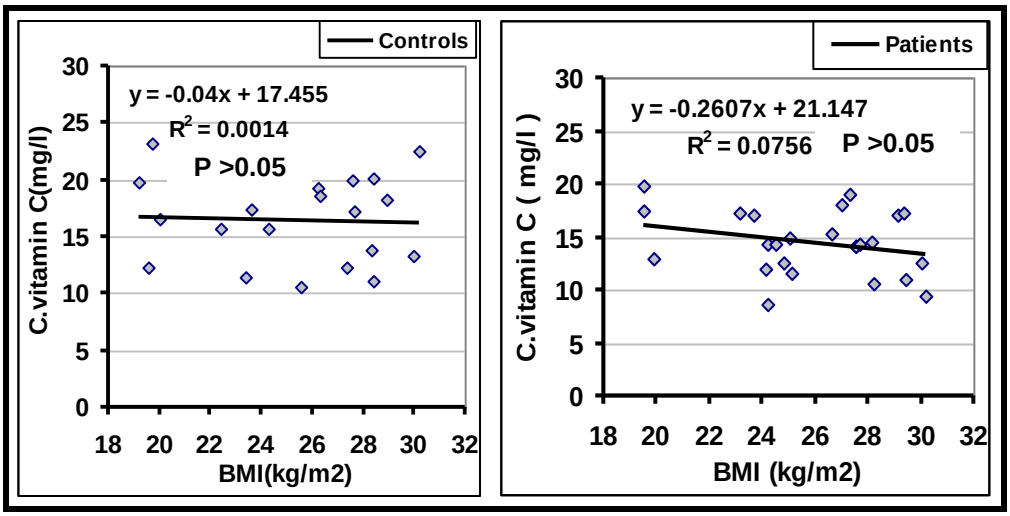


Figure (4 . 37)The correlation between BMI (kg/m^2) and vitamin C levels in cervical secretions (mean ±S.D mg/l) in infertile women with unexplained infertility and fertile control.

4.3.5.5The Relationship between duration of infertility and vitamin C

Vitamin C levels in sera and cervical secretions of infertile women with unexplained infertility show insignificant negative correlation ($p > 0.05$) with duration of infertilityas shown in Figure(4.38):

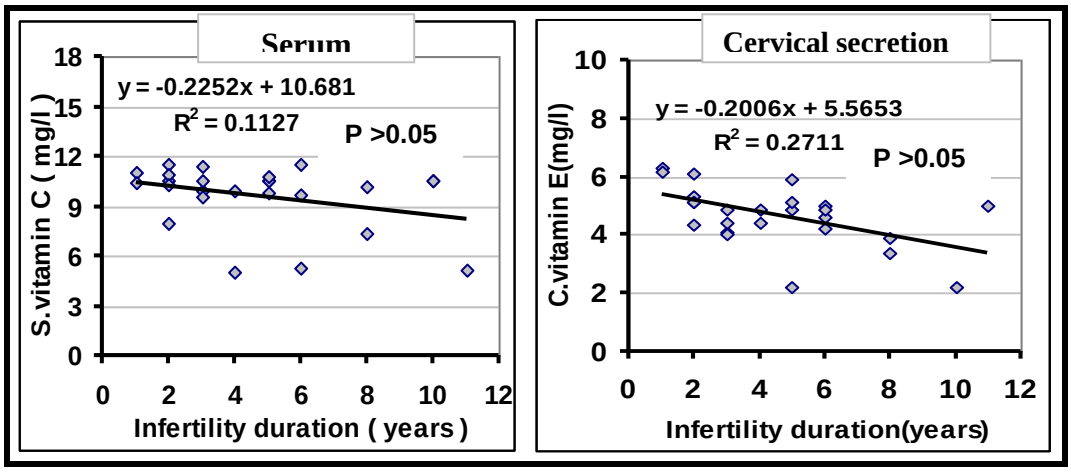


Figure (4. 38) Relationship between duration of infertility in years and vitamin C levels (mean ±S.D mg/l) in sera and cervical secretions of in infertile women with unexplained infertility.

4.3.6 Measurement of vitamin E levels

Vitamin E levels in sera and cervical secretions of infertile women with unexplained infertility show a significant decrease ($p<0.05$ and $p< 0.001$) respectively when compared with fertile control as shown in Table (4.5):

Table (4.5) Vitamin E levels in sera and cervical secretions (mg/l) of infertile women with unexplained infertility and fertile control.

Sample	Control Mean ±SD mg/l	Patients Mean ±SD mg/l	P value
Serum Vitamin E	**7.337 ± 3.535**	**5.272 ± 1.228**	$p<0.05$
C.mucus Vitamin E	**7.023 ± 0.754**	**4.644 ± 1.343**	$p< 0.001$

4.3.6.1 Vitamin E levels and types of infertility

Vitamin E levels in sera and cervical secretions of infertile women with unexplained infertility show insignificant differences($p>0.05$) between primary and secondary infertility as shown in Figure(3.39):

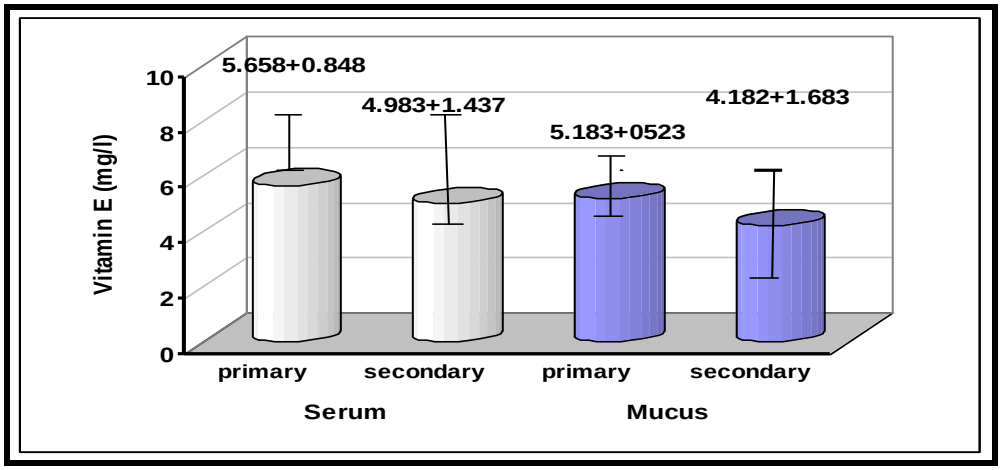


Figure (4.39) The vitamin E levels in sera and cervical secretions (mean ±S.D mg/l) in primary and secondary infertile women with unexplained fertility($p>0.05$).

4.3.6.2 Vitamin E levels and smoking

Vitamin E levels in sera and cervical secretions show insignificant differences between passive and not smoker in infertile women with unexplained infertility and fertile control ($p> 0.05$) as shown in Figure(4.40) and (4.41):

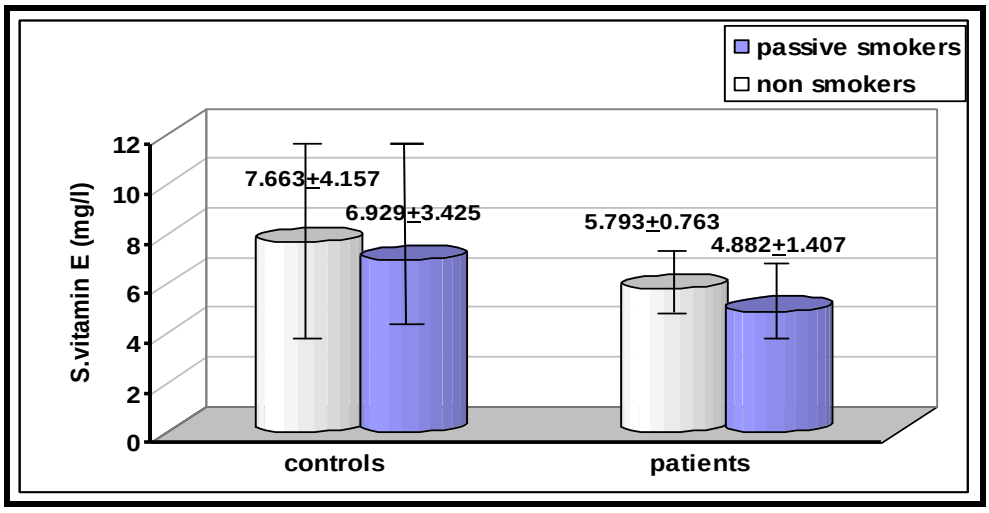


Figure (4 .40)The serum vitamin E levels (mean ±S.D mg/l) in passive and non smoker of infertile women with unexplained infertility and fertile control($p>0.05$).

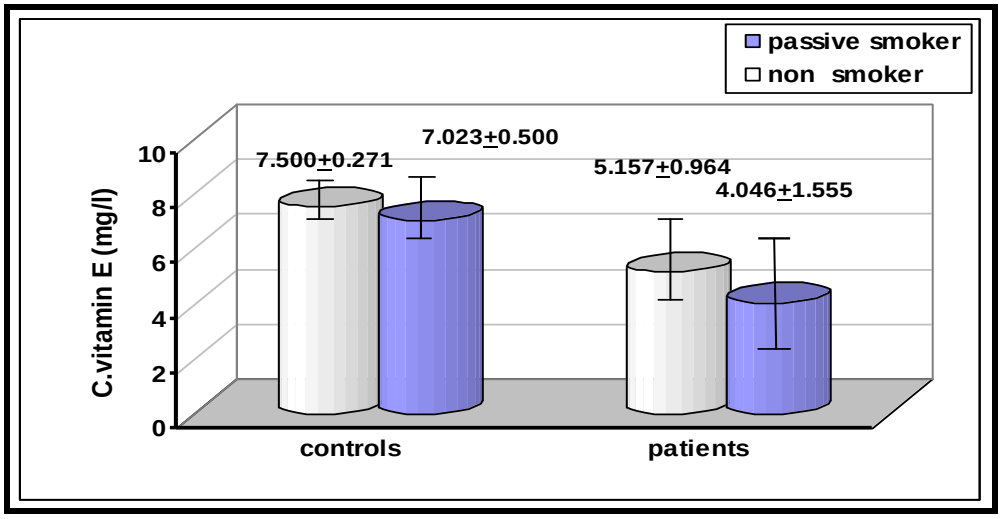


Figure (4.41)The vitamin E level in cervical secretions (mean ±S.D mg/l) in passive and non smoker of infertile women with unexplained infertility and fertile control(p>0.05) .

