SECTION- II

MATERIALS AND METHODS
Study population

The study subjects were classified according to the diagnosis based on their fertility condition.

Infertile subjects: Infertile subjects were selected when two consecutive semen samples (with minimum three to five days of sexual abstinence) were abnormal with respect to the routine parameters mentioned before.

Control subjects: This group was recruited with proven fertility. This group either already fathered a child naturally or semen parameters were normal.

Inclusion criteria

All males aged between 21-50 years, clinically diagnosed with infertility or sub-fertility including azoospermia, oligospermia, aspermia, asthenospermia, teratozoospermia or combined conditions were included as cases and males with proven fertility who passed all the criteria of the WHO (2010) guideline dealing with spermiogram were included as controls.

Exclusion criteria

All males below 21 and above 50 years of age were not considered for the study. Men with obesity, cardiovascular problem, HIV positive and Hepatitis (HBsAg) positive were excluded from the study.

Source of the samples

The subjects in the present investigation including infertile males and control group were recruited through Mediwave IVF and fertility research hospital, Mysore.
**Sample size**

A total of 274 clinically diagnosed infertile males as well as 130 healthy fertile males were recruited as controls from the Mediwave IVF and fertility research hospital, Mysore. The age of the subjects ranges between 21 to 50 years old.

**Ethical clearance**

The study was approved by the Institutional Human Ethical Committee (IHEC) of University of Mysore numbered IHEC-UOM No. 54/Ph. D/ 2011-12. Informed consent in English and regional language (Kannada) to participate in the study was obtained from the subjects or their spouse.

**Physical examination**

A general physical examination is an integral part of the evaluation of male infertility. In addition to the general physical examination, particular focus should be given to the genitalia including 1) examination of the penis; including the location of the urethral meatus; 2) palpation of the testes and measurement of their size; 3) presence and consistency of both the vasa and epididymis; 4) presence of a varicocele; 5) secondary sexual characteristics including body habitus, hair distribution and breast development; and 6) digital rectal exam. The diagnosis of congenital bilateral absence of the vasa deferentia (CBAVD) is established by physical examination.

**Semen Collection and Analysis**

After 3-5 days of ejaculatory abstinence the semen samples were collected in a sterile plastic container by the process of masturbation from the subjects (WHO, 2010). Semen samples were collected in the laboratory room in a clean, dry, biologically inert container. In case of oligozoospermic or azoospermic patients, three semen samples were collected 3 times on different days with three days abstinence
and thorough examination was carried out. The collected samples were allowed to liquefy at 37°C for 30 minutes and analyzed within one hour after collection. The semen samples were centrifuged at 3,000 rpm for 10 minutes and the seminal plasma was separated and stored under -80°C for further analysis. Macro and microscopic assessment of the semen was carried out to measure semen volume, sperm count, concentration, sperm motility, viability, morphology and leukocyte count according to the World Health Organization (WHO, 2010) Guidelines.

**SEMEN ANALYSIS:**

**Examination of semen by physical characteristics (WHO, 2010)**

**Coagulation:** Semen will be ejaculated in a gel state which starts to liquefy after the ejaculation. Absence of coagulation is indicative of a congenital absence of vas deferens and seminal vesicles.

**Liquefaction:** The gel state of the semen will be liquefied between 20 to 30 min after ejaculation in normal men. Abnormality in the liquefaction is indicative of a problem with the prostate and / or the seminal vesicles.

**Odour:** A normal sample has a distinctive smell which is unpleasant. Occasionally a sample may smell of spices such as garlic and cloves which is quite normal. Malodorous, pungent smelling semen is indicative of an infection, while odourless semen is associated with abnormal prostate function.

**Color:** Most samples have a whitish opacity on ejaculation but after liquefaction they acquire a grayish, translucent color. Creamy, white samples are indicative of a high sperm concentration while those having low sperm concentrations will appear clear.
**Volume:** The volume of the ejaculate will be measured to the nearest 0.1ml in a graduated centrifuge tube. The volume of the sample would be categorized as normal ranges in between (1.5ml to 4.5ml), high (>4.5ml) or low (> 1.5ml). If the volume is less than 1 ml it is important to establish whether it was a complete sample.

**pH:** pH should be determined immediately after liquefaction by placing a drop of semen on to pH paper (range pH 6.4 to 8.0). Normal values range between 7.2 and 7.8 but pH will be higher if samples are left standing prolonged periods.

