The review of literature of the present study entitled “Assessment of Oocyte Quality with AMH, Inhibin B in Serum and Follicular Fluid and Predicting Pregnancy outcome with Sperm DNA Fragmentation in Art Cycles” were discussed under the following headings.

2.1 Gonads and gametes
2.2 Male reproductive system
2.3 Causes of male infertility
2.4 Production and maturation of sperm
   2.4.1 Spermatogenesis
   2.4.2 Spermiogenesis
   2.4.3 Maturation and capacitation of sperm
2.5 Structure of a mature sperm
2.6 Semen parameters
   2.6.1 Semen analysis
   2.6.2 Importance of sperm morphology
2.7 Sperm DNA damage
   2.6.1 Importance of sperm DNA
   2.6.2 Methods of Sperm DNA assessment
   2.6.3 Acridine orange method
2.6.4 Human sperm DNA and chromatin structure
2.6.5 Etiology of sperm DNA damage
2.6.6 Influence of sperm DNA damage in natural and Assisted pregnancy

2.8 Female reproductive system

2.9 Causes of female reproductive system

2.10 Oogenesis

2.11 Menstrual cycle

2.12 Ovarian reserve and formation of ovarian follicle

2.13 Structure and fate of ovum

2.14 Hormonal control of ovarian and uterine cycles

2.15 Role of hormones in female reproductive system
   2.15.1 Follicle stimulating hormone
   2.15.2 Leutinizing hormone
   2.15.3 Estradiol
   2.15.4 Progesterone
   2.15.5 Antimullarian hormone(AMH)
   2.15.6 Inhibin B

2.1 GONADS AND GAMETES

   The cells that carry out the special function of reproduction are called gametes. The development of a new individual begins when one male gamete (sperm or spermatozoon) meets and fuses with one female gamete (ovum or oocyte). The process of fusion of male and female gametes is called fertilization. The fused ovum and spermatozoon form the zygote. The zygote later develops into an embryo and then into a fetus.
The male sex cells (spermatozoa) are produced in the male gonads (testes) while the female sex cells (ova) are produced in female gonads (ovaries). The formation of spermatozoa in the testis is called spermatogenesis while the formation of ova in the ovary is called oogenesis. The two are collectively referred to as gametogenesis (Pal and Singh, 2007).

2.2 MALE REPRODUCTIVE SYSTEM

The male reproductive system consists of the testes and a series of ducts and glands. Sperm are produced in the testes and are transported through the reproductive ducts. These ducts include the epididymis, ductus deferens, ejaculatory duct and urethra. The reproductive glands produce secretions that become part of semen, the fluid that is ejaculated from the urethra. These glands include the seminal vesicles, prostate gland, and bulbourethral gland.

The testes (singular, testis) are located in the scrotum (a sac of skin between the upper thighs). In the male fetus, the testes develop near the kidneys, and then descend into the scrotum just before birth. Each testis is about 1 1/2 inches long by 1 inch wide. Testosterone is produced in the testes which stimulates the production of sperm as well as give secondary sex characteristics beginning at puberty.

The two testicles are each held in a fleshy sac called the scrotum. The major function of the scrotal sac is to keep the testes cooler than thirty-seven degrees Celsius (ninety-eight point six degrees Fahrenheit). The external appearance of the scrotum varies at different times in the same individual depending upon temperature and the subsequent contraction or relaxation of two muscles. These two muscles contract involuntarily when it is cold to move the testes closer to the heat of the body in the pelvic region. This causes the scrotum...
to appear tightly wrinkled. On the contrary, they relax in warm temperatures causing the testes to lower and the scrotum to become flaccid. The temperature of the testes is maintained at about thirty-five degrees Celsius (ninety-five degrees Fahrenheit), which is below normal body temperature. Temperature has to be lower than normal in order for spermatogenesis (sperm production) to take place. The two muscles that regulate the temperature of the testes are the dartos and cremaster muscles.

The dartos muscle is a layer of smooth muscle fibers in the subcutaneous tissue of the scrotum (surrounding the scrotum). This muscle is responsible for wrinkling up the scrotum, in conditions of cold weather, in order to maintain the correct temperature for spermatogenesis.

The cremaster muscle is a thin strand of skeletal muscle associated with the testes and spermatic cord. This muscle is a continuation of the internal oblique muscle of the abdominal wall, from which it is derived as Seminiferous Tubules

Each testis contains over 100 yards of tightly packed seminiferous tubules. Around 90% of the weight of each testis consists of seminiferous tubules. The seminiferous tubules are the functional units of the testis, where spermatogenesis takes place. Once the sperms are produced, they moved from the seminiferous tubules into the rete testis for further maturation. In between the seminiferous tubules within the testes, are interstitial cells, or, Cells of Leydig. They are responsible for secreting the male sex hormones (i.e., testosterone). Sertoli cell (a kind of sustentacular cell) is a 'nurse' cell of the testes which is part of a seminiferous tubule. It is activated by follicle-stimulating hormone, and has FSH-receptor on its membranes. Its main function is to nurture the
developing sperm cells through the stages of spermatogenesis. Because of this, it has also been called the "mother cell." It provides both secretary and structural support.

Other functions during the maturation phase of spermiogenesis, the Sertoli cells consume the unneeded portions of the spermatozoa. The sperm are transported out of the testis and into the epididymis through a series of efferent ductules. The testes receive blood through the testicular arteries (gonadal artery). Venous blood is drained by the testicular veins. The right testicular vein drains directly into the inferior vena cava. The left testicular vein drains into the left renal vein.

The seminiferous tubules join together to become the epididymis. The epididymis is a tube that is about 20 feet long that is coiled on the posterior surface of each testis. Within the epididymis the sperm complete their maturation and their flagella become functional. This is also a site to store sperm until the next ejaculation. Smooth muscle in the wall of the epididymis propels the sperm into the ductus deferens.

The ductus (vas) deferens, also called sperm duct, or, spermatic deferens, extends from the epididymis in the scrotum on its own side into the abdominal cavity through the inguinal canal. The inguinal canal is an opening in the abdominal wall for the spermatic cord (a connective tissue sheath that contains the ductus deferens, testicular blood vessels, and nerves. The smooth muscle layer of the ductus deferens contracts in waves of peristalsis during ejaculation.

The pair of seminal vesicles is posterior to the urinary bladder. They secrete fructose to provide an energy source for sperm and alkalinity to enhance
sperm mobility. The duct of each seminal vesicle joins the ductus deferens on that side to form the ejaculatory duct.

There are two ejaculatory ducts. Each receives sperm from the ductus deferens and the secretions of the seminal vesicle on its own side. Both ejaculatory ducts empty into the single urethra.

The prostate gland is a muscular gland that surrounds the first inch of the urethra as it emerges from the bladder. The smooth muscle of the prostate gland contracts during ejaculation to contribute to the expulsion of semen from the urethra.

The bulbo urethral glands also called Cowper's glands are located below the prostate gland and empty into the urethra. The alkalinity of seminal fluid helps neutralize the acidic vaginal pH and permits sperm mobility in what might otherwise be an unfavorable environment.

The penis is an external genital organ. The distal end of the penis is called the glans penis and is covered with a fold of skin called the prepuce or foreskin. Within the penis are masses of erectile tissue. Each consists of a framework of smooth muscle and connective tissue that contains blood sinuses, which are large, irregular vascular channels.

The urethra, which is the last part of the urinary tract, traverses the corpus spongiosum and its opening, known as the meatus, lies on the tip of the glans penis. It is both a passage for urine and for the ejaculation of semen (Valerie et al., 2006)

**Figure 1 : The Male Reproductive System**
2.3 CAUSES OF MALE INFERTILITY

The most common cause for male infertility is a problem with the sperm - either low sperm count or sperm with poor quality. Sperm with poor quality cannot move rapidly enough or in the right direction, or may be abnormally shaped. Some conditions that may contribute to sperm problems include:

- **Under-developed testes** - usually arising after a mumps infection, a hernia surgery, an injury or birth defect.
- **Swollen veins in the scrotum.**
- **Undescended testes** - a problem often present from birth in which the testes remain in the body cavity. Normally they descend into the scrotum before birth.
- **Infections**, such as gonorrhea or **tuberculosis**, that block the ducts through which the sperm travel.
- **Exposure to metals** such as leads, or chemicals such as pesticides.
- **Certain medications**, such as Tagamet (cimetidine), Dilantin (phenytoin), Folex (methotrexate), Axulfidine (sulfasalazine), corticosteroids and chemotherapy drugs such as Cytoxan and Neosar (cyclophosphamide).