4.3.6.3 The Relationship between age and vitamin E

Vitamin E levels in sera and cervical secretions of infertile women with unexplained infertility show insignificant negative correlation (p>0.05) with age increment when compared with fertile controls as shown in Figure (4.42) and (4.43):

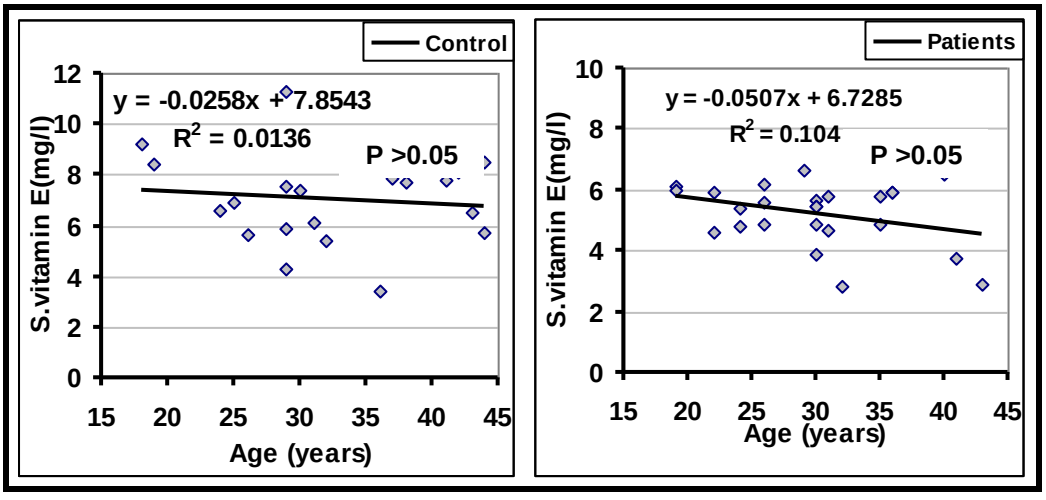


Figure (4.42) Relationship between age (years) and serum vitamin E levels (mean ±S.D mg/l) in infertile women with unexplained infertility and fertile control.

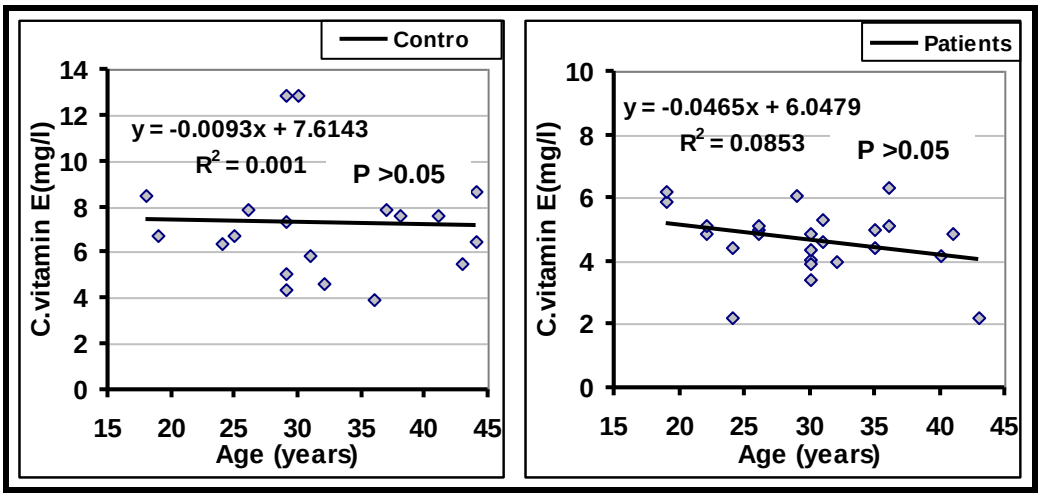


Figure (4.43) Relationship between age (years) and vitamin E levels in cervical secretions (mean ±S.D mg/l) in infertile women with unexplained infertility and fertile control.

4.3.6.4 The Relationship between body mass index and vitamin E

Vitamin E levels in sera and cervical secretions of infertile women with unexplained infertility show insignificant decrease ($p<0.05$) with body mass index increment when compared with fertile controls as shown in Figure (4.44) and (4.45):

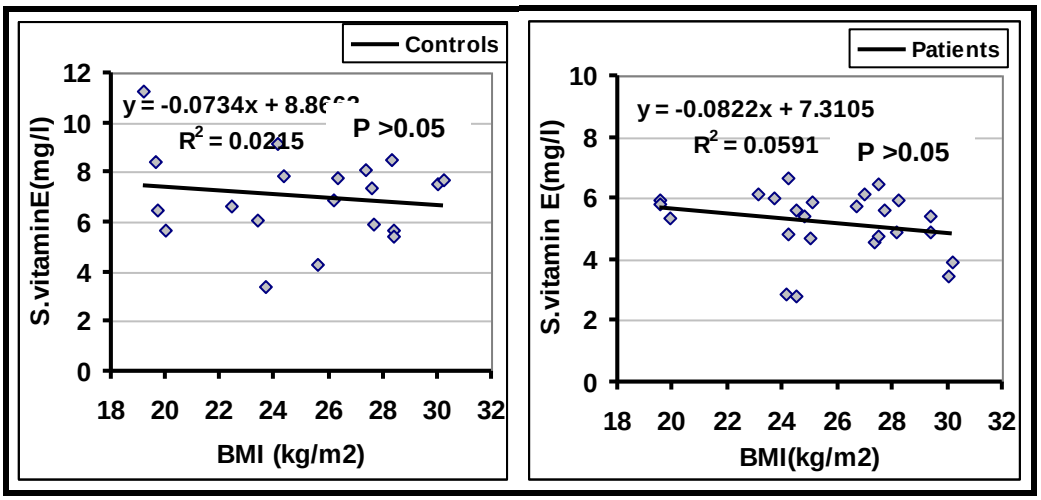


Figure (4. 44) Relationship between BMI (kg/m^2) and serum vitamin E levels (mean ±S.D mg/l) in infertile women with unexplained infertility and fertile control.

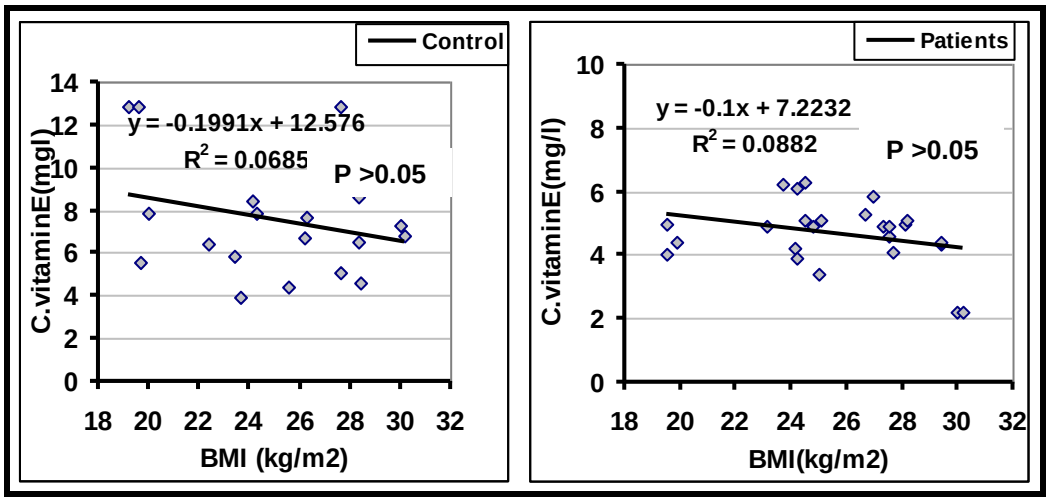


Figure (4. 45) The correlation between BMI (kg/m^2) and vitamin E levels in cervical secretions (mean ±S.D mg/l) in infertile women with unexplained infertility and fertile control.

4.3.6.5The Relationship between duration of infertility and vitamin E

Vitamin E levels in sera and cervical secretions of infertile women with unexplained infertility show insignificant negative correlation ($p > 0.05$) with duration of infertility as shown in Figure(4-46):

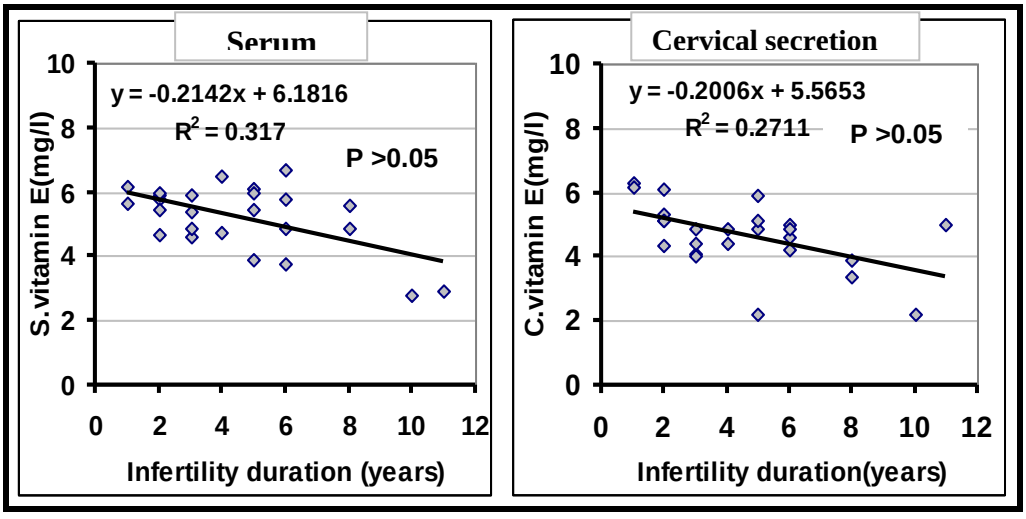


Figure (4.46) Relationship between duration of infertility (years) and vitamin E levels in sera and cervical secretions (mean ±S.D mg/l) in infertile women with unexplained infertility .

Chapter Five

Discussion

5.1 History and physical examination

From history , it has been found that more patients are inhabited in urban areas than rural areas as shown in figures(4.1)and this result may be due to environmental and nutritional variations between these areas.

Also from the history and physical examination, it has been statistically found that there's no significant differences ($p > 0.05$) between patients with unexplained infertility and fertile controls in menstrual phase duration ,date of menarche and BMI as shown in figures(4.2),(4.3)and(4.4) respectively .

This means (in addition to other findings such as regular cycle , dysmenorrhea , normal ova size in ultrasound) that the women with unexplained infertility have normal hormonal function in reproductive system.