**Examination of semen by microscopy**

Around 10 to 15 µl of liquefied semen was placed on to a clean glass slide using a Pasteur pipette. Then covered with cover slip and examined under phase contrast microscopy using a 40x objective or under light microscopy with the condenser lowered. The following aspects studied from the microscopy were as follows:

**Motility:** A simple method for grading motility was recommended by WHO (1999). That distinguishes spermatozoa based on the motility rate such as progressive or non-progressive motility from those which are immotile. The motility of each spermatozoan was graded as follows

1. Rapid linear and progressive (grade a)
2. Sluggish, linear and progressive (grade b)
3. Non progressive (grade c)
4. Immotile (grade d)

Within a given microscopic field, all spermatozoa with grade a and b were counted first. Subsequently spermatozoa with non-progressive motility and immotile
spermatozoa were counted in the same field. Motility of at least 200 different spermatozoa were observed and expressed in percentage.

**Sperm Vitality:**

Sperm vitality was estimated by using eosin and nigrosin staining to assess the membrane integrity of the cells. The percentage of viable cells normally exceeds that of motile cells.

**Vitality test using eosin–nigrosin**

This one-step staining technique uses nigrosin to increase the contrast between the ackground and the sperm heads, which makes them easier to discern. It also permits slides to be stored for re-evaluation and quality-control purposes (Bjorndahl *et al.*, 2003).

**Procedure**

A drop of 1% aqueous solution of eosin-Y and 10% aqueous solution of Nigrosin was taken in a tube. A drop of well mixed semen was added and mixed well using Pasteur pipette. A wet preparation of this mixture was observed under optical microscope. Dead sperms were stained red and live ones will remain unstained. At least 200 spermatozoa were counted and the incidence of live versus dead spermatozoa was estimated and expressed in percentage.

**Sperm and germ cell morphology by Papanicolaou staining:**

Papanicolaou staining is the widely used procedure for examination of germ cell morphology since it distinguishes clearly between basophilic and acidophilic cell components and allows a detailed examination of the nuclear chromatin pattern. This method gives a optimal results for analysis of sperm morphology and immature male germ cell.
(Stains used: Orange-G, EA-36, Haematoxylin)

Procedure: Wet smear of the semen sample was prepared in such a way that all the sperms lie in a single focal plane. Slides were air dried and fixed in ether-alcohol (1:1) mixture for 20 minutes.

Staining Method:

- 95% Alcohol - 1x 10 dip
- 50% Alcohol - 1x 10 dip
- Running water - 2 minutes
- Haematoxylin - 2 minutes
- Running water - 2 minutes
- Alcoholic ammonia - 1x 10 dip
- 70% Alcohol - 2x 10 dip
- 95% Alcohol - 2x 10 dip
- Orange G - 4 minutes
- 95% Alcohol - 2x 10 dip
- EA 36 - 4 minutes.
- 95% Alcohol - 2x 10 dip
- 100% Alcohol - 3x 10 dip
- Xylene - 1x 10 dip

After 30 minutes, slide was cleared in Xylene and mounted in DPX. Slides were analyzed under optical microscope using an oil immersion x100 objective and enumerated different types of sperm morphology abnormality.
Estimating Sperm concentration (sperm count) by cytometry

Solutions:

Dilution media was prepared by dissolving 50 g of sodium bicarbonate in 10ml of 40% formalin, 5ml of a saturated aqueous solution of gentian violet was added and make up to final volume of 1000 ml with distilled water.

Procedure:

Around 10 - 15µl of semen sample was taken onto a cytometer and a cover slip was placed over it. The sample was allowed to settle down for about 5 minutes. High (>100 X 106. ml-1) density semen samples would require further dilution while low (<10 X 106. ml-1) density samples would require lesser dilutions. Number of spermatozoa in the central square of the Neubauer counting chamber was counted. The number of squares was examined for sperm enumeration will depend on the average number of spermatozoa present in a square. If there are <10 spermatozoa per squares should be examined, the occurrence of 10 – 40 spermatozoa per square would necessitate counting of 10 squares, 5 squares should be examined if there are >40 spermatozoa per square.

Statistical analysis

The obtained data were subjected to statistical analysis using statistical software SPSS version 16.0. Data were expressed as Mean± Standard deviation. Student t-test, one way analysis of variance (ANOVA) was done to compare means. Statistical significance was analyzed by Chi-square test. Pearson correlation was performed to assess the linear relationship between semen parameters.
HORMONE ANALYSIS OF THE SUBJECTS THROUGH ELISA

All study subjects have undergone sex hormone analysis including FSH, LH, Testosterone, Esteradiol and Prolactin using ELISA kit method (ERBA, DRG, ).