- **Injury to the testicles**

- **Chronic prostate infections**

Other common causes of male infertility are:

- **Autoimmunity**, in which antibodies or cells of the man's immune system attack sperm cells, mistaking them for toxic invaders.

- The antibodies attach themselves to the sperm and may cause them to stick together, or may stop them from penetrating the cervical mucous or the egg.

- **Retrograde ejaculation**. In retrograde ejaculation the muscles of the urethra do not force the sperm out. Instead, the sperm travel backward into the bladder. Causes of retrograde ejaculation include:

  - **Drugs such as tranquilizers** or high blood pressure medicines.
  
  - **Diseases such as diabetes or multiple sclerosis**.

  - Neck, bladder or prostate surgery.

  - Spinal cord injury.

In a small percentage of cases, male infertility is caused by:

- Sexual difficulties such as impotence, premature ejaculation, or painful intercourse. These problems can often be easily treated.
• Genetic defects or structural problems. In germ-cell aplasia, for instance, the sperm-producing germ cells do not develop correctly. Defects in the Y chromosome or in certain genes may also play a part in infertility.

• Rarely, a hormonal difficulty that decreases or stops the man's production of sperm. Hormonal problems may be present from birth or can develop from brain or pituitary gland tumors or radiation treatment. Sometimes, hormonal difficulties are induced by excessive exercise, malnutrition or other illnesses.

2.4 PRODUCTION OF SPERM AND MATURATION

2.4.1 Spermatogenesis

In a human male, the formation of gametes (spermatozoa) takes place only during the reproductive period, which begins at the age of puberty (12 to 16 years) and continues even through old age. Spermatozoa are formed in the walls of the seminiferous tubules of the testes. If we look at one of these tubules under a microscope, we find that there are many cells of different sizes and shapes. Most of these represent stages in the formation of spermatozoa, but some (called Sertoli cells) have only a supporting function. The various cell-stages in Spermatogenesis are as follows (the number of chromosome each stage)

• The spermatogonia (type A) or germ cells (44 + X + Y) divide mitotically, to give rise to more spermatogonia of type A, and also to spermatogonia of type B

• The spermatogonia (type B) (44 + X + Y) enlarge, or undergo mitosis, to form primary spermatocytes.
• The primary spermatocytes (44 + X + Y) now divide so that each of them forms two secondary spermatocytes. This is the first meiotic division: it reduces the number of chromosomes to half.

• Each secondary spermatocyte has 22 + X or 22 + Y chromosomes. It divides to form two spermatids. This is the second meiotic division and this time there is no reduction in chromosome number.

• Each spermatid (22 + X or 22 + Y) gradually changes its shape to become a spermatozoon. This process of transformation of a circular spermatid to a spermatozoon is called spermiogenesis.

2.4.2 Spermiogenesis

The process by which a spermatid becomes a spermatozoon is called spermiogenesis (or spermateleosis). The spermatid is a more or less circular cell containing a nucleus, Golgi apparatus, centriole and mitochondria. All these components take part in forming the spermatozoon. The nucleus forms the head. The Golgi apparatus is transformed into the acrosomic cap. The centriole divides into two parts that are at first close together: the axial filament appears to grow out of them. One centriole becomes spherical and comes to lie in the neck. According to some workers, the other centriole forms the basal body, but according to some others it forms the annulus. The part of the axial filament between the neck and the annulus, becomes surrounded by mitochondria, and together with these forms the middlepiece. The remaining part of the axial filament elongates to form the principal piece or tail. Most of the cytoplasm of the spermatid is shed, but the cell membrane persists as a covering for the spermatozoon.

The process of spermatogenesis, including spermiogenesis, requires about two months for its completion.
2.4.3 Maturation and capacitation of spermatozoa

When first formed in seminiferous tubules, spermatozoa are immature. They are non-motile and incapable of fertilizing an ovum. A current of fluid in seminiferous tubules carries spermatozoa from the testis to the epididymis. Here they are stored and undergo maturation. As spermatozoa pass through the epididymis they undergo a process of maturation.

Changes take place in glycoproteins of the plasma membrane covering the sperm head. Spermatozoa acquire some motility after maturation but become
fully motile only after ejaculation when they get mixed up with secretion of the prostate gland and seminal vesicles.

Spermatozoa acquire the ability to fertilize an ovum after they have been in the female genital tract for some time. This final step in their maturation is called capacitation. In the process of capacitation, the glycoprotein coat and seminal proteins lying over the surface of the spermatozoon are altered. Spermatozoa usually undergo capacitation in the uterus or uterine tube, under the influence of substances secreted by the female genital tract. When a spermatozoon comes in contact with the zona pellucida, changes take place in the membranes over the acrosome and enable the release of lysosomal enzymes. This is called the acrosome reaction. Some enzymes help in digesting the zona pellucida and in penetration of the spermatozoa through it. Changes in the properties of the zona pellucida constitute the zona reaction.

2.5 STRUCTURE OF MATURE SPERMATOZOOON

A spermatozoon is a highly specialized, free swimming, actively motile cell. The spermatozoon has a head, a neck, a middle piece and a principal piece. An axial filament passes through the middle piece and extends into the tail. The spermatozoon measures about 60 um in length.

The Head

The head of the human spermatozoon is oval in shape and measures 4 um in length. It is derived from the nucleus, which consists of 23 highly condensed chromosomes. The head is covered by a cap-like structure called the acrosome (also called the acrosomic cap, or galea capitis). The acrosome contains enzymes that help in penetration of the spermatozoon into the ovum during fertilization.
The Neck

The neck is narrow: it contains a funnel-shaped basal body and a spherical centriole. The basal body is also called the connecting piece because it helps to establish an intimate connection between the head and the remainder of the spermatozoon.

The Axial Filament

The axial filament begins just behind the centriole. It passes through the middle piece and most of the tail. At the point where the middle piece joins the tail, the axial filament passes through a ring-tike structure called the annulus. The part of the axial filament, which lies in the middle piece, is surrounded by a spiral sheath made up of mitochondria.

The axial filament is actually composed of several fibrils. There is a pair of central fibrils, surrounded by nine pairs (doublets) arranged in a circle around the central pair. The whole system of fibrils is kept in position by a series of coverings. Immediately outside the fibrils there is a fibrous sheath. In the region of the middle piece the fibrous sheath is surrounded by spirally arranged mitochondria. Finally the entire spermatozoon is enclosed in a plasma membrane (Pal and Singh 2007)

Figure 3: The structure of a mature spermatozoon
2.6 SEMEN PARAMETERS

2.6.1 Semen analysis

Semen analysis comprises a set of descriptive measurements of spermatozoa and seminal fluid parameters that help to estimate semen quality. Conventional semen analysis includes measurement of particular aspects of spermatozoa such as concentration, motility and morphology and of seminal plasma. Quantification and identification of non-spermatozoal cells and detection of antisperm antibodies are also part of basic semen analysis. Normal values of semen parameters issued by the World Health Organization (WHO) in 1992 are generally used as reference values (Table 1).
Results and Discussion

Assessment of Oocyte Quality with AMH, Inhibin B, in Serum and Follicular Fluid and Predicting Pregnancy outcome with Sperm DNA Fragmentation in Art Cycles

Table 1. Normal values of semen variables (WHO 1992)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Normal Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume</td>
<td>2.0 ml or more</td>
</tr>
<tr>
<td>pH</td>
<td>7.2-8.0</td>
</tr>
<tr>
<td>Sperm concentration</td>
<td>20x10^6 spermatozoa/ml or more</td>
</tr>
<tr>
<td>Total sperm count</td>
<td>40x10^6 spermatozoa per ejaculate or more</td>
</tr>
<tr>
<td>Motility</td>
<td>50% or more with forward progression(categories a and b) or 25% or more with rapid progression(category a) within 60 minutes of ejaculation</td>
</tr>
<tr>
<td>Morphology</td>
<td>30% or more with normal forms</td>
</tr>
<tr>
<td>Vitality</td>
<td>75% or more live, i.e., excluding dye</td>
</tr>
<tr>
<td>White blood cells</td>
<td>fewer than 1x10^6/ml</td>
</tr>
</tbody>
</table>