5.2 Hematological tests

Good knowledge about ABO blood group system has expended to include numerous rays of antigenic determinants on erythrocyte. The antigenic determinants of blood group are produced by allele at a single gene locus or at other gene loci (Parslow, *et al.*,2001)

The result of ABO erythrocyte surface antigen and their distribution among unexplained infertile women show that blood group A is present to be more distributed (40%)than other types of ABO blood group system when compared with fertile group who have mostly blood group B(36%) as shown in Figure (4.5). This result may be due to genetic predisposition between types of blood group and unexplained infertility.

5.3 Biochemical tests

5.3.1 Measurement of serum proteins

Measurement of total serum protein concentration provides a general information reflecting disease states in many organ systems (Bishop, *et al*. ,2000 and Varley, *et al*.,1991).

In this study, the total serum protein ,serum albumin and serum globulin shows insignificant difference between women with unexplained infertility and fertile group ($p > 0.05$) as shown in Figure (4.6).

This means that there is a normal amount of carrier protein for sex hormones in women with unexplained infertility.

5.3.2 Malondialdehyde levels in sera and cervical secretions

The results of the present study show that the total MDA levels in sera and cervical secretions of infertile women with unexplained infertility show a significant increase ($p<0.001$) when compared with fertile controls as shown in Table (4.1) . The data of the present study are agreed with the results of Polak, *et al*.(1999) ; Agarwal, *et al*.(2003)and (2006); as well as Savita, *et al*. (2009).

Agarwal, *et al***.**(2005) suggest that women with idiopathic infertility have reduced concentrations of antioxidants in peritoneal fluid and increased ROS-induced lipid peroxidation damage resulting in infertility.

Dong, *et al*. **(**2001),on the other hand, have found significantly high levels of nitric oxide in peritoneal fluid of patients with unexplained infertility.

Increased level of MDA in sera and cervical secretions in females with unexplained infertility can be attributed largely to higher rate of lipid peroxidation result in structural and functional damage to cellular membrane of both ova and transported sperms. Accumulated lipid

peroxides may cause not only tissue damage but also some biological events that lead to apoptosis and accelerate termination of pregnancy (Sugino, *et al*. ,2000).

Free radicals in cervical secretion and free radicals that diffuse into the fallopian tubes from peritoneal fluid may cause damage to sperm; which are known to be sensitive to OS because of their plasma membranes contain large quantities of polyunsaturated fatty acids and their cytoplasm contains low concentrations of the scavenging enzymes (Saleh and Agarwal ,2002).

Therefore; ROS may affect the quality and number of spermatozoa reaching the ovum in the female reproductive tract (Aitken , *et al*. ,2003).

At high concentrations, ROS may cause a series of damages to spermatozoa that include membrane lipid peroxidation, oxidation of protein, loss of motility fertilizing potential, and DNA fragmentation (Talevi ,*et al*. ,2007).

Therefore; spermatozoa and oocyte have built in a mechanism to prevent excessive production of ROS at the time of sperm-oocyte fusion. This may be done by the release of antioxidant enzymes (Maiorino and Ursini 2002)and if there is an abnormality in the production of antioxidant enzymes, ROS generation can continue uninterruptedly and damage both spermatozoa and oocyte.

Severe OS can lead to infertility because of the negative impact on fusion events such as acrosome reaction and sperm–oocyte fusion (Aitken, *et al*., 2003).

So the high rate of free radicals production in female with unexplained infertility may be generated from increases metabolism and depletion of protective antioxidants.

5.3.2.1 Malondialdehyde levels and types of infertility

Malondialdehyde levels in sera and cervical secretions of infertile women with unexplained infertility show insignificant differences ($p > 0.05$) between primary and secondary infertilityas shown in Figure (4.7). The same findings are reported by Pyari, *et al*. (2006) who find that insignificant differences between primary and secondary infertility in MDA blood level and MDA in endometrial biopsy and this result may be due to the factors that lead to increase of free radicals level which are happened nearly equal in both primary and secondary infertility types.

5.3.2.2 Malondialdehyde levels and smoking

The total MDA levels in sera and cervical secretions of infertile women with unexplained infertility show insignificant differences ($p>0.05$)between passive and not smoker in infertile women with unexplained infertility and fertile controls as shown in Figure(4.8)and (4.9).

Paszkowski, *et al*. (2002) report that prolonged smoking history is associated with elevated levels of OS. In addition, they find that smokers have been reported to have higher ROS in follicular fluid and have a lower in vitro fertilization success rate.

Dumollard, *et al*. (2007) observe that intrafollicular exposure to cigarette smoking metabolites is associated with a significant increase in follicular lipid peroxidation.

The result in the present study may be firstly due to environmental factors (which contain many sources of exogenous pro-oxidants) and secondly the patients in the present study are passive not active smokers.

5.3.2.3 The Relationship between age and Malondialdehyde

In the present study, there is significant and positive correlations observed between total MDA levels in sera , cervical secretions of female

with unexplained infertility and the age increment ($p<0.05$, $r= +0.858$; $p<0.05$, $r = +0.866$)respectively when compared with fertile control group as shown in Figure (4.10) and (4.11). This outcome is in good agreement with the outcomes reported by Sohal ,*et al*.(1995) who find that O_2^- and H_2O_2 production are increased with age, especially in isolated mitochondria from the aged heart.

Sadraie, *et al*.(2000); Moffatt, *et al*.(2002) and Takahashi, *et al.* (2003) find that free radical activity of human Follicular fluid increases with age as does apoptosis of human granulosa and cumulus cells.

Agarwal, *et al*. (2005) suggest that OS modulates the age-related decline in fertility and there is an age related decline in the number and quality of follicles in females. Oxidative damage could also account for the mutations in DNA that accumulate with age (Wei, *et al*. ,1998) and this may lead to abnormal gametes and infertility.

Oxidative stress increases with age may be due to the steady-state in accumulation of free radicals which occurs as a consequence of aerobic metabolism which thought to be an important mechanism underlying the age-related increase in pathology as well as the progressive decline in the functional efficiency of various cellular processes(Sohal,1993).

5.3.2.4 The Relationship between body mass index and malondialdehyde

This study shows insignificant positive correlation ($p>0.05$,$r = +0.344$; $p>0.05$, $r= +0.266$) between total MDA levels in sera , cervical secretions of infertile patients and the BMI when compared with fertile control groups respectively as shown in Figure (4.12) and (4.13). This result goes parallel with the results of Trevisan, *et al*. (2001) who suggest that BMI is modestly but positively correlated with OS in women. This result may be

due to the increasing level of polyunsaturated lipid with BMI increment which leads to increase free radicals level.

5.3.2.5 The Relationship between duration of infertility and Malondialdehyde

The total MDA levels in sera and cervical secretions of infertile women with unexplained infertility shows insignificant positive correlation ($p>0.05$, $r = +0.499$; $p>0.05$, $r = +0.493$) with duration of infertility as shown in Figure (4.14) and (4.13) and this result is in a good agreement with the results obtained by Jackson, *et al*.(2005) who find that there is no statistically significant associations between serum MDA level and time to get pregnancy.

The follicular fluid microenvironment and oxidative stress status provides a window to oocyte quality, success of fertility and fertility outcomes (Bedaiwy ,*et al*.,2005).

This result may be due to prolonged exposure to stressful events, dietary regimen and therapeutic drugs that may lead to increase OS.

5.3.3 Measurement of catalase activity

Catalase level in sera and cervical secretion of infertile women with unexplained infertility show a significant decrease ($p< 0.05$) when compared with fertile controls as shown in Table (4.2) and this result is in agreement with the results of Pyari, *et al*. (2006) who find that CAT level is significantly low in endometrium and blood of infertile women compared to those in controls. The lowest levels have been found in cases of unexplained infertility. Similarly Polak, *et al*. (2001**)** find that the total antioxidant status is significantly lower in peritoneal fluid from women with unexplained infertility. In another study, wang ,*et al*. (1997)find that the concentrations of antioxidants in idiopathic infertility patients significantly reduced compared to those of fertile patients .

Catalase is enzymatic antioxidant ;located mainly in the tubal fluid. Neutralizes intracellular and extracellular hydrogen peroxide (Ruder ,*et al*.,2008).Higher ROS levels in patients with unexplained infertility may lead to increase ROS-scavenging process which ultimately lead to reduce levels of antioxidants such as catalase enzyme. Catalase Promotes an increase in the proportion of zygotes undergoing at least one cleavage (Ruder ,*et al*.,2008). So any change in its concentration may lead to disruption of zygotes cleavage which leads to infertility.

Ali, *et al*.(2000) show that the usage of catalase in media of in vitro fertilization gives better quality embryos.

5.3.3.1 Catalase levels and types of infertility

Catalase levels in sera and cervical secretions of infertile women with unexplained infertility show insignificant differences($p>0.05$) between primary and secondary infertility as shown in Figure (4.15). This result is in disagreement with the results obtained by Pyari, *et al*. (2006) who find that catalase levels in the endometrial biopsy and blood are significantly higher in women with secondary unexplained infertility when compared to those with primary unexplained infertility. This result may be due to affect of catalase level by other factors such as age ,BMI, free radical production, environmental conditions and antioxidant dietary supplementation.

5.3.3.2 Catalase levels and smoking

Catalase levels in sera and cervical secretions of infertile women with unexplained infertility show insignificant differences($p> 0.05$) between passive and not smoker in infertile women with unexplained infertility and fertile controls as shown in Figure (4.16) and (4.17). Hence the duration of exposure to smoke, degree of air ventilation and other environmental factors may lead to such results.

5.3.3.3 The Relationship between age and Catalase enzyme

Catalase levels in sera and cervical secretions of infertile women with unexplained infertility show insignificant negative correlation ($p>0.05$, $r = -0.391$; $p>0.05$, $r = -0.363$)respectively with age increment when compared with fertile controls as shown in Figure (4.18) and (4.19). As in the study of Carbone, *et al*., (2003) who find that the specific activity of catalase is lower in older women compared with the younger ones.

This means that the free radicals increase with age which ultimately leads to decrease the levels of antioxidants.

5.3.3.4 The Relationship between body mass index and Catalase enzyme

Catalase levels in sera and cervical secretions of infertile women with unexplained infertility show insignificant negative correlation ($p>0.05$, $r = -0.113$; $p>0.05$, $r = -0.255$) respectively with BMI increment when compared with fertile controls as shown in Figure (4.20) and (4.21).

This result may be due to the increasing level of polyunsaturated lipid with BMI increment which leads to increase free radical and decrease antioxidant levels.