Serum Follicle Stimulating Hormone (FSH):

Human follicle-stimulating hormone (FSH, follitropin) is a glycoprotein produced and secreted by the basophilic cells of the anterior lobe of the pituitary gland. Secretion of FSH is stimulated by gonadotropin-releasing hormone (GnRH). In men, determination of FSH is useful in the diagnosis infertility, hypogonadism, gynaecomastia and tumours in the reproductive organs.

Material required:

- A micro titre plate calibrated reader (eg., the DRG instruments micro titer plate reader).
- Calibrated variable precision micropipettes
- Absorbent paper
- Distilled water
- Semi logarithmic Figure paper or software for data reduction

Preparation of Sample

Dilute samples with concentrations above 135mlU/mL 1:1 with test distilled water

Procedure: 5 standards and 1 blank were included

- Micro wells from pouch were removed, and required micro well was taken and unused stripes were returned in the sealed pouch to refrigerator. Around 25µl of
calibrators and patient samples were pipette into the wells and incubated at room temperature for 15 minutes.

- 100µl of Enzyme conjugate was added to the wells except for blank well and incubated 15 minutes at room temperature.
- After 15 minutes, 300µl of distilled water was added (decant or aspirate). This step was repeated for 4 additional times.
- 100µl of Substrate solution was pipette into each micro well using the same order and timing as for the addition of the substrate solution.
- 100µl of stop solution was added into each micro well using the same order and timing as for the addition of the substrate solution.
- Absorbance of each micro well at 450 nm against blank was taken using a micro plate reader (BIOTEK ELX800-MS).

**Normal Reference Value for men : Range 2.0-14.0 mIU/mL**

**Serum Lutenizing hormone (LH):**

The LH test is based on simultaneous binding of human LH to two monoclonal antibodies, one immobilized micro well plates, and the other conjugate with horseradish peroxidase (HRP). After incubation the bound separation is performed by a simple solid-phase washing, and then the substrate solution is added. After an appropriate time has elapsed for maximum color development, the enzyme reaction is stopped and the absorbance was determined. The color intensity is proportional to the LH concentration in the sample.
Material required:

- A micro titter plate calibrated reader (e.g. the DRG instruments micro titter plate reader).
- Calibrated variable precision micropipettes
- Absorbent paper
- Distilled water
- Semi logarithmic Figure paper or software for data reduction

Preparation of samples:

Samples with concentration over 200 mlU/ml, dilute the sample 1:1 with standard A.

Test procedure: 5 samples and 1 blank was included,

- Micro wells from pouch were removed, taken required micro well were taken and returned unused stripes in the sealed pouch to the refrigerator. The micro wells were carefully placed into the extra provided holder.
- 25µl of standards and 25µl patient samples were pipette into the wells and incubated for 10 minutes at room temperature
- 100 µl Enzyme conjugate was added to the well except for blank well and incubated for 60 minutes at room temperature.
- Approximately 300µl of distilled water was added, decant (tap or blot) or aspirate. This step was repeated for 4 additional times.
- 100µl of substrate solution was pipetted into each micro well in the same order and timing as for the enzyme conjugate, including the blank well and incubated for 10 minutes at room temperature in the dark.
• 100µl of stop solution was added into each micro well using the same order and timing as for the addition of the substrate solution.

• Absorbance of each micro well at 450 nm against blank was taken using a micro plate reader (BIOTEK ELX800-MS).

**Normal reference values for men:** Range 4.0 to 10.0 mlU/mL

**Serum Testosterone:**

Testosterone is a C19 steroid with an unsaturated bond between c-4 and c-5, a ketone group in c-3 and a hydroxyl group in the beta position at c-17. This steroid hormone has a molecular weight of 288.47. Testosterone is the most important androgen secreted into the blood. In males, testosterone is secreted primarily by the Leydig cells of the testis. Testosterone is responsible for the development of secondary male sex characteristics and its measurements are helpful in evaluating the hypogonadal states. In men, high levels of testosterone are associated to the hypothalamic pituitary unit diseases, testicular tumors, congenital adrenal hyperplasia and prostate cancer. Low levels of testosterone can be found in patients with the following diseases: Hypopituitarism, Klinefelter’s syndrome, Testicular feminization, Orchidectomy and Cryptorchidism, enzymatic defects and some autoimmune diseases.