Table 2. Nomenclature for semen variables (WHO 1992)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normozoospermia</td>
<td>Normal ejaculate as defined in table I</td>
</tr>
<tr>
<td>Oligozoospermia</td>
<td>Sperm concentration fewer than 20x10^6/ml</td>
</tr>
<tr>
<td>Asthenozoospermia</td>
<td>Fewer than 50% spermatozoa with forward progression(categories a and b) or fewer than 25% spermatozoa with category a movement</td>
</tr>
<tr>
<td>Teratozoospermia</td>
<td>Fewer than 30% spermatozoa with normal morphology</td>
</tr>
<tr>
<td>Oligoasthenoteratozoospermia</td>
<td>Signifies disturbance of all three variables (combination of only two prefixes can be used)</td>
</tr>
<tr>
<td>Azoospermia</td>
<td>No spermatozoa in the ejaculate</td>
</tr>
</tbody>
</table>
2.6.2 Importance of sperm morphology

A spermatozoon is considered normal when the head has a smooth, oval configuration with a well-defined acrosome comprising about 40% - 70% of the sperm head. In addition, there must be neck, midpiece or tail defects and no cytoplasmic droplets of more than one half the size of the sperm head. At least 100 but preferably 200 spermatozoa normal, with another abnormality present, large heads or amorphous heads all with or without tail, neck or midpiece defects, loose heads, immature germinal cells and unknown cells were recorded separately and reported. In classical in-vitro fertilization a decrease in sperm concentration, progressive motility and particularly sperm cell morphology has been shown to be significant value in predicting fertilization and pregnancy rates.

Miller et al., (2001) showed that sperm cell morphology could be significantly correlated with blastocyst development and Salumats et al., (2002) concluded blastocyst cleavage rate was also determined by sperm cell morphology. Host et al., (2001) however have shown that WHO criteria correlated with fertilization rate, embryo development, and score or pregnancies in couples undergoing IVF.

A number of more recent studies also indicate that sperm morphology does in fact influence ICSI fertilization and subsequent pregnancy outcome. Levran et al., (1998) recorded the sperm morphology of each injected sperm cell, and found that fertilization declined with the following morphological appearance; short acrosome region, round head, and amorphous head defect. No pregnancies occurred in the amorphous head defect group. De Vos et al., (2003) also evaluated the influence of individual spermatozoa on ICSI fertilization and

<table>
<thead>
<tr>
<th>Aspermia</th>
<th>no ejaculate</th>
</tr>
</thead>
</table>

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pregnancy outcome. Their results showed a lower fertilization, pregnancy and implantation rate after injection of morphologically abnormal spermatozoa.

Figure 4: Normal spermatozoa

Figure 5: Amorphous head

Figure 6: Tapered head

Figure 7: Tapered:Pyriform (dumpbell) severe
Results and Discussion

Assessment of Oocyte Quality with AMH, Inhibin B, in Serum and Follicular Fluid and Predicting Pregnancy outcome with Sperm DNA Fragmentation in Art Cycles

Figure 8: Small acrosome area

Figure 9: Vacuolated head

Figure 10: Bent mid piece

Figure 11: Irregular mid-piece

Figure 12: Cytoplasmic droplets

Figure 13: Coiled tail
2.7 SPERM DNA DAMAGE

Infertility is a major clinical problem with male factor responsible for 40% percent cases of total infertility. Current estimates indicate that nearly 20 percent cases of idiopathic infertility have high level of sperm DNA fragmentation, a negative factor for infertility. Full sperm DNA integrity usually is defined as the absence of DNA nicks or single stranded breaks, double stranded breaks, and chemical modifications of the DNA. Of these double stranded DNA breaks are the most mutagenic, because in the pronuclues stage zygote, the two genomes are separated, hence DNA template information for error free repair of the double strand breaks is absent.
Much higher degree of negative association between the degree of DNA damage with various indices of fertility such as fertilization rate, embryo cleavage rate, implantation rate, pregnancy rate and live birth rate have been observed. The spontaneous abortion rate is about 1.7 fold higher when more than 30 percent of sperms contain fragmented DNA. The chances of live birth and conception decreases drastically when DNA fragmentation index (DFI) is > 30 percent. (Evenson et al., 2002)

2.7.1 Importance of sperm DNA

Sperm DNA is increasingly being recognized as an important measure of fertilizing efficiency that has better diagnostic and prognostic capabilities than standard sperm parameters like sperm morphology, concentration and motility. Routine semen parameter does not reflect the quality of sperm DNA. Men with normal spermatograms may still be infertile: the cause could be related to abnormal sperm DNA (Agarwal 2004). Sperm DNA integrity may be evaluated in addition to routine sperm parameters to indicate the quality of DNA.

According to recent studies when sperm deformity index is greater than 1.6, the chances of ART failure increases (Agarwal et al., 2003). Small DNA damages in sperm are repaired by pre and post replication repair mechanisms, but large DNA damages not be repaired. Thus, though infertile men may have sperms with normal morphology, these germ cells may harbour damaged DNA. This results in pregnancy loss or birth of offsprings with major or minor congenital malformation, severe dysmorphogenesis or may lead to increased predisposition to certain cancers like retinoblastoma. Thus DNA integrity studies are of foremost important evaluation of all infertile males prior to assisted conception.

2.7.2 Methods of sperm DNA fragmentation assessment
There are different methods to detect the sperm DNA fragmentation. These techniques help to detect subtle defects in the chromatin structure or DNA integrity, and thereby assist in semen quality assessment.

These assays include simple staining techniques such as acidic aniline blue and toluidine blue stains, fluorescent staining techniques such as sperm chromatin in dispersion test, chromomycin A3. DNA breakage detection fluorescent in situ hybridization assay and in-situ nick translation and flow cytometric based sperm chromatin structure assay. The other method includes acridine orange method and TUNEL assay.

**Table 3 : Basic assay for Sperm DNA fragmentation Assessment**

<table>
<thead>
<tr>
<th>Type of assay</th>
<th>Basis of assay</th>
<th>Measured parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DIRECT ASSAYS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. TUNEL</td>
<td>Adds labeled nucleotides to free DNA ends</td>
<td>% of cells with labeled DNA</td>
</tr>
<tr>
<td></td>
<td>Template independent</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Labels SS and DS breaks</td>
<td></td>
</tr>
<tr>
<td>2. COMET</td>
<td>Electro phoresis of single sperm cells</td>
<td>% of sperm with long tails (tail length, % of DNA in tail)</td>
</tr>
<tr>
<td></td>
<td>DNA fragments form tail</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Intact DNA stays in head</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Alkaline COMET</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Alkaline conditions, denatures all DNA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Identifies both SS and DS breaks</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Neutral COMET</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Does not denature DNA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Identifies DS breaks, may be some SS breaks</td>
<td></td>
</tr>
<tr>
<td>3. IN SITU NICK TRLS</td>
<td>Incorporates biotinylated dUTP at SS DNA breaks with DNA polymerase I</td>
<td>% of cells with incorporated dUTP (fluorescent cells)</td>
</tr>
<tr>
<td></td>
<td>Template- dependent</td>
<td></td>
</tr>
<tr>
<td>Type of assay</td>
<td>Basis of assay</td>
<td>Measured parameter</td>
</tr>
<tr>
<td>------------------------------------</td>
<td>---------------------------------------</td>
<td>----------------------------------------------------------</td>
</tr>
<tr>
<td><strong>INDIRECT ASSAYS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. DNA BREAK DETECTION FISH</td>
<td>Denatures nicked DNA</td>
<td>Amount of fluorescence proportional to number of DNA breaks</td>
</tr>
<tr>
<td></td>
<td>Whole genome probes binds to SS DNA</td>
<td></td>
</tr>
<tr>
<td>2. SCD</td>
<td>Individual cells immersed in agarose</td>
<td>% of sperm with small or absent halos</td>
</tr>
<tr>
<td></td>
<td>Denatured with acid then lysed</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Normal sperm produce halo</td>
<td></td>
</tr>
<tr>
<td>3. Acridine orange mild treatment assays (e.g: SCSA.SDFA)</td>
<td>Mild acid treatment denatures DNA with SS or DS breaks</td>
<td>DFI—the percentage of sperm with a ratio of red to (red + green) fluorescence greater than the main cell population</td>
</tr>
<tr>
<td></td>
<td>Acridine orange binds to DNA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DS DNA (non denatured) fluoresces green</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SS DNA (denatured) fluoresces red</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Flow cytometry counts thousands of cells</td>
<td></td>
</tr>
<tr>
<td>4. ACRIDINE ORANGE ASSAY</td>
<td>Same as above hand-counting of green and red cells</td>
<td>% of cells with red fluorescence</td>
</tr>
</tbody>
</table>