5.3.3.5 The Relationship between duration of infertility and catalase enzyme levels

Catalase levels in sera and cervical secretions of infertile women with unexplained infertility show insignificant negative correlation with duration of infertility ($p>0.05$, $r = -0.118$; $p>0.05$, $r = -0.160$)respectively as shown in Figure (4.22).This result matches with the results obtained by Jackson, *et al*. (2005)who find that there is no statistically significant

associations between serum catalase level and time to get pregnancy. Catalase is enzymatic antioxidant ;located mainly in the tubal fluid. Neutralizes intracellular and extracellular hydrogen peroxide ,decreases its level ,lead to increase free radicals level and increase probability of sperm and ova damage which ultimately lead to increase duration of infertility.

5.3.4 Measurement of glutathione levels

Numerous laboratory studies have suggested that glutathione is a critical factor in protecting organisms against toxicity and disease. Blood and serum glutathione concentrations may serve as an indicator of GSH status, and thus diseases risk in human.

In female reproductive tract , glutathione is located mainly in the tubal fluid and oocyte/embryo. It neutralizes superoxide anion, metabolizes hydrogen peroxide and hydroxyl radical, improves the development of zygotes through the 2-cell block to the blastocyst stage, protects embryos against ROS, improves bovine embryo production, and prevents oxygen-induced embryonic malformation(Ruder , *et al*.,2008).

Studied cases of GSH levels in serum and cervical secretion of infertile women with unexplained infertility show a significant decrease ($p< 0.001$,$P<0.05$)respectively when compared with fertile controls. as shown in Table (4.3), this result agrees with the results obtained by Agarwal, *et al*.(2008).

It is suggested from animal studies that the concentration of GSH in the oocyte is important to reduce the disulfide bonds during sperm nucleus decondensation and enable pronucleus(PN) formation, decapitation and pronuclear apposition (Bedaiwy, *et al*., 2004).This is indecated by the observation that GSH antagonists disturbed the maturation of oocytes by compromising the decondensation of the sperm

nucleus and thus preventing PN apposition. Furthermore, supplementation of GSH during in vitro maturation of oocytes resulted in improved male PN formation, normal fertilization and embryo development (Ebisch,*et al*.,2006).

Oocytes are rich in glutathione reductase (Urner and Sakkas,2005**).** A glutathione deficiency can lead to instability of the mid-piece of sperm, resulting in defective motility (Ursini, *et al*.,1999). It protects plasma membrane from lipid peroxidation, scavenges superoxide and prevents O_2^-formation.

In a study consisting of infertile men , GSH led to significant improvement in the sperm quality (Lenzi, *et al*.,2004).

The depletion of GSH levels in the present study, when compared with healthy control, supports the hypothesis that considers GSH a protective factor against the development of different types of diseases one of them is infertility.

5.3.4.1 Glutathione levels and types of infertility

Glutathione levels of sera and cervical secretions of unexplained infertile women show insignificant differences between primary and secondary infertility ($p > 0.05$) as shown in Figure (4.23).These results may be due to other factors such as prolonged stress ,prolonged treatment and environmental factors that may lead to increase OS and decrease glutathione in similar manner in both types of female infertility

5.3.4.2 Glutathione levels and smoking

Glutathione levels of sera and cervical secretions of unexplained infertile women show insignificant differences between passive and not smoker in infertile women with unexplained infertility and fertile controls ($p > 0.05$) as shown in Figure (4.24) and (4.5) .

Paszkowski, *et al*.(2002) report that smokers have diminished follicular fluid activity of the antioxidant enzyme glutathione peroxidase as compared with non smoker.

The result of the present study may be due to indirect smoking and the effect of environmental pollution which make indifference between passive smoker and non smoker in GSH levels.

5.3.4.3 The Relationship between age and glutathione levels

Glutathione levels of infertile studied patients of serum and cervical secretion show significant negative correlation with age increment when compared with fertile controls ($p<0.05$, $r = -0.820$; $p<0.05$, $r = -0.708$) respectively as shown in Figure (4.26) and (4.27) and this result matches with the results obtained by Carbone, *et al*.(2003) who find that GSH is higher in the younger than older women.

Other investigations with aging rats demonstrate that reduced expression of glutathione reductase with aging leads to decrease the GSH (Yeh, *et al*.,2005).

5.3.4.4 The Relationship between body mass index and Glutathione levels

Both serum and cervical secretion of GSH levels of infertile women with unexplained infertility present with insignificant negative correlation with body mass index when compared with fertile controls ($p>0.05$, $r = -0.247$; $p>0.05$, $r = -0.120$) respectively as shown in Figure (4.28) and (4.29).

The reason behind such a result might be due to the increased level of polyunsaturated lipid with BMI increment which leads to increase OS and decrease the antioxidant levels.

5.3.4.5 The Relationship between duration of infertility and glutathione levels

Glutathione levels in sera and cervical secretions of unexplained infertile women show significant negative correlation with the increasing of infertility duration ($p<0.05$, $r = -0.800$; $p<0.05$, $r = -0.763$) respectively as shown in Figure (4.30). This result approves with the results of Jackson, *et al*. (2005) who report that significantly increased serum glutathione reductase is associated with decreased time to get pregnancy. No statistically significant associations are found with glutathione peroxidase. So this result is due to decrease of glutathione reductase enzyme with increase duration of infertility which lead to decrease of glutathione level.

5.3.5 Measurement of vitamin C activity

In female reproductive tract, vitamin C is located mainly in the ovary.

Vitamin C levels in sera and cervical secretions of infertile women with unexplained infertility show insignificant decrease ($p>0.05$) when compared with fertile controls as shown in Table (4.4)

Many studies indicate that the addition of ascorbic acid does prevent oocyte membrane damage and increases basement membrane turnover (Tatemoto, *et al*.,2005), leading to increased follicle integrity and survival. Other researchers propose that certain concentrations of alpha-tocopherol or ascorbic acid facilitate meiotic maturation of cumulus free oocytes and can protect cumulus cell DNA damage and apoptosis (Tao, *et al*.,2004).

Supplementation inhibits follicular apoptosis and causes premature resumption of meiosis (Ruder ,*et al* 2008).

5.3.5.1 Vitamin C levels and types of infertility

Vitamin C levels in serum and cervical secretion of infertile women with unexplained infertility show insignificant differences between primary and secondary infertility ($p>0.05$) as shown in Figure (4.31).Therefore, this result may be explained by the fact of dietary vitamin C intake in which each type of female infertility are near equal.

5.3.5.2 Vitamin C levels and smoking

Vitamin C levels in sera and cervical secretions of infertile women with unexplained infertility show insignificant differences between passive and not passive smoker in infertile women with unexplained infertility and fertile controls ($p> 0.05$) as shown in Figure (4.32) and (3.33).

Ayaori ,*et al*. (2000) report that plasma ascorbic acid is inversely associated with smoking.

Vitamin C helps to combat the pollution surrounding us. Moreover; it has been shown to reduce the DNA damage (Dietrich, *et al* .,2003).

No dietary difference between passive smoker and not smoker might be the cause behind such a result..

5.3.5.3 The Relationship between age and vitamin C

A significant negative correlation between vitamin C levels in sera, cervical secretions of infertile patients and the age increment when compared with fertile controls ($p<0.05$, $r = -0.814$, $p<0.05$, $r = -0.761$) as shown in Figure (4.34) and (4.35). Tarín ,*et al*.(1998) find that supplementation of vitamin C reduces the risk of ovulating aneuploid and diploid oocytes in aged female mice. Furthermore, dietary supplementation of mice with the antioxidants vitamins C and E, resulted in improvements in both the quantity and quality of oocytes when compared to unsupplemented older mice (Tarín ,*et al*,2002). This

reduction of vitamin C with age may be due to increase OS with age increment which leads consequently to depletion of antioxidants.

5.5.4 The Relationship between body mass index and vitamin C

Vitamin C levels in serum and cervical secretion of infertile women with unexplained infertility show insignificant negative correlation with BMI increment when compared with fertile controls ($p>0.05$, $r = -0.346$; $p>0.05$, $r = -0.274$)respectively as shown in Figure (4.36) and (4.37).This result may be regarded to increase of polyunsaturated lipid , increase lipid peroxidation which consequently leads to decrease antioxidants levels.

5.5.4 The Relationship between duration of infertility and vitamin C

Vitamin C levels in sera and cervical secretions of infertile women with unexplained infertility show insignificant negative correlation with duration of infertility ($p>0.05$, $r = -0.335$; $p>0.05$, $r = -0.217$) respectively as shown in Figure(4.38) and this result is in agreement with a result obtained by Crha ,*et al*.(2003) who report higher pregnancy rate with vitamin C supplementation when compared to the a controling group.

5.3.6 Measurement of vitamin E levels

Vitamin E is a major chain breaking antioxidant in membranes, located mainly in the ovary specially in follicular fluid.

Vitamin E levels in sera and cervical secretions of infertile women with unexplained infertility show a significant decrease ($p<0.05$;$p< 0.001$) respectively when compared with fertile controls as shown in Table (4.5). This result matches with the results got by Makinde and Adedeji(1994) and Mehendale, *et al*.(2009) who state that plasma vitamin E level is greater in fertile women than in infertile women.

Savita, *et al*.(2009) suggest that the increased OS are associated with the decrease of antioxidants and associated with infertility.

Vitamin E directly neutralizes superoxide anion, hydrogen peroxide, and hydroxyl radical ;so increase these types of free radical may lead to depletion of vitamin E. Also vitamin E increases number of embryos developing to the expanded blastocysts and increases viability of embryos exposed to heat shock. So any change in its concentration may have a role in infertility(Ruder ,*et al*.,2008).

Vitamin E may increase oocyte quality. In a human trial, infertile couples given vitamin E show a significant increase in fertility (Bayer,1960).

5.3.6.1 Vitamin E levels and types of infertility

Vitamin E levels in sera and cervical secretions of infertile women with unexplained infertility show insignificant differences between primary and secondary infertility($p>0.05$)as shown in Figure(4.39). This result may be due to the lack of vitamin E in diet as well as to other factors such as environmental factors which nearly affect both types of female infertility equally .

5.3.6.2 Vitamin E levels and smoking

Vitamin E levels in serum and cervical secretion of infertile women with unexplained infertility show insignificant differences between passive and not passive smoker in infertile women with unexplained infertility and fertile controls ($p> 0.05$) as shown in Figure (4.40) and (4.41) . Tiboni, *et al.* (2004) state that no smoking-related differences in follicular fluid or plasma concentrations of vitamin E between fertile and infertile women as well as to Dietrich ,*et al.* (2003) research who find that no significant differences in plasma concentrations of alpha-tocopherol between fertile and infertile smoker. This result may

be due to the same level of vitamin E in diet in both passive smoker and non smoker.

5.3.6.3 The Relationship between age and vitamin E

Vitamin E levels in serum of infertile women with unexplained infertility show a insignificant negative correlation ($p>0.05$, $r = -0.332$; $p>0.05$, $r = -0.292$)respectively with age increment when compared with fertile controls as shown in Figure (4.42) and (4.43).This result approves with the results obtained by Yeh, *et al.*(2005) who say that decrease in vitamin E and other antioxidant defense with age.

In this respect, Makinde and Adedeji (1994) show that vitamin E values are increased with age up to the 25-29 year age group. Other evidences researching by Carbone , *et al*. (2003) show that the aged ovaries have elevated vitamin E content. Thus, a shift toward a higher concentration of vitamin E (and low serum vitamin E level) may occur to help protect the aging ovary during luteolysis and compensate for the decline in the luteal cell ability to quench ROS, as evidenced by lower glutathione reductase enzyme. Other researchers, such as Ruder,*et al*.(2008) find that vitamin E supplementation reduces the risk of ovulating aneuploid and diploid oocytes in aged female mice and increases survival of explanted rat conceptuses in vitro. In general, when supplemental vitamins E is given to older mice, the age-associated reduction in ovulation is partially prevented (Tarin, *et al*., 2002).

5.3.6.4 The Relationship between body mass index and vitamin E

Vitamin E levels in serum and cervical secretion of infertile women with unexplained infertility show insignificant decrease ($p>0.05$, $r = -0.243$; $p>0.05$, $r = -0.296$)respectively with body mass index when compared with fertile controls as shown in Figure (4.44) and (4.45). Increase BMI may lead to increase unsaturated lipid which lead to higher

ROS levels production . Higher ROS levels production would lead to reduce levels of antioxidants such as vitamin E that would ultimately reduce ROS-scavenging ability and prevent the neutralization of toxic ROS effects (Wang, *et al*.,1997) .

5.3.6.5 The Relation between duration of infertility and vitamin E

Vitamin E levels in serum and cervical secretion of infertile women with unexplained infertility show insignificant negative correlation with duration of infertility ($p>0.05$, $r = -0.563$; $p>0.05$, $r = -0.520$) respectively as shown in Figure(4.46).

This result may be due to the fact that the vitamin E level may be affected by the level of free radical production and level of vitamin E intake during time of infertility.

Antioxidant levels in patients with unexplained infertility are significantly lower than those of healthy controls (Polak, *et al*.,2001).

Ruder, *et al*., (2009) find that the use of multivitamins, impacts the generation of ROS and may play a beneficial role in female fertility.

Chapter six

Conclusions and Recommendations

6.1 Conclusions

The present study shows that the oxidative stress plays an important role in human fertility:

- Malondialdehyde level is significantly increased in sera and cervical mucus secretion in patients with unexplained infertility.
- There is a direct significant relationship between MDA level and age increment in patients with unexplained infertility.
- Catalase ,glutathione and vitamin E levels are significantly decreased in sera and cervical mucus secretion in patients with unexplained infertility.
- There is an inverse significant relationship between GSH level with age and duration of infertility increment in patients with unexplained infertility.
- There is an inverse significant relationship between vitamin C level and age increment in patients with unexplained infertility.

6.2 Recommendations

- Malondialdehyde level should be carried out as primary test for assessment OS status.
- Assessment of OS as a cause of unexplained female infertility must be carried out to discriminate OS infertility from other causes of infertility.
- Studying the role of oxidants in multiple sites such as ovary, peritoneal cavity and uterus separately.
- Measurement of biomarkers of OS is subject to interlaboratory variations, and interobserver differences. A uniform method with comprehensive assessment of the OS biomarkers should be used so that the results can be compared across the studies.
- Study the role of oxidants in other types of infertility.
- Strategies to overcome OS in-vitro conditions and balancing between in vivo and in vitro environments can be utilized in assisted reproductive technique , to successfully treat infertility.

References

Abi,H.(1974). Method of enzymatic analysis. New York. Academic press,2:674-684.

Agarwal, A.; Saleh, R.A. and Bedaiwy, M.A.(2003). Role of reactive oxygen species in the pathophysiology of human reproduction. Fertil. Steril. J., 79: 829-43.

Agarwal, A.; Shyam, S.R. and Allamaneni, M.D. (2004). Oxidants and antioxidants in human fertility. Middle East Fertil. Soci. J.,9(3)187-197.

Agarwal, A. and Allamaneni, S.S.(2004). Role of free radicals in female reproductive diseases and assisted reproduction.Reproductive BioMedicine Online, 9:338–347.

Agarwal, A., Gupta, S. and Sharma, R .(2005). Oxidative stress and its implications in female infertility - a clinician's perspective. Reprod. Biomed. Online ,11(5): 641-650.

Agarwal, A., Gupta, S. and Sharma, R .(2005a). Role of oxidative stress in female reproduction. Reproductive Biology and Endocrinology, **3**:28.

Agarwal ,A.; Gupta, S.; Krajcir, N. and Sikka,S.(2006). The role of free radicals and antioxidants in reproduction. Current Opinion in Obstetrics and Gynecology, 18:325–332.

Agarwal,A.;Gupta,S.;Sekhon,L..and.Shaha,R.(2008). Redox Considerations in Female Reproductive Function and Assisted Reproduction: From Molecular Mechanisms to Health Implications. Antioxidants and redox signaling J., 10(8) :1376-1396.

Agarwal, A ;Gupta, S.; Malhotra, N.; Sharma, D. and Chandra, A. (2009). Oxidative Stress and its Role in Female Infertility and Assisted Reproduction: Clinical Implications. International Journal of Fertil. and Steril., 2(4): 147-164.

Aitken, R.J.; Baker, M.A. and Sawyer D.(2003). Oxidative stress in the male germ line and its role in the aetiology of male infertility

and genetic disease. Reprod. Biomed. Online ,7:65-70.

Alberts, B.; Johnson, A.; Lewis, J.; Raff, M.; Roberts, K. and Walter, P.(2002). Peroxisomes, in Molecular Biology of the Cell . 4th ed. Garland .

Alexander, P.Q. and Dokras , A.(2008). Diagnosis and Treatment of Unexplained Infertility. Obstet. Gynecol. J.,1(2):69-79.

Ali, J., Shahata, M.A. and Al-Natsha, S.D.(2000). Formulation of a protein-free medium for human assisted reproduction. Human Reprod., 15: 145–156.

Allaire, C.(2006). Endometriosis and infertility. Reprod. Med. J., 51:164–168.

Ames, B. N.; Profet, M. and Gold, L. S. (1990). Proc. Natl. Acad. Sci. USA ,87: 7777-7781.

American Society for Reproductive Medicine (ASRM).(2004). Definition of 'infertility'. Fertil. and Steril. J. ,82 (1): 206.

Ayaori, M.; Hisada, T. and Suzukawa, M.(2000). Plasma levels and redox status of ascorbic acid and levels of lipid peroxidation products in active and passive smokers. Environ. Health Perspect.,108:105–8.

Balusik, L.D.(2003). The Female Reproductive System & Cycle: Perspectives from the West & the East.

Battino, M.; Ferreiro, M.S.; Galalrdo, I. ; Newman, H.N. and Bullon, P.(2002). The antioxidant capacity of saliva. J. Clin. Periodontol.,29:189-94.

Bayer, R. (1960). Treatment of infertility with vitamin E. Int. J. Ferti., 5:70–78.

Bedaiwy, M.A.; Falcone ,T.; Sharma, R.K.; Goldberg, J.M.; Attaran, M., Nelson, D.R. and Agarwal, A. (2002). Prediction of endometriosis with serum and peritoneal fluid markers: a prospective controlled trial. Hum. Reprod. , 17:426–431.

Bedaiwy, M.A.; Falcone, T. and Mohamed, M.S.(2004). Differential growth of human embryos in vitro: role of reactive oxygen species. Fertil.and Steril., 82: 593–600.

Bedaiwy ,M.A.; Agarwal ,A. and Falcone ,T. (2005). Relationship of follicular fluid oxidative stress parameters and outcome of intracytoplasmic sperm injection. Fertil. Steril. J., 4:S250.44.

Behrman, H.R.; Kodaman, P.H.; Preston , S.L. and Gao, S.(2001). Oxidative stress and the ovary. J. of the Society for Gynecol.Investigation,8: S40–42.

Benov, L. and Fridovich, I.(1998). Growth in iron-enriched medium partially compensates Escherichia coli for the lack of manganese and iron superoxide dismutase. Bio.l Chem. J., 273: 10313-10316.

Bergamini ,C.M.; Gambetti, S.; Dondi, A. and Cervellati, C.(2004). Oxygen,reactive oxygen species and tissue damage. Curr. Pharm. Des.,10: 1611-26.

Bishop, M.L.; Engelkirk,D. and Fody, E.P.(2000). Clinical Chemistry, 4th ed. Philadelphia .

Boivin, J.; Bunting, L.; Collins, JA. and Nygren, KG.(2007). International estimates of infertility prevalence and treatment-seeking: potential need and demand for infertility medical care. Hum. Reprod.,22:1506–1512.

Boon, E.M.; Downs, A. and Marcey, D.(2007). Catalase: H_2O_2: H_2O_2 Oxidoreductase. Catalase Structural Tutorial .Text.2-11.

Bosco ,L., Ruvolo ,G. and Morici ,G.(2005). Apoptosis in human unfertilized oocytesafter intracytoplasmic sperm injection. Fertil. Steril. J., 84:1417–1423.

Boyer, R.(2000). Modren Expermental Biochemistry, 3ed .,San Francisco, Addisoi .Wesley Longman .

Brioukhanov ,A.; Netrusov, A. and Eggen,R. (2006). The catalase and superoxide dismutase genes are transcriptionally up-regulated upon oxidative stress in the strictly anaerobic archaeon Methanosarcina barkeri.. Microbiology, **152**: 1671–1677.

Bruce,N. ;Mark, K. and Tory, M.(1993). Oxidants, antioxidants, and the degenerative diseases of aging(cancer/mutation/endogenous DNA adducts/oxygen radicals). Proc. Natl. Acad. Sci. USA, 90: 7915-7922.

Burtis, C.A. and Ashwood, E.R. (1999). Textbook of Clinical Chemistry, 3rd ed., Philadelphia, W.B.Saunders Co.

Cameron,**I.T. and Campbell ,S.(1998). Nitric oxide** in the endometrium. European Society of Hum. Reprod. and Embryology,4(5):565.

Carbone, M.C.; Tatone, C. and Monache, S.(2003). Antioxidant enzymatic defences in human follicular fluid: characterization and age-dependent changes. Hum. Reprod., 9:639–643 .

Chan, A.C.(1993). Partners in defense, vitamin E and vitamin C. J Physiol Pharmacol , 71:725-731.

Chavarro, J.E.; Rich–Edwards, J.W.; Rosner, B.A. and Willett, W.C.(2007). Diet and lifestyle in the prevention of ovulatory disorder infertility. Obstet. Gyneco., l 110: 1050–1058.