### 2.7.3 Acridine orange assay

The AO assay measures the susceptibility of sperm nuclear DNA to acid induced denaturation in-situ by quantifying the metachromatic shift of AO fluorescence from green (native DNA) to red (denatured DNA). The fluorochrome AO intercalates into double stranded DNA as a monomer and binds to single stranded DNA as a monomer and binds to single stranded DNA as an aggregate. The monomeric AO bound to nature fluorescence green, whereas the
aggregated AO on denatured DNA fluorescence red. The AO assay is a biologically stable measure of sperm quality. The interassay variability is less than 5% rendering the technique highly reproducible. A strong positive correlation exists between the AO assay and other techniques used to evaluate SS DNA.(Hoshi et al. 1996)

**Figure 18: Acridine orange stain showing Green signal (DS DNA) and Red signal (denatured DNA)**

2.7.4 Human Sperm DNA and Chromatin Structure

Unlike the relatively loose structure of chromatin in somatic cells, sperm chromatin is very tightly compacted by virtue of the unique associations between the DNA and sperm nuclear proteins (predominantly protamines; Ward and Coffey, 1991; Brewer et al., 1999). During the later stages of spermatogenesis, the spermatid nucleus is remodeled and condensed, and this is associated with the displacement of histones by transition proteins and then by
protamines (Steger et al., 2000). The DNA strands are tightly wrapped around the protamine molecules, forming tight and highly organized loops (Brewer et al., 1999). Intermolecular and intramolecular disulfide cross-links between the cysteine-rich protamines are responsible for the compaction and stabilization of the sperm nucleus, and it is thought that this nuclear compaction is important to protect the sperm genome from external stresses such as oxidation or temperature elevation (Kosower et al., 1992).

In humans, sperm chromatin is tightly packaged by protamines, but up to 15% of the DNA remains packaged by histones at specific DNA sequences (ie, there is a nonrandom association between histones and DNA sequences; Gatewood et al., 1987). The histone-bound DNA sequences are less tightly compacted, and it is thought that these DNA sequences and/or genes may be involved in fertilization and early embryo development (Gatewood et al., 1987; Gineitis et al., 2000). Infertile men have a higher sperm histone to protamine ratio when compared with fertile controls (Oliva, 2006 and Zhang et al., 2006). An excess of nuclear histones may result in poorer chromatin compaction and an increased susceptibility to DNA damage (Cho et al., 2001, 2003 and Aoki et al., 2005, 2006).

2.7.5 Etiology of Sperm DNA Damage

The etiology of sperm DNA damage is multifactorial: it may be due to intrinsic (eg, protamine deficiency, excess reactive oxygen species [ROS] levels, and apoptosis), or extrinsic factors (eg, testicular hyperthermia, environmental toxins). Sperm DNA damage is clearly associated with male infertility (and abnormal spermatogenesis) but a small percentage of spermatozoa from fertile men also possess detectable levels of DNA damage (Kodama et al., 1997; Evenson et al., 1999; Spano et al., 2000; Zini et al., 2001).
2.7.6 Influence of Sperm DNA Damage on Natural and Assisted Pregnancy

Several studies suggest that sperm DNA damage is associated with lower rates of natural insemination and intrauterine insemination (IUI) pregnancies. Indeed, couples in whom the husband has a high percentage of spermatozoa with DNA damage have very low potential for natural fertility and a prolonged time to pregnancy (Evenson et al., 1999; Spano et al., 2000; Loft et al., 2003). A recent meta-analysis indicates a strong association between sperm DNA damage and failure to achieve a natural pregnancy (Evenson and Wixon, 2008). High levels of sperm DNA damage have generally been associated with lower IUI pregnancy rates (Duran et al., 2002; Muriel et al., 2006; Bungum et al., 2007).

Numerous studies have examined the influence of sperm DNA integrity on pregnancy rates after standard IVF. A systematic review and meta-analysis of IVF studies indicates that sperm DNA damage is associated with lower IVF pregnancy rates. (Collins et al., 2008).

A systematic review and meta-analysis of ICSI studies indicates that sperm DNA damage is not associated with ICSI pregnancy rates. These observations are in keeping with a recent meta-analysis (Collins et al., 2008) and suggest that sperm DNA damage has no measurable impact on pregnancy rates at ICSI. It is possible that the stringent process of sperm and embryo selection at ICSI (in humans) mitigates the potential adverse effect(s) of sperm DNA damage on reproductive outcomes (Gandini et al., 2004). Several studies have reported a relationship between sperm DNA damage and pregnancy loss after both standard IVF and IVF/ICSI. A systematic review and meta-analysis of IVF and ICSI studies shows that sperm DNA damage is associated with a significant increase in the rate of pregnancy loss after IVF and ICSI (P< .0001). These data suggest
that the adverse effect of sperm DNA damage on live birth rates is probably more significant than is reflected by the clinical pregnancy rates alone.

2.8 FEMALE REPRODUCTIVE SYSTEM

The female reproductive system is made up of the vagina, uterus and ovaries. The vagina leads into the female reproductive system and connects to the uterus through the cervix, which serves as a sort of barrier between the two organs. The ovaries contain eggs, the female sex cells, and also produce the female sex hormones estrogen and progesterone. Unlike the male reproductive system, which consistently produces sperm, the female reproductive system contains a limited amount of eggs. About once a month, the ovaries release one of these eggs, a process called ovulation. The egg travels down into the fallopian tubes, which connect to the uterus. If the egg doesn't come into contact with sperm, it's released out of the body through the vagina along with blood and mucus in a process called menstruation.

Figure 19: The female Reproductive system
2.9 CAUSES OF FEMALE INFERTILITY

Infertility in a woman may stem from many causes, such as hormonal deficiencies, problems in the reproductive organs, and some illnesses. Complications from surgery and certain medications may also impair fertility.

The most likely causes for female infertility are:

- **Pelvic Inflammatory Disease** (PID) - PID is the most common cause of infertility worldwide. It's an infection of the pelvis or one or more of the reproductive organs, including the ovaries, the fallopian tubes, the cervix or the uterus. Sometimes PID spreads to the appendix or to the entire pelvic area.

  PID usually stems from the same bacteria that cause sexually transmitted diseases, such as gonorrhea or chlamydia. Chlamydia, in fact, causes 75 percent of fallopian tube infections.
PID may also develop from bacteria that reach the reproductive organs through abortion, hysterectomy, childbirth, sexual intercourse, use of an intrauterine (IUD) contraceptive device or a ruptured appendix.

Not only does PID cause infertility, but it may also lead to ectopic pregnancy and blood poisoning, a potentially fatal complication.

- **Polycystic ovarian syndrome** (PCOS) - This condition affects 5 million American women and is another major cause of infertility. In PCOS, the ovaries produce high amounts of male hormones, especially testosterone. LH levels also remain abnormally high while FSH levels are abnormally low; thus, the follicles do not produce eggs. Instead they form fluid-filled cysts that eventually cover the ovaries.

Recent research indicates that PCOS is caused by the failure of muscle, fat and liver cells to accept glucose (the cellular fuel made from the food that humans eat). As a result, the pancreas begins churning out large amounts of insulin, a hormone that usually ushers glucose from the blood to the body's cells. This extra insulin plays havoc with the ovaries, as well as other parts of a woman's body.

PCOS not only causes infertility, but also increases the risk of diabetes, cancer and even heart disease. The symptoms include:

- Excessive facial hair
- Thinning hair
- Acne
- Depression
- Unexplained weight gain
• Irregular or no periods
• High insulin or cholesterol readings

- **Endometriosis** - This disease is another common cause of female infertility.