Cheeseman ,K.H. and Slater, T,F. (1993). An introduction to free radical biochemistry. Med Bull , 49:481–493.

Chen, Y.(2007). Hepatocyte-specificGclc deletion leads to rapid onset of steatosis with mitochondrial injury and liver failure. Hepatology ,45: 1118.

Chitra ,S. and Devi, S. (2008). Effect of alpha tocopherol on proxidant and antioxidant enzymes in radiation treated oral squamous cell carcinoma. Indian J.Med.Sci.,62(4):141-148.

Christophersen, A. G.; Jun, H.; Jŕgensen, K., and Skibsted, L. H. (1991). Photobleaching of astaxanthin and canthaxanthin: quantum-yields dependence of solvent, temperature, and wavelength of irradiation in relation to packageing and storage of carotenoid pigmented salmonoids. Z. Lebensm. Unters. Forsch., 192:433-439.

Cohen , G.; Dembiec , D. and Marcus , J.(1970). Measurement of catalase activity .Analyt.Biochem., 34 : 30-38.

Crha, I.; Hruba, D.; Ventruba, P.; Fiala, J.; Totusek, J.; and Visnova, H.(2003). Ascorbic acid and infertility treatment. Cent Eur J Public Health ,11: 63–67.

Crosignani ,P.G.; Walters, D.S. and Soliani, A.(1992). Addendum to the ESHRE multicentre trial: a summary of the abortion and birth statistics, Hum. Reprod., 7:286- 1992 .

Crosignani, P.G.; Collins, J.; Cooke, I.D.; Diczfaluzy ,E. and Rubin, B.(1993). Unexplained infertility. Hum. Reprod., 8:977.

Czeizel, A.E.; Metneki, J. and Dudas, I. (1994). Higher rate of multiple births after periconceptional vitamin supplementation. N Engl J Med, 330:1687–1688.

Dacie ,S.J.V. and Lewis,S.M.(2005). Practical hematology.9th ed. Churchil, living stone , London.

Davis, M.B.; Austin , J.; and Partridge, D.A.(1991). Vitamin C: Its Chemistry and Biochemistry. Cambridge: Royal Society of Chemistry.

Dalvit ,G.; Llanes, S.P.; Descalzo, A.; Insani, M.; Beconi, M. and Cetica,P.(2005). Effect of alpha-tocopherol and ascorbic acid on bovine oocyte in vitro maturation. Reprod Domest Anim, 40: 93–97.

Daniel,W.W.(1999). Probability and t distribution biostatistics:A foundation for analysis in health science.7th ed.83-123.John willey and Sons ,INC-USA.

De Bruin, J.P.; Dorland, M.; Spek, E.R.; Posthuma, G.; van Haaften, M.; Looman, C.W. and te Velde, E.R.(2002). Ultrastructure of the resting ovarian follicle pool in healthy young women. Biol Reprod.,**66**:1151–1160.

De.Cherney, A.;Nathan, L.; Eskandari, M. and Cadieux, M.(2003). Current Obstetric &Gynecologic Diagnosis & Treatment. McGraw Hill, New York, 6:979-990.

De Souza, M.J. **and Williams, .I.(2004)**. Physiological Aspects and Clinical Sequelae of Energy Deficiency and Hypoestrogenism in Exercising Women. Human Reproduction Update, 10(5):433-448.

Dietrich, M.; Block, G.; Norkus, E.P.; Hudes, M.; Trabe, M.G. and Cross L. (2003). Smoking and exposure to environmental tobacco smoke decrease some plasma antioxidants and increase gamma-tocopherol in vivo after adjustment dietary antioxidant intakes. Amer. J Clin. Nutr., 77(1):160-166.

Dong, M.; Shi, Y. and Cheng, Q.(2001). Increased nitric oxide in peritoneal fluid from women with idiopathic infertility and endometriosis. J Reprod Med , 46:887-91.

Doumas, B. T.; Watson, W.A. and Biggs, H.G.(1971). Clin. Chem. Acta., 31: 87.

Duckitt, K. 2003. Infertility and subfertility. Clin. Evid.,2044-2073.

Dudas, I.; Rockenbauer, M. and Czeizel, A.E.(1995). The effect of preconceptional multivitamin supplementation on the menstrual cycle. Arch Gynecol. Obstet. J. , 256:115–123.

Duleba, A.J.; Foyouzi,N. and Karaca, M.(2004). Proliferation of ovarian theca-interstitial cells is modulated by antioxidants and oxidative stress. Human Reproduction, 19: 1519–1524.

Dumollard, R.; Duchen, M. and Carroll, J.(2007). The role of mitochondrial function in the oocyte and embryo. Curr. Top Dev. Biol. ,77:21–49.

Ebisch, M.W.; **Peters**, **W.H.M.**; **Thomas, C.M.G**.; **Wetzels**, **A.M.M.**; **Peer**, **P.G.M.** and **Theunissen**., **R.P.M.**(2006). Homocysteine, glutathione and related thiols affect fertility parameters in the (sub)fertile couple. Hum. Reprod., 21(7):1725-1733.

Ebisch ,I.M.; Thomas, C.M.; Peters ,W.H.; Braat, D.D. and Steegers-Theunissen, R.P.(2007). The importance of folate, zinc and antioxidants in the pathogenesis and prevention of subfertility. Hum. Reprod. Update , 13:163–174.

Edmonds, K.(2007). Dewhurst's text book of obstetric and gynecology.7th ed.Massachusetts.USA,440-458.

Edward, L. M.(2008). Unexplained Infertility. Fertility Centers of Illinois.

Evers, J.L.H.2002. Female subfertility. Lancet ;360:151–159.

Fissore, R. A.; Kurokawa, M.;Knott, J.; Zhang, M. and Smyth, J.(2002). Mechanisms underlying oocyte activation and postovulatory ageing. Reproduction J., 124:745 -754.

Franceschi ,R. T.(1992). The role of ascorbic acid in mesenchymal differentiation. Nutr. Rev. , 50:65-70 .

Fujii ,J.; Iuchi, Y. and Okada, F.(2005). Fundamental roles of reactive oxygen species and protective mechanisms in the female reproductive system. Reprod. Biol. Endocrinol., 3:43–53.

Gaby, S.K. and Singh, V.N. (1991). "Vitamin C," – Vitamin intake and health: A Scientific Review, Gaby, S.K.,Bendich, A., Singh, V. and Machlin, L. (eds.). Marcel. Dedder., N.Y.

Ganong,M.D. ;William, S and Deloris, .J .(2005). Review of Medical Physiology. 22 th ed. United States of California .San Francisco.

Gardiner, C.S.; Salmen, J.J.; Brandt, C.J.and Stover, S.K.(1998). Glutathione is present in reproductive tract secretions and improves development of mouse embryos after chemically induced glutathione depletion. Biol. Reprod.,59:431–436.

Giordano, G.(2007). Organophosphorus insecticides chlorpyrifos and diazinon and oxidative stress in neuronal cells in a genetic model of glutathione deficiency. Toxicol. Appl. Pharmacol. ,219 (2-3): 181.

Gleicher, N.and Barad, D.(2006). Unexplained infertility: does it really exist? Hum. Reprod. J.,21: 1951–1955.

Gold, L. S.; Slone, T. H.; Stern, B. R.; Manley, N. B. and Ames, B. N. (1992). Science, 258:261-265.

Goldman, M.B.; Missmer, S.A. and Barberi ,R.L. (2000). Infertility. In:Women and Helath . San Diego: Academic Press., 196–214.

Golubovsky , M.(2008). Unexplained infertility in Charles Darwin's family: genetic aspect. Human Reproduction , 23(5):1237-1238.

Goto, T.; Jones, G.M.; Lolatgis, N.; Pera, M.F.; Trounson ,A.O. and Monk M.(2002). Identification and characterisation of known and novel transcripts expressed during the final stages of human oocyte maturation. Mol. Reprod .Dev., 62:13–28.

Griveau ,J.F and Le Lannou, D.(1997). Reactive oxygen species and human spermatozoa: physiology and pathology. Int. J. Androl., 20:61–69.

Groff, J.L.; Gropper S.S. and Hunt S.M.(1995). The Water Soluble Vitamins. In: Advanced Nutrition and Human Metabolism. Minneapolis : West Publishing Company, 222-237.

Guerin, P.; El Mouatassim, S. and Menezo, Y. (2001). Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. Hum. Reprod. Update, 7:175–189.

Guyton ,A.C. and Hull, J.E.(2006). Text book of medical physiology. 11th ed. W.B. Saunders , Phyladelphia.1011-1026.

Hakim ,R.B.; Gray, R.H. and Zacur ,H. (1998). Alcohol and caffeine consumption and decreased fertility. Fertil Steril ,70:632–637.

Hashim, S.A. and Schuttringer, G.R(1966). Rapid determination of tocopherol in macro-and micro quantities of plasma results obtained in various nutrition and metabolic studies. Am.J. Clin.Nutr.,19 (2):137-145.

Herrera, E. and Barbas, C. (2001). Vitamin E: action, metabolism and perspectives. J. Physiol. Biochem., 57 (2): 43 – 56.

Ikeda, S.; Kitagawa, M.; Imai, H. and Yamada, M.(2005). The roles of vitamin A for cytoplasmic maturation of bovine oocytes. J. Reprod. Dev. ,51:23–35.

Iqbal ,K.; Khan ,A. and Khattak, A.K.(2004). Biological Significance of Ascorbic Acid (Vitamin C) in Human Health .Pakistan J. of Nutrition,3 (1): 5-13.

Jackson, L.W.; Schisterman ,E.F.; Browne, R.W. and Armstrong, D. (2005). Oxidative stress and female fecundity. Society of Pediatric and Perinatal Reproductive Epidemiologic Research.

Janicki,D.(2008).oxidative stress .pittsburgh mind body center.

José M. ;Matés, A. and Sánchez-Jiménez,F.(1999). Antioxidant Enzymes and their Implications in Pathophysiologic Processes, 4: 339-345.

Kawakami ,E.; Kashiwagi, C.; Hori, T. and Tsutsui, T. (2001) .Effects of canine oviduct epithelial cells on movement and capacitation of homologous spermatozoa in vitro. Anim. Reprod. Sci., 68:121–131.

Krajcir, N.; Chowdary, H.; Gupta, S. and Agarwal , A.(2008). Female Infertility and Assisted Reproduction: Impact of Oxidative Stress. Current Women's Health Reviews, 4, 9-15 9.

Lauffer, R. B. (1992). Iron and Human Disease .CRC, Boca Raton, FL.