Endometriosis refers to a condition in which sections of the uterine lining implant in the vagina, ovaries, fallopian tubes or pelvis. These implants eventually form cysts that grow with each menstrual cycle, and may eventually turn into blisters and scars. The scars can then block the passage of the egg.

- **Other sexually transmitted diseases** such as genital herpes can decrease fertility.

- **Ovary Problems** - Decreased production of any one of the five hormones that regulate a woman's reproductive cycle may result in infertility. Problems within the ovaries may inhibit reproduction as well. Instead of releasing an egg, the ovarian follicle remains empty, fails to rupture or traps the egg.

- **Hormonal Problems** - Adrenal or thyroid deficiencies may cause hormonal and ovarian problems.

Some women produce excess amounts of prolactin, a hormone that normally stimulates the production of breast milk. Prolactin can also prevent ovulation. High levels of prolactin in a woman who is not nursing may indicate a pituitary tumor. It can also result from the use of **oral contraceptives**.

- **Immune System Problems** - Women may develop antibodies or immune cells that attack the man's sperm, mistaking it for a toxic invader. Certain
autoimmune diseases, in which the woman's immune cells attack normal cells in her own body, may also contribute to ovarian problems.

- **Luteal Phase Defect** - In a luteal phase defect, a woman's corpus luteum - the mound of yellow tissue produced from the egg follicle - may fail to produce enough **progesterone** to thicken the uterine lining. Then the fertilized egg may be unable to implant.

- **Fibroids** - Fibroids, or benign growths, may form in the uterus near the fallopian tubes or cervix. As a result, the sperm or fertilized egg cannot reach the uterus or implant there. Fibroids in the uterus are very common in women over age 30.

- **Other Uterine Problems** - Abnormal reproductive organs or endometritis (an abnormal swelling of the uterine lining) may make it difficult for the fertilized egg to implant.

- **Surgical Complications** - Scar tissue left after abdominal surgery can cause problems in the movement of the ovaries, fallopian tubes, and uterus, resulting in infertility. Frequent abortions may also produce infertility by weakening the cervix or by leaving scar tissue that obstructs the uterus.

- **Uterine muscle problems** - Some women may produce weak, infrequent or abnormal contractions in the uterus. During ovulation, these contractions usually push the sperm up to the fallopian tubes.

- **Poor quality cervical mucus** - Sometimes a woman's mucus fails to thin around the time of ovulation, and consequently it prevents the sperm from traveling through it. A cervical infection may also be the cause.

- **Illness** - Certain diseases, such as diabetes, kidney disease or high blood pressure may cause **infertility**. Ectopic pregnancy and some urinary tract infections may also elevate the risk of infertility.
Results and Discussion

Assessment of Oocyte Quality with AMH, Inhibin B, in Serum and Follicular Fluid and Predicting Pregnancy outcome with Sperm DNA Fragmentation in Art Cycles

• **Medications** - Many medicines, such as hormones, antibiotics, antidepressants, and pain killers may bring on temporary infertility. Commonly used medications such as aspirin and ibuprofen can also impair fertility if taken mid-cycle. Acetaminophen (Tylenol) pills can reduce the amount of estrogen and luteinizing hormones in the body, impairing fertility.

• **Premature Menopause** - Some women may experience premature menopause, when their ovaries stop producing eggs. Often the cause is excessive exercise or anorexia.

Other causes: some other contributors to infertility include excessive exercise, stress or anorexia.

**2.10 OOGENESIS**

The female gonad is the ovary. It has an outer part called the cortex and an inner part, the medulla. The cortex contains many large round cells called oogonia. All the oogonia to be used throughout the fertile life of a woman are produced at a very early stage (possibly before birth) and do not multiply thereafter.

Ova are derived from oogonia. In the late fetal period primary oogonia enlarge to form primary oocytes. At the time of birth all primary oocytes are in the prophase of the first meiotic division. Their number is about 40,000.

The primary oocytes remain, in prophase and do not complete their first meiotic division until they begin to mature and are ready to ovulate. The reproductive period of a female is between 12 to 50 years of age. With each menstrual cycle, a few primary oocytes (about 5 begin to 30) begin to mature and complete the first meiotic division shortly before ovulation.
The first meiotic division of a primary oocyte produces two unequal daughter cells. Each daughter cell has the haploid number of chromosomes. The large cell, which receives most of the cytoplasm, is called the secondary oocyte and the smaller cell is known as the first polar body.

**Figure 20: Oogenesis**
The secondary oocyte immediately enters the meiotic cell division. Ovulation takes place while the oocyte is in metaphase. The secondary oocyte remains arrested in metaphase till fertilization occurs.

The Second meiotic division is completed only if fertilization occurs. This division results two unequal daughter cells. The smaller daughter cell is called the second polar body. The first polar body may also divide during the second meiotic division.

If fertilization does not occur, the secondary oocyte fails to complete the second meiotic division, and degenerates in about 24 hours after ovulation.

In each menstrual cycle, 5 to 30 primary oocytes start maturing, but only one of them reaches maturity and is ovulated. The remaining degenerate.

During the entire reproductive life of a female only around 400 ova are discharged (out of 40,000 primary oocytes available) (Pal and Singh 2007)

2.11 MENSTRUAL CYCLE

The menstrual cycle is a cycle of physiological changes that can occur in fertile females. Overt menstruation (where there is blood flow from the uterus through the vagina) occurs primarily in humans. The menstrual cycle, under the control of the endocrine system, is necessary for reproduction. It is commonly divided into three phases: the follicular phase, ovulation, and the luteal phase; although some sources use a different set of phases: menstruation, proliferative phase, and secretory phase. The length of each phase varies from woman to woman and cycle to cycle, though the average menstrual cycle is 28 days. Menstrual cycles are counted from the first day of menstrual bleeding.
contraception interferes with the normal hormonal changes with the aim of preventing reproduction.

Stimulated by gradually increasing amounts of estrogen in the follicular phase, menses slow then stop, and the lining of the uterus thickens. Follicles in the ovary begin developing under the influence of a complex interplay of hormones, and after several days one or occasionally two become dominant (non-dominant follicles atrophy and die). Approximately mid-cycle, 24–36 hours after the Luteinizing Hormone (LH) surges, the dominant follicle releases an ovum, or egg in an event called ovulation. After ovulation, the egg only lives for 24 hours or less without fertilization while the remains of the dominant follicle in the ovary become a corpus luteum; this body has a primary function of producing large amounts of progesterone. Under the influence of progesterone, the endometrium (uterine lining) changes to prepare for potential implantation of an embryo to establish a pregnancy. If implantation does not occur within approximately two weeks, the corpus luteum will involute, causing sharp drops in levels of both progesterone and estrogen. These hormone drops cause the uterus to shed its lining in a process termed menstruation.

In the menstrual cycle, changes occur in the female reproductive system. A woman's first menstruation is termed menarche, and occurs typically around age 12. The end of a woman's reproductive phase is called the menopause, which commonly occurs somewhere between the ages of 45 and 55.

The menstrual cycle can be divided into several different phase. Menstruation is also called menstrual bleeding, menses, or a period. The flow of menses normally serves as a sign that a woman has not become pregnant. During the reproductive years, failure to menstruate may provide the first indication to a woman that she may have become pregnant.
Follicular phase

This phase is also called the proliferative phase because a hormone causes the lining of the uterus to grow, or proliferate, during this time. Through the influence of a rise in follicle stimulating hormone (FSH) during the first days of the cycle, a few ovarian follicles are stimulated. These follicles, which were present at birth and have been developing for the better part of a year in a process known as folliculogenesis, compete with each other for dominance. Under the influence of several hormones, all but one of these follicles will stop growing, while one dominant follicle in the ovary will continue to maturity. The follicle that reaches maturity is called a tertiary, or Graafian, follicle, and it forms the ovum.

As they mature, the follicles secrete increasing amounts of estradiol, an estrogen. The estrogens initiate the formation of a new layer of endometrium in the uterus, histologically identified as the proliferative endometrium. The estrogen also stimulates crypts in the cervix to produce fertile cervical mucus, which may be noticed by women practicing fertility awareness.

Ovulation

During the follicular phase, estradiol suppresses production of luteinizing hormone (LH) from the anterior pituitary gland. When the egg has nearly matured, levels of estradiol reach a threshold above which they stimulate production of LH. These opposite responses of LH to estradiol may be enabled by the presence of two different estrogen receptors in the hypothalamus: estrogen receptor alpha, which is responsible for the negative feedback estradiol-LH loop, and estrogen receptor beta, which is responsible for the positive estradiol-LH relationship. In the average cycle this LH surge starts around cycle day 12 and may last 48 hours.
The release of LH matures the egg and weakens the wall of the follicle in the ovary, causing the fully developed follicle to release its secondary oocyte. The secondary oocyte promptly matures into an ootid and then becomes a mature ovum. The mature ovum has a diameter of about 0.2 mm.

Which of the two ovaries—left or right—ovulates appears essentially random; no known left and right co-ordination exists. Occasionally, both ovaries will release an egg; if both eggs are fertilized, the result is fraternal twins.

After being released from the ovary, the egg is swept into the fallopian tube by the fimbria, which is a fringe of tissue at the end of each fallopian tube. After about a day, an unfertilized egg will disintegrate or dissolve in the fallopian tube.

Fertilization by a spermatozoon, when it occurs, usually takes place in the ampulla, the widest section of the fallopian tubes. A fertilized egg immediately begins the process of embryogenesis, or development. The developing embryo takes about three days to reach the uterus and another three days to implant into the endometrium. It has usually reached the blastocyst stage at the time of implantation.

In some women, ovulation features a characteristic pain called mittelschmerz (German term meaning middle pain). The sudden change in hormones at the time of ovulation sometimes also causes light mid-cycle blood flow.

**Luteal phase**

The luteal phase is also called the secretory phase. An important role is played by the corpus luteum, the solid body formed in an ovary after the egg has been released from the ovary into the fallopian tube. This body continues to
grow for some time after ovulation and produces significant amounts of hormones, particularly progesterone. Progesterone plays a vital role in making the endometrium receptive to implantation of the blastocyst and supportive of the early pregnancy; it also has the side effect of raising the woman's basal body temperature. There is a noted secretion of prolactin towards the end of the secretory phase.

After ovulation, the pituitary hormones FSH and LH cause the remaining parts of the dominant follicle to transform into the corpus luteum, which produces progesterone. The increased progesterone in the adrenals starts to induce the production of estrogen. The hormones produced by the corpus luteum also suppress production of the FSH and LH that the corpus luteum needs to maintain itself. Consequently, the level of FSH and LH fall quickly over time, and the corpus luteum subsequently atrophies. Falling levels of progesterone trigger menstruation and the beginning of the next cycle. From the time of ovulation until progesterone withdrawal has caused menstruation to begin, the process typically takes about two weeks, with ten to sixteen days considered normal. For an individual woman, the follicular phase often varies in length from cycle to cycle; by contrast, the length of her luteal phase will be fairly consistent from cycle to cycle.

Figure 21: The menstrual cycle
The loss of the corpus luteum can be prevented by fertilization of the egg; the resulting embryo produces human chorionic gonadotropin (hCG), which is very similar to LH and which can preserve the corpus luteum. Because the hormone is unique to the embryo, most pregnancy tests look for the presence of hCG.

2.12 OVARIAN RESERVE AND FORMATION OF OVARIAN FOLLICLE

According to the traditional standpoint the human ovary hold a decreasing reserve of oocytes from fetal life until the women enters menopause. The oocyte quantity reaches its peak already before birth around 20 weeks of gestation, with approximately seven million follicles. At birth the number has decreased to around one to two million and at the onset of puberty only 300 to 400,000 follicles are left. When a woman enters menopause at an average age of
51 years approximately 1000 follicles remain in the ovary (Faddy 2000). During the reproductive life of women, approximately 400 oocytes ovulate and at the same time hundreds of oocytes go through atresia and degenerate (Gougeon 1996).

**Formation of Ovarian Follicles**

Ova develop from oogonia present in the cortex of the ovary. The oogonia are surrounded by other cells that form the stroma. These stromal cells form ovarian or Graafian follicles that surround ova and protect them. The stages in the formation of a follicle are as follows:

**Figure 22: Formation of mature follicle from ovary**
• Some cells of the stroma become flattened and surround an oocyte. These flattened cells ultimately form the ovarian follicle and are, therefore, called follicular cells.

• The flattened follicular cells become columnar. Follicles up to this stage of development are called primordial follicles.

• A homogeneous membrane, the zona pellucida appears between the follicular cells and the oocyte.

• The follicular cells proliferate to form several layers. These constitute the membrana granulosa. The cells may now be called granulosa cells.
• A cavity (or antrum) appears within the membrana granulosa. With its appearance a follicle is formed (follicle = small sac)

• The cavity of the follicle rapidly increases in size. As a result, the wall of the follicle (formed by the granulosa cells) becomes relatively thin. The oocyte now lies eccentrically in the follicle, surrounded by some granulosa cells that are given the name cumulus oophoricus (or cumulus ovariucus). The cells that attach it to the wall of the follicle are given the name discus proligerus.

• As the follicle expands, the stromal cells surrounding the membrana granulosa become condensed to form a covering called the theca interna (theca = cover). The cells of the theca interna later secrete a hormone called oestrogen; and they are then called the cells of the thecal gland.

• Outside the theca interna some fibrous tissue becomes condensed to form another covering for the follicle called the theca externa. The ovarian follicle is now fully formed.

Ovulation

The shedding of the ovum from the ovary is called ovulation. The ovarian follicle is at first very small compared to the thickness of the cortex of the ovary. As it enlarges, it becomes so big that it not only reaches the surface of the ovary, but also forms a bulging in this situation. Ultimately, the follicle ruptures and the ovum is shed from the ovary. Just before ovulation the follicle may have a diameter of 15 mm. The stroma and theca on this side of the follicle become very thin. An avascular area (stigma) appears over the most convex point of the follicle. At the same time, the cells of the cumulus oophoricus become loosened by accumulation of intercellular fluid between them.
2.14 STRUCTURE AND FATE OF OVUM

Structure of the Ovum

The ovum that is shed from the ovary is not fully mature. It is in fact a secondary oocyte that is undergoing division to shed off the second polar body. The ovum is surrounded by the zona pellucida. Some cells of the corona radiata can be seen sticking to the outside of the zona pellucida. No nucleus is seen, as the nuclear membrane has dissolved for the second meiotic division. A spindle is, however, present. Between the cell membrane (or vitelline membrane) and the zona pellucida, a distinct perivitelline space is seen. The first polar body lies in this space. Note that the ovum is a very large cell and measures more than 100 \( \mu \text{m} \) in diameter. In contrast, most other cells of the body measure less than 10 \( \mu \text{m} \). (One \( \mu \text{m} \) is one thousandth of a millimetre).

Fate of the Ovum
The ovary is closely embraced by the fimbriated end of the uterine tube. The ovum is, therefore, easily carried into the tube, partly by the follicular fluid discharged from the follicle and partly by the activity of ciliated cells lining the tube. The ovum slowly travels through the tube towards the uterus, taking three to four days to do so. If sexual intercourse takes place at about this time, the spermatozoa deposited in the vagina swim into the uterus and into the uterine tube. One of these spermatozoa may fertilize the ovum. If this happens, the fertilized ovum begins to develop into an embryo. It travels to the uterus and gets implants in its wall. On the other hand if the ovum (secondary oocyte) is not fertilized it dies in 12 to 24 hours. It passes through the uterus into the vagina and is discharged.

**Figure 24 : Overall view of the process of fertilization and implantation**

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HORMONAL CONTROL OF OVARIAN AND UTERINE CYCLES
These cycles are under the control of various hormones. The hypothalamus acts as a major centre for the control of reproduction. It secretes the Gonadotropin – releasing hormones (GnRH), which in turn control the secretion of gonadotrophic hormones by the anterior pituitary gland (adenohypophysis).

There are two gonadotrophic hormones. They are the follicle stimulating hormone (FSH) and the luteinizing hormone (LH). In the first half of the menstrual cycle the GnRH acts on the anterior pituitary to release FSH. The FSH acts on the ovary and stimulates the formation and maturation of ovarian follicles. The maturing ovarian follicles now start secreting oestrogens. The repair and proliferation of endometrium takes place under the influence of oestrogens. The endometrial stroma progressively thickens the glands in it elongate and the spiral arteries begin to grow towards the surface epithelium.