Lenzi,A.; Sgro, P.; Salacone, P.; Paoli, D.;Gilio, B.; Lombardo, F.; Santulli, M.; Agarwal, A. and Gandini, L.(2004). A placebo-controlled double-blind randomized trial of the use of combined l-carnitine and l-acetyl-carnitine treatment in men with asthenozoospermia. Fertil. Steril. , 81:1578–1584.

Luberda, Z.(2005). The role of glutathione in mammalian gametes. Reprod. Biol.J. , 5:5–17.

Lukaski, H.C.(2004). Vitamin and mineral status: effects on physical performance. Nutrition, 20: 632-644.

Lunec, J. (1990). Review Article , Ann Clin. Biochem. , 27:173 .

Maiorino, M. and Ursini, F.(2002). Oxidative stress, spermatogenesis and fertility. Biol. Chem.,383:591-7.

Makinde ,K.A. and Adedeji, O.O. (1994). Comparative study of vitamin E levels of Nigerian men and age-matched fertile and infertile women. J. Nutr. Med. , 4:39-42.

McConnachie ,L.A.;Mohar, I. and Hudson, F.N.(2007). Glutamate cysteine ligase modifier subunit deficiency and gender as determinants of acetaminophen-induced hepatotoxicity in mice. Toxicol. Sci., 99 (2): 628–36.

Menkveld, R.;Wong, W.Y.; Lombard, CJ.; Wetzels, AMM.; Thomas, CMG., Merkus, M. and Steegers-Theunissen, P.M.(2001). Semen

parameters, including WHO and strict criteria morphology, in a fertile and subfertile population: an effort towards standardization of in-vivo thresholds. Hum. Reprod.,16:1165–1171.

Mehendale, S.S.; KilariBams, A.S.; Deshmukh, C.S.; Dhorepatil, B.S.; Nimbargi, V.N. and Joshi, S.R.(2009). Oxidative stress-mediated essential polyunsaturated fatty acid alterations in female infertility.Hum. Fertil.J.,12(1):28-33

Moffatt ,O.; Drury, S.; Tomlinson ,M.; Afnan, M. and **Sakkas, D.(2002).** The apoptotic profile of human cumulus cells changes with patient age and after exposure to sperm but not in relation to oocyte maturity. Fertil. Steril.J. , 77:1006–1011.

Nathan,L.(2007). Current obstetric and gynecologic diagnosis and treatment.9th ed.USA.979-990.

Packer, L.; Weber, S.U. and Rimbach, G. (2001). "Molecular aspects of alpha-tocotrienol antioxidant action and cell signalling. J. Nutr., 131 (2): 369–373.

Parslow,T.G.;Stites,D.P.;Abbal,I.T.and.John,B.I.(2001). Medical immunology ,10 th ed., lange Medical Books, McGraw –Hill/ Medical publishing division ,New York.

Paszkowski, T.; Traub, A.I.; Robinson, S.Y. and McMaster, D. (1995). Selenium dependent glutathione peroxidase activity in human follicular fluid. Clin. Chim. Acta., 236: 173–180.

Paszkowski ,T. and Clarke, R.N.(1999). The Graafian follicle is a site of L-ascorbate accumulation. J. Assist. Reprod. Genet.,16:41-5.

Paszkowski ,T.; Clarke, R. and Hornstein, M. (2002). Smoking induces oxidative stress inside the Graafianfollicle. J. Obstet. Gynecol. Reprod. Biol.,17(4):921-925.

Pierce, J.D.; Cackler, A.B. and Arnett, M.G.2004. Why should you care about free radicals? .RN.j., 67:38–42.

Pendyala, G.; Thomas, B. and Kumari, S.(2008). The challenge of antioxidants to free radicals in periodontitis. Human Reprod.;12(3):79-83.

Pereira ,C.F. and de Oliveira, C.R. (2000). "Oxidative glutamate toxicity involves mitochondrial dysfunction and perturbation of intracellular Ca2+ homeostasis". Neuroscience Research, 37 (3): 227–36.

Perry,R.L.; Barratt1,C.L;Warren,M.A. and Cooke,L.D.(1996). Comparative study of the effect of human cervical mucus and a cervical mucus substitute, Healonid, on capacitation and the acrosome reaction of human spermatozoa in vitro. Hum.Reprod. ;.11 (5): 1055-1062.

Polak, G.; Rola, R. and Gogacz, M.(1999). Malonyldialdlehydle and total antioxidant status in peritoneal fluid of infertile women. GinekolPol. ,70:135-40.

Polak ,G.; Montewka, M. and Gogacz ,M. (2000). Total antioxidant status and activity of an extracellular superoxide dismutase in peritoneal fluid and plasma from women with unexplained infertility. Ginekol. Pol.,71:571-6.

Polak, G.; Montewka ,M.; Gogacz, M.; Blaszkowska, I. and Kotarski,J.(2001). Total antioxidant status of peritoneal fluid in infertile women. Eur. J. Obstet. Gynecol. Reprod. Biol., 94: 261-3.

Pollack,M. and Leeuwenburgh,C.(2004). Molecular mechanisms of oxidative stress in aging: free radicals, aging, antioxidants and disease.

Prcker,L.;Rosei,P.;Tritschler,H.J.;King,G..and.Rzzi,R.(2000). Antioxidants in diabetes management. Marceld Ekkerin, C. New york.

Pyari,J.S.; Rekha,S. and Agarwal.A.(2006). Free radicals in female infertility. J. Obstet. Gynecol. India , 56(1): 64-67.

Quinlan,T.; Spivack,S. and Mossman,B.T(1994). Regulation of Antioxidant Enzymes in Lung after Oxidant Injury. Environmental Health Perspectives, 102(2):77-81.

Rifal, B. and Tietz,A.(1999). Text Book of Clinical Chemistry, 3 ed ed., Saunders Publadelshia.

Rigotti, A .(2007). Absorption, transport, and tissue delivery of vitamin E. Mol. Aspects Med., 28 (5-6): 423–36.

Rod,R.S.; Trent,D.S. and Philip ,D.A.(1996). Anatomy and physiology.3rd ed. Mosby. New York . Phyladelphia.957-970.

Ruder,E.; Hartman, J.; Blumberg ,J. and Goldman ,M.(2008). Oxidative stress and antioxidants: exposure and impact on female fertility. Human Reproduction and Embryology J.

Ruder, E.H.; Hartman ,T.J. and Goldman, M.B.(2009). Impact of oxidative stress on female fertility. Curr. Opin. Obstet. Gynecol.;Jun,21(3):219-22.

Sadraie, S.H.; Saito, H.; Kaneko, T.; Saito, T. and Hiroi, M. (2000). Effects of aging on ovarian fecundity in terms of the incidence of apoptotic granulosa cells. J. Assist Reprod. Genet. ; 17:168–173.

Saleh ,R.A. and Agarwal, A.(2002). Oxidative stress and male infertility:from research bench to clinical practice. J. Androl, 23:737-52.

Saleh ,R.A.; Agarwal, A.; Sharma, R.K.;Nelson, D.R. and Thomas, A.J.(2002). Effect of cigarette smoking on levels of seminal oxidative stress in infertile men: a prospective study. Fertil. Steril. J.,78:491-9.

Savita, S. M.; Anitha, S. K.; Chaya ,S. D. and Bharati,A . (2009). Oxidative stress-mediated essential polyunsaturated fatty acid alterations in female infertility .Human Fertility,12(1) 28 – 33.

Scully, R.E. and Cohen, R.B.(1964). Oxidative-enzyme activity in normal and pathologic human ovaries. Obstet. and Gynecol., 24: 667–681.

Speroff, L.; Glass, R.H. and Kase, N.G. (1999). Female infertility. Clinical Gynecologic Endocrinology and Infertility, 6th edn. Lippincott Williams & Wilkins, Philadelphia, PA, USA.

Sharma ,R.K. and Agarwal, A.(1996). Role of reactive oxygen species in male infertility. Urology J.,48:835-50.

Shatavi, S.V.; Lanes, B.L. and Judith, L.(2006). Association of unexplained infertility with gonadotropin and ovarian antibodies. Am J Reprod Immunol , 56:286–291.

Sies ,H. (1999). Glutathione and its role in cellular functions. Free Radical Biology and Medicine ,27: 916–921.

Siristatidis ,C. and Bhattacharya,S.(2007). Unexplained infertility: does it really exist? Does it matter?. Human Reproduction J., 22(8)2084-2087.

Smith ,S.; Pfiefer, S.M. and Collins, J. (2003). Diagnosis and management of female infertility. J.A.M.A. ,290:17.

Sohal, R.S.(1993). The free radical hypothesis of aging: an appraisal of the current status. Aging Clin Exp Res.,5:3-17.

Sohal, R.S.; Sohal, B.H. and Orr,W.C. (1995). Free Radicals. Biol Med,19(4):499-504.

Stone,I.(1972). The Natural History of Ascorbic Acid in the Evolution of Mammals and Primates.

Suarez ,S.S. and Pacey ,A.A.(2005). Sperm transport in the female reproductive tract. Human Reproduction Update, 12(1):23-37.

Svirbelf, L. and Szent Gyorgyi,A.(2007). The Chemical Nature Of Vitamin C. Part of the National Library of Medicine collection.

Sugino, N.; Nakata ,M. and Kashida, A.(2000). Decreased superoxide dismutase expression and increased concentration of lipid peroxide and prostaglandin F2" in the deciduas of failed pregnancy. Mol. Hum. Reprod., 6(7): 642-647.

Sugino, N.; Karube-Harada, A.; Taketani, T.; Sakata, A. and Nakamura, Y.(2004). Withdrawal of ovarian steroids stimulates prostaglandin F2alpha production through nuclear factorkappaB activation via oxygen radicals in human endometrial stromal cells: potential relevance to menstruation. J. Reprod. Dev., 50: 215-25.

Sun,J.G.; Jurisicova, A. and Casper, R.F.(1997). Detection of deoxyribonucleic acid fragmentation in human sperm: correlation with fertilization in vitro.Biol. Reprod., 56:602–7.

Takahashi ,T.,; Takahashi, E.; Igarashi ,H.; Tezuka, N. and Kurachi, H.(2003). Impact of oxidative stress in aged mouse oocytes on calcium oscillations at fertilization. Mol. Reprod. Dev., 66: 143–152.

Talevi ,R. **;Zagami** ,M.; **Castaldo , M.** and **Gualtieri,R.(2007).** Redox Regulation of Sperm Surface Thiols Modulates Adhesion to the Fallopian Tube Epithelium. Biology of reproduction , **76**:728–735.

Tao ,Y.; Zhou ,B.; Xia, G. and Wang, F.(2004). Exposure to L-ascorbic acid or alpha-tocopherol facilitates the development of porcine denuded oocytes from metaphase I to metaphase II and prevents cumulus cells from fragmentation. Reprod. Domest. Anim., 39: 52–57.