The level of oestrogen rises to a peak about two days before ovulation. This leads to sudden increase in the level of LH secreted by the anterior pituitary (LH surge) about 24 to 36 hours before ovulation. The LH surge leads to ovulation; and the Graafian follicle is transformed into the corpus luteum.

The LH stimulates the secretion of progesterone by the corpus luteum. Though the secretion of progesterone predominates, some oestrogen is also produced. The combined action of oestrogen and progesterone stimulates the endometrial glands to secrete glycogen rich mucoid material.

If fertilization does not occur the granulosa cells produce the protein inhibin, which acts on the anterior pituitary and inhibits the secretion of gonadotrophins. This leads to regression of the corpus luteum.
Due to the regression of the corpus luteum there is a fall in the blood level of oestrogen and progesterone. The withdrawal of these hormones causes the endometrium to regress and triggers the onset of menstruation.

If fertilization occurs the corpus luteum does not regress. It continues to secrete progesterone and oestrogen. The secretory phase of endometrium continues and menstruation does not occur.

**Figure 25  Hormonal levels in menstrual cycle**

### 2.15 ROLE OF HORMONES
2.15.1 Follicle stimulating hormone

Follicle-stimulating hormone (FSH) is a hormone found in humans and other animals. It is synthesized and secreted by gonadotropes of the anterior pituitary gland. FSH regulates the development, growth, pubertal maturation, and reproductive processes of the body. FSH and Luteinizing hormone (LH) act synergistically in reproduction. FSH is a glycoprotein. Each monomeric unit is a protein molecule with a sugar attached to it; two of these make the full, functional protein. Its structure is similar to those of LH, TSH, and hCG. The protein dimer contains 2 polypeptide units, labeled alpha and beta subunits. The alpha subunits of LH, FSH, TSH, and hCG are identical, and contain 92 amino acids. The beta subunits vary. FSH has a beta subunit of 118 amino acids (FSHB), which confers its specific biologic action and is responsible for interaction with the FSH-receptor. The sugar part of the hormone is composed of fructose, galactose, mannose, galactosamine, glucosamine, and sialic acid, the latter being critical for its biologic half-life. The half-life of FSH is 3-4 hours. Its molecular wt is 30000. FSH stimulates the growth and recruitment of immature ovarian follicles in the ovary. In early (small) antral follicles, FSH is the major survival factor that rescues the follicles from atresia (programmed death of the somatic cells of the follicle and oocyte). In the luteal-follicle phase transition period the serum levels of progesterone and estrogen (primarily estradiol) decrease and no longer suppress the release of FSH, consequently FSH peaks at about day three (day one is the first day of menstrual flow). The cohort of small antral follicles is normally sufficiently in number to produce enough Inhibin B to lower FSH serum levels.

In addition, there is evidence that gonadotrophin surge-attenuating factor produced by small follicles during the first half of the follicle phase also exerts a negative feedback on pulsatile luteinizing hormone (LH) secretion amplitude,
thus allowing a more favorable environment for follicle growth and preventing premature luteinization. As a woman nears perimenopause the number of small antral follicles recruited in each cycle diminishes and consequently insufficient Inhibin B is produced to fully lower FSH and the serum level of FSH begins to rise. When the follicle matures and reaches 8-10 mm in diameter it starts to secrete significant amounts of estradiol. Normally in humans only one follicle becomes dominant and survives to grow to 18-30 mm in size and ovulate, the remaining follicles in the cohort undergo atresia. The sharp increase in estradiol production by the dominant follicle (possibly along with a decrease in gonadotrophin surge-attenuating factor) cause a positive effect on the hypothalamus and pituitary and rapid GnRH pulses occur and an LH surge results.

The increase in serum estradiol levels cause a decrease in FSH production by inhibiting GnRH production in the hypothalamus. The decrease in serum FSH level causes the smaller follicles in the current cohort to undergo atresia as they lack sufficient sensitivity to FSH to survive. Occasionally two follicles reach the 10 mm stage at the same time by chance and as both are equally sensitive to FSH both survive and grow in the low FSH environment and thus two ovulations can occur in one cycle possibly leading to non identical (dizygotic) twins (Fower et al., 2003: dipiro etal 2007)

2.15.2 Leutinizing hormone

Luteinizing hormone (LH, also known as lutropin) is a hormone produced by the anterior pituitary gland. In females, an acute rise of LH called the LH surge triggers ovulation and development of the corpus luteum.

LH is a heterodimeric glycoprotein. Each monomeric unit is a glycoprotein molecule; one alpha and one beta subunit make the full, functional
protein. Its structure is similar to the other glycoprotein hormones, follicle-stimulating hormone (FSH), thyroid-stimulating hormone (TSH), and human chorionic gonadotropin (hCG). The protein dimer contains 2 glycopeptidic subunits, labeled alpha and beta subunits, that are non-covalently associated (i.e. without any disulfide bridge linking them).

The alpha subunits of LH, FSH, TSH, and hCG are identical, and contain 92 amino acids in human. The beta subunits vary. LH has a beta subunit of 121 amino acids (LHB) that confers its specific biologic action and is responsible for the specificity of the interaction with the LH receptor. This beta subunit contains an amino acid sequence that exhibits large homologies with that of the beta subunit of hCG and both stimulate the same receptor, however, the hCG beta subunit contains an additional 24 amino acids, and the two hormones differ in the composition of their sugar moieties. The different composition of these oligosaccharides affects bioactivity and speed of degradation. The biologic half-life of LH is 20 minutes, shorter than that of FSH (3–4 hours) or hCG (24 hours).

In females, at the time of menstruation, FSH initiates follicular growth, specifically affecting granulosa cells. With the rise in estrogens, LH receptors are also expressed on the maturing follicle that produces an increasing amount of estradiol. Eventually at the time of the maturation of the follicle, the estrogen rise leads via the hypothalamic interface to the “positive feed-back” effect, a release of LH over a 24-48 hour period. This 'LH surge' triggers ovulation thereby not only releasing the egg, but also initiating the conversion of the residual follicle into a corpus luteum that, in turn, produces progesterone to prepare the endometrium for a possible implantation. LH is necessary to maintain luteal function for the first two weeks. In case of a pregnancy luteal function will be further maintained by the action of hCG (a hormone very
similar to LH) from the newly established pregnancy. LH supports thecal cells in the ovary that provide androgens and hormonal precursors for estradiol production.

2.15.3 Estradiol

**Estradiol** (E2 or 17β-estradiol) (also oestradiol) is a sex hormone. Estradiol is the predominant sex hormone present in females; however, it is present in males, although at lower levels, as well. It represents the major estrogen in humans. Estradiol has not only a critical impact on reproductive and sexual functioning, but also affects other organs including the bones.

Estradiol, like other steroids, is derived from cholesterol. After side chain cleavage and utilizing the delta-5 pathway or the delta-4 pathway, androstenedione is the key intermediary. A fraction of the androstenedione is converted to testosterone, which in turn undergoes conversion to estradiol by an enzyme called aromatase. In an alternative pathway, androstenedione is "aromatized" to estrone, which is subsequently converted to estradiol.

During the reproductive years, most estradiol in women is produced by the granulosa cells of the ovaries by the aromatization of androstenedione (produced in the theca folliculi cells) to estrone, followed by conversion of estrone to estradiol by 17β-hydroxysteroid dehydrogenase. Estradiol is produced not only in the gonads: In both sexes, precursor hormones, to be specific testosterone, are converted by aromatization to estradiol. In particular, fat cells are active to convert precursors to estradiol, and will continue to do so even after menopause. Estradiol is also produced in the brain and in arterial walls.

In the female, estradiol acts as a growth hormone for tissue of the reproductive organs, supporting the lining of the vagina, the cervical glands, the
endometrium, and the lining of the fallopian tubes. It enhances growth of the myometrium. Estradiol appears necessary to maintain oocytes in the ovary. During the menstrual cycle, estradiol that is produced by the growing follicle triggers, via a positive feedback system, the hypothalamic-pituitary events that lead to the luteinizing hormone surge, inducing ovulation. In the luteal phase estradiol, in conjunction with progesterone, prepares the endometrium for implantation. During pregnancy, estradiol increases due to placental production. In baboons, blocking of estrogen production leads to pregnancy loss, suggesting that estradiol has a role in the maintenance of pregnancy.

2.15.4 Progesterone

Progesterone also known as P4 (pregn-4-ene-3,20-dione) is a C-21 steroid hormone involved in the female menstrual cycle, pregnancy (supports gestation) and embryogenesis of humans and other species. Progesterone belongs to a class of hormones called progestogens, and is the major naturally occurring human progestogen. Like other steroids, progesterone consists of four interconnected cyclic hydrocarbons. Progesterone contains ketone and oxygenated functional groups, as well as two methyl branches. Like all steroid hormones, it is hydrophobic progesterone, like all other steroid hormones, is synthesized from pregnenolone, which in turn is derived from cholesterol.

Cholesterol undergoes double oxidation to produce 20, 22-dihydroxycholesterol. This vicinal diol is then further oxidized with loss of the side chain starting at position C-22 to produce pregnenolone. Biologically, this reaction is catalyzed by cytochrome P450scc. The conversion of pregnenolone to progesterone takes place in two steps. First, the 3-hydroxyl group is oxidized to a keto group and second, the double bond is moved to C-4, from C-5 through a keto/enol tautomerization reaction. This reaction is catalyzed by 3beta-
hydroxysteroid dehydrogenase/delta (5)-delta(4) isomerase. Progesterone in turn is the precursor of the mineralocorticoid aldosterone, and after conversion to 17-hydroxyprogesterone (another natural progestogen) of cortisol and androstenedione. Androstenedione can be converted to teatosterone, estrone and estradiol. In women, progesterone levels are relatively low during the preovulatory phase of the menstrual cycle, rise after ovulation, and are elevated during the luteal phase. Progesterone levels tend to be < 2 ng/ml prior to ovulation and > 5 ng/ml after ovulation. If pregnancy occurs, progesterone levels are initially maintained at luteal levels. With the onset of the luteal-placental shift in progesterone support of the pregnancy, levels start to rise further and may reach 100-200 ng/ml at term. Whether a decrease in progesterone levels is critical for the initiation of labor has been argued and may be species-specific. After delivery of the placenta and during lactation, progesterone levels are very low.

Progesterone is sometimes called the "hormone of pregnancy", and it has many roles relating to the development of the fetus:

Progesterone converts the endometrium to its secretory stage to prepare the uterus for implantation. At the same time progesterone affects the vaginal epithelium and cervical mucus, making it thick and impermeable to sperm. If pregnancy does not occur, progesterone levels will decrease, leading, in the human, to menstruation. Normal menstrual bleeding is progesterone-withdrawal bleeding. During implantation and gestation, progesterone appears to decrease the maternal immune response to allow for the acceptance of the pregnancy. Progesterone decreases contractility of the uterine smooth muscle. In addition progesterone inhibits lactation during pregnancy. The fall in progesterone levels following delivery is one of the triggers for milk production. A drop in
progesterone levels is possibly one step that facilitates the onset of labor. The fetus metabolizes placental progesterone in the production of adrenal steroids. (Bowen 2008)

2.15.5 Anti mullerian hormone

Anti Mullerian Hormone (AMH) is a homodimeric disulfide-linked glycoprotein with a molecular weight of 140 kDa. The gene is located on the short arm of chromosome 19 in humans, 19p 13.3 (Cohen-haguenauer et al., 1987)

Serum and follicular anti mullerian hormone measurements: Very recent evidence indicates that antimullerian hormone (AMH), a member of the transforming growth factor beta super family produced by the granulose cells of ovarian follicles mainly from the primary to the preantral and early antral stages of folliculogenesis and independently from FSH( Elver-Geiva 2005) is a unique biomarker of ovarian follicular status. Serum day 3 AMH levels have been strongly correlated with ovarian reserve (De Vat et al., 2002: Fanchin et al., 2003) and ovarian response to controlled ovarian hyperstimulation (Seifer et al., 2002: van Rooj et al., 2002 and Hazout et al., 2004) showing a better cycle to cycle reproducibility than serum inhibin B and FSH levels (Fanchin and Taieb etal 2005). Furthermore, a positive correlation between serum AMH measured around the time of HCG administered and the number of oocytes retrieved, the fertilization rates, the embryo score, and the implantation rates was recently reported(Silberstein 2006). Similarly lower serum concentrations of AMH were correlated with reduced fertilization rates and increased miscarriage rates, suggesting serum AMH as a predictor of oocyte quality (Lekamge 2007). On the contrary other recent studies did not confirm these results (Takahashi 2008). The observation in the animal model (Baarends et al., 1995: Fanchin and louafi et al.,
and in the women (Weenen 2004) that AMH is not expressed by the granulosa cells of atretic follicles suggests a more interesting role of this biomarker measured in the follicular fluid to predict the oocyte competence (Fanchin 2005).

In a very recent study the AMH levels in follicular fluid from women undergoing IVF with fertilized oocytes were statistically higher than in the follicular fluid of patients with follicular fluid AMH levels positively correlated with oocyte quality (Takahashi et al., 2008). Similarly, follicular fluid AMH concentration observed in natural IVF cycles were strongly and positively correlated with embryo implantation, suggesting follicular fluid AMH as a better predictor of oocyte quality than serum AMH (Fanchin 2007). According to these results, it can be suggested that follicular fluid AMH concentrations may be used for oocyte selection in stimulated cycles. However, although these data are very interesting and promising, further prospective randomized studies are needed to draw any conclusion.

2.15.6 Inhibin B

Inhibins are members of the transforming growth factor-β (TGF-β) superfamily (Massague, 1990; Kingsley, 1994). Structurally, inhibins are heterodimeric glycoprotein with a molecular weight of ~31–32 kDa. Bioactive inhibins consist of an α-subunit and one of two β-subunits, β_A or β_B, linked by disulphide bonds (inhibin A = αβ_A and inhibin B = αβ_B) (Groome et al., 1996). Physiologically, inhibins are secreted by the granulosa cells of ovarian follicles and selectively inhibit the pituitary FSH secretion (Klein et al., 1996).

Figure 26: Structure of Inhibin B, Inhibin A and activin
In follicular phase, inhibin B is secreted from developing preantral and small antral follicles by the stimulation of FSH (Burger et al., 1998 and Fraser et al., 1999), and further enhanced by the combination of FSH and insulin-like growth factor-I (IGF-I) (Welt and Schneyer, 2001). In contrast, inhibin A is not secreted from preantral follicles, but in small antral follicles under the stimulation of FSH (Welt and Schneyer, 2001). Collectively, inhibin B appears to play a more important paracrine role in developing follicles and a greater regulatory role with respect to FSH secretion than inhibin A (Magoffin and Jakimiuk, 1997).

In women with ovulatory cycles, the circulating inhibin B levels increase slowly and steadily in the early follicular phase, and reach a peak in the mid-follicular phase, then decrease progressively in the late follicular phase before ovulation. Shortly after the LH surge, there is a short-lived peak in the circulating inhibin B, which in turn declines to a low concentration for the remainder of the luteal phase (Groome et al., 1996). In contrast, the inhibin A levels are low in the early follicular phase, rise at the pre-ovulatory phase, and reach a peak in the mid-luteal phase (Groome et al., 1996 and Wang et al., 2000).

Figure 27: Inhibin level changes throughout the menstrual cycle. Inhibin B dominates the follicular phase of the cycle, while Inhibin A dominates the luteal phase.
Previous studies have shown that serum inhibin B is believed to be of predictive value in monitoring ovarian stimulation treatment for IVF (Hayes et al., 1998 and Elder-Geva et al., 2000). In addition, serum inhibin A may also act as a useful marker for monitoring the effects of gonadotrophin stimulation (Lockwood et al., 1996). Furthermore, concentrations of inhibin in FF, though not specified inhibin A or B, are greater in the fertilized group as compared with the unfertilized (Fowler et al., 1995), and are suggested to be used as an index of follicular maturation (Franchimont et al., 1990). However, the relationship between inhibin levels in follicular fluid (FF) and the quality of oocytes and embryos has not yet been explored.