Tarin ,J.J.(1996). Potential effects of age-associated oxidative stress onmammalian oocytes/embryos. Mol. Hum. Reprod., 2: 717–724.

Tarin, J.; Ten, J.; Vendrell, F.J.; de Oliveira, M.N. and Cano, A. (1998). Effects of maternal ageing and dietary antioxidant supplementation on ovulation, fertilisation and embryo development in vitro in the mouse. Reprod. Nutr. Dev. , 38:499–508.

Tarin ,J.J.; Perez-Albala ,S. and Cano, A.(2002). Oral antioxidants counteract the negative effects of female aging on oocyte quantity and quality in the mouse. Mol. Reprod. Dev. , 61:385–397.

Tatemoto, H.; Sakuraim N.and Muto, N.(2000). Protection of porcine oocytes against apoptotic cell death caused by oxidative stress during In vitro maturation: role of cumulus cells. Biology of Reproduction ,63 805–810.

Tatemoto, H.; Ootaki, K.; Shigeta ,K. and Muto, N.(2005). Enhancement of developmental competence after in vitro fertilization of porcine oocytes by treatment with ascorbic acid 2-O-alpha-glucoside during in vitro maturation. Biol. Reprod., 65: 1800–1806.

Tiboni ,G.M.; Bucciarelli, T.;Giampietro, F.; Sulpizio, M. and DiIlio, C.(2004). Influence of cigarette smoking on vitamin E, vitamin A, beta-carotene and lycopene concentrations in human pre-ovulatory follicular fluid. Int. J. Immunopathol. Pharmacol. , 17:389–393.

Tirouvanziam ,R.; Conrad, C.K.; Bottiglieri, T.; Herzenberg, L.A., Moss, R.B. and Herzenberg, L.A.(2006). High-dose oral N-acetylcys;eine, a glutathione prodrug, modulates inflammation in cystic fibrosis. PNAS, 103 (12): 4628–33.

Tremellen ,K.(**2008)**. Oxidative stress and male infertility—a clinical perspective .Hum. Reprod. J. , 14(3):243-258.

Trevisan ,M.; Browne, R.; Ram, M.; Muti, P.; Freudenheim, J.; Carosella, A.M. and Armstrong, D.(2001). Correlates of markers of oxidative status in the general population. Am. J. Epidemiol. , 154:348–356.

Tsuji, M.; Ito, Y.; Terada, N. and Mori H.(1989). Ovarian aromatase activity in scorbutic mutantrats unable to synthesize ascorbic acid. Acta. Endocrinol. (Copenh) , 121:595-602.

Tummon, I.S.;Macklin ,V.M.; Radwanska, E.; Binor, Z. and Dimowski, W.P.(1988). Occult ovulatory dysfunction in women with minimal endometriosis or unexplained infertility, Fertil. Steril., 50:716.

Turton. M. and Luderer. U. (2006). Opposing Effects of Glutathione Depletion and FSH on Reactive Oxygen Species and Apoptosis in Cultured Preovulatory Rat Follicles. Endocrinology, 147: 1224-1236.

Uerin, P.;El Mouatassim, S. and Menezo ,Y. (2001). Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. Hum. Reprod. Update, 7:175–189.

Urner, F. and Sakkas, D. (2005). Involvement of the pentose phosphate pathway and redox regulation in fertilization in the mouse. Mol. Reprod. Dev., 70,494 -503.

Ursini, F.; Heim, S.; Kiess, M.; Maiorino, M.; Roveri, A.,; Wissing, J. and Flohe, L.(1999). Dual function of the selenoprotein PHGPx during sperm maturation. Science,**285**:1393–1396.

Valentine ,A. A.;Linda ,P. H.;David, J. C. and Julian, M. J.(2004). Differences in time to natural conception between women with unexplained infertility and infertile women with minor

endometriosis European Society of Human Reprod. and Embryo.,19(1): 96-103.

Van Langendonckt, A.; Casanas-Roux, F.; Donnez, J.(2002). Oxidative stress and peritoneal endometriosis. Fertil. Steril. J.,77:861–870.

Varley, H.; Gowenlock, A.H. and Bell, M.(1991). Practical Clinical Biochemistry Vol.1, 5th ed., London.

Vega, M.;Johnson, M.C.; Diaz ,H.A.; Urrutia, L.R.; Troncoso, J.L. and Devoto, L.(1998). Regulation of human luteal steroidogenesis in vitro by nitricoxide. Endocrine ,8:185-19120.

Wang,Y.; Sharma, R.K.; Falcone, T.; Goldberg, J. and Agarwal, A.(1997). Importance of reactive oxygen species in the peritoneal fluid of women with endometriosis or idiopathic infertility. Fertil. Steril. J. , 68:826–830.

Wei, Y.H.; Lu, C.Y. and Lee, H.C.(1998). Oxidative damage and mutation to mitochondrial DNA and age-dependent decline of mitochondrial respiratory function. Ann. N. Y. Acad. Sci. ,854:155–70.

Westphal, L.M.; Polan, M.L.; Trant, A.S. and Mooney, S.B. (2004), A nutritional supplement for improving fertility in women: a pilot study, Reprod. Medicine J., 49 (4):289-93.

Wiener-Megnazi, Z.; Vardi, L.; Lissak, A.; Shnizer, S.; Reznick, A.Z,; Ishai, D.; Lahav-Baratz, S.; Shiloh, H.; Koifman, M. and Dirnfeld, M.(2004). Oxidative stress indices in follicular fluid as measured by the thermochemiluminescence assay correlate with outcome parameters in in vitro fertilization. Fertil. Steril. J., 82(3):1171–1176.

White, C. W. (2007). Product and process for liquefaction of mucus or sputum.United.States.Patent,7:195.

World Health Organization(WHO).(1991). Infertility: A Tabulation of Available Data on Prevalence of Primary and Secondary Infertility.

World Health Organization(WHO).(1992). The influence of varicocele on parameter of infertility in a large group of men presenting to infertility clinic.Fertil.steril. J.,57:1289-1293.

World Health Organization(WHO).(1999). Laboratory manual for the examination of human semen and sperm–cervical mucus interaction. Cambridge University Press, Cambridge , 60–61.

Yeh, J.; Bowman, M.J.; Browne, R.W. and Chen, N..2005. Reproductive aging results in a reconfigured ovarian antioxidant defense profile in rats. Fertil. Steril. J. ;84(2):1109–1113.

Yoshida, M.; Ishigaki, K.; Nagai ,T.; Chikyu, M. and Pursel ,V.G.(1993). Glutathione concentration during maturation and after fertilization in pig oocytes: relevance to the ability of oocytes to form male pronucleus. Biol. Reprod. ,49:89–94.

Yu, S.L. and Yap, C.(2003). Investigating the infertile couple. Ann Acad. Med. Singapore , 32:611-613.

Zini ,A.; Garrels, K. and Phang, D.(2000). Antioxidant activity in the semen of fertile and infertile men. Urology J.,55:922-6.

Zuelke, K.A.; Jeffay ,S.C.; Zucker, R.M. and Perreault, S.D.(2003). Glutathione (GSH) concentrations vary with the cell cycle in maturing hamster oocytes, zygotes, and pre-implantation stage embryos. Mol. Reprod. Dev. , 64:106–112.

Summary

Infertility is one of the most important and underappreciated reproductive health problems in developing countries.

The causes of infertility can be found in about 90% of cases, while about 10% of patients don't know why they can not conceive .The causes of this case (unexplained infertility) seems to be heterogeneous, with suggested potential causes ranging from disturbances in endocrinological, immunological , genetic and reproductive physiological factors.

One of the main causes of unexplained infertility is the reactive oxygen species (ROS). Therefore this study is aimed to estimate and calculate the ROS and to determine the relationship between free radicals, antioxidant enzymes activities and unexplained infertility. To carry out this aim, ROS has been estimated in both sera and cervical mucus secretions among healthy fertile women (control) and patients with unexplained infertility ; in form of malondialdehyde (MDA). In addition, catalase (CAT), glutathione level (GSH), vitamin C and vitamin E (as antioxidant agents) for both fertile women and infertile women studied groups have estimated and calculated.

Moreover, other assessing physiological parameters have been done. These include menstrual phase duration ,date of menarche ,body mass index, ABO blood group percentage ,total serum protein ,serum albumin and serum globulin levels in fertile women and patients with unexplained infertility.

The study groups were attended to Babylon Maternity and Pediatric Hospital and privet clinics. All studied patients are suffering from primary or secondary unexplained infertility types and are diagnosed by gynecologist. The study is carried out on (30) apparently healthy fertile women as a control group, their mean age (30.133±8.011years) and (60) infertile women as patient group , their mean age (29.866 ±7.195 years).

The results of the tests have shown that the malondialdehyde (MDA) were increased significantly ($p<0.01$) in serum and cervical mucus secretion(3.105± 1.441µM and 6.729±3.055)µM in patients group comparing with control group which where (1.524 ±0.518 µM and 3.201± 1.028µM) respectively.

Catalase levels decrease significantly($p<0.05$) in serum and cervical mucus secretion (0.303± 0.286 k/ml and 0.331±0.302 k/ml) in patients group comparing with(0.479±0.342 k/ml and 0.508±0.301 k/ml) in control group.

The glutathione levels, on the other hand, decrease significantly ($p<0.05$) in serum and cervical mucus secretion (17.977±8.506 µM and 51.602 ±27.335µM) in patients group comparing with (27.384 ±13.547µM and 65.327±18.217µM) in control group.

Vitamin C levels decrease insignificantly($p>0.05$) in serum and cervical mucus secretion(9.944 ± 1.549mg/l and14.233 ±3.458mg/l) in patient group comparing with(10.383±3.655mg/l and(16.447± 4.042mg/l) in control group.

Vitamin E levels decrease significantly ($p<0.05$) in serum and cervical mucus secretion(5.272 ± 1.228mg/l and 4.644 ± 1.343 mg/l) in patient groups comparing with (7.337±3.535 mg/l and 7.023± 0.754 mg/l) in control groups.

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List of Abbreviations

Symbol	Word or phrase
α	Alpha
ASRM	American Society for Reproductive Medicine
BMI	body mass index
C°	Degree centigrade
DNA	Deoxyribonucleic acid
FSH	Follicle stimulating hormone
K	katal
LH	Luteinizing hormone
mg/l	milligram per liter
µmole	Micromole
MW	Molecular weight
nm	Nanometer
nm	nanometer
nmole	Nanomole
OS	Oxidative stress
SFA	seminal fluid analysis
TSP	Total serum protein
TSH	Thyroid stimulating hormone
WHO	World Health Organization
UV	Ultraviolet

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