**Material required:**

• A micro titer plate calibrated reader (e.g., the DRG instruments micro titre plate reader).

• Calibrated variable precision micropipettes

• Absorbent paper

• Distilled water

• Semi logarithmic Figure paper or software for data reduction
Procedure:

• 25 µl of each standard, control samples were dispense with new disposable tips into appropriate wells.

• 200 µl enzyme conjugate was dispensed into each well and thoroughly mixed for 10 seconds. (It is important to have completed mixing in this step) and incubated for 60 minutes at room temperature.

• After 60 minutes of incubation briskly shook out the contents of the wells and rinsed the wells for 3 times with diluted wash solution.

• 200 µl of substrate solution was added to each well and incubated for 15 minutes at room temperature.

• After incubation the enzymatic reaction was stopped by adding 100µl of stop solution to each well.

• Absorbance (OD) of each well at 450(±10) nm was determined using micro plate reader (BIOTEK ELX800-MS).

• Normal reference values formen : Range : 2.0 to 6.9 ng/ml

Serum Prolactin

Prolactin is a hormone secreted from the lactotrophs of the anterior pituitary consisting of a single polypeptide chain containing approximately 200 amino acids. Prolactin may be involved in steroidogenesis in the gonads, acting synergistical with luteinizig hormone. High levels of prolactin appers to inhibit steroidogenesis as well as inhibiting LH and follicuole stimulatin hormones synthesis at the pituitary gland.

Material required:

• A micro titter plate calibrated reader (e.g. the DRG instruments micro titter plate reader).
• Calibrated variable precision micropipettes
• Absorbent paper
• Distilled water
• Semi logarithmic Figure paper or software for data reduction

**Procedure:**

• 25 µl of each standard, control samples were dispense with new disposable tips into appropriate wells.

• 100 µl enzyme conjugate was added to each well and and incubated for 60 minutes at room temperature.

• After 60 minutes of incubation briskly shook out the contents of the wells and rinsed the wells for 3 times with diluted wash solution.

• 100 µl of TMB substrate solution was added to each well and incubated for 15 minutes at room temperature.

• After incubation the enzymatic reaction was stopped by adding 50µl of stop solution to each well. Absorbance of each well at 450(±10) nm was determined using micro plate reader (BIOTEK ELX800-MS).

**Normal reference values for men:** Range 1.82 to 17.0 mlU/mL

**Serum Estrodiol**

**Material required:**

• A micro titter plate calibrated reader (e.g. the DRG instruments micro titter plate reader).

• Calibrated variable precision micropipettes

• Absorbent paper

• Distilled water
• Semi logarithmic Figure paper or software for data reduction

Procedure:

• 25 µl of each standard, control samples were dispense with new disposable tips into appropriate wells.
• 200 µl enzyme conjugate was added to each well and and incubated for 2 hours at room temperature.
• After 2 hours of incubation briskly shook out the contents of the wells and rinsed the wells for 3 times with diluted wash solution (1 ml wash buffer + 39 ml of distilled water).
• 100 µl of substrate solution was added to each well and incubated for 15 minutes at room temperature.
• After incubation the enzymatic reaction was stopped by adding 50 µl of stop solution to each well. Absorbance of each well was determined using micro plate reader (BIOTEK ELX800-MS)

Normal values in men: Range: 11 to 36 pg/ml

COLOUR DOPPLER SCANNING AND TRANS RECTAL ULTRASOUND SCANNING (TRUS) OF THE STUDY SUBJECTS

The principal application of transrectal ultrasonography in the infertile men is to evaluate patency of distal ductal system (Vas deferens) and internal genital organs. Although gold standard for evaluation of male ductal system is vasography, TRUS has the advantage of being non-invasive. Transrectal ultrasound allows visualization of seminal vesicles, prostate and ejaculatory ducts.

Preparation, positioning, and contraindications

For Transrectal scanning fasting is not advised, but the patient is instructed to empty his bladder before the examination. It is advisable to do the scanning in empty
rectal condition. For this, a purgative (Dulcolax 2 tablets) is given to the patient to be taken in the previous night. He is advised to empty the bowel before scanning.

Subjects were asked to remove their inner garments. Scanning can be done in left lateral, lithotomy, or knee-elbow positions. Lithotomy was most comfortable position for subjects in our study and this was done. Knee joints were supported using supportive strands attached to a gynecological examination table. Two leggings were used to cover the legs for patient’s and examiner’s convenience. To make the US room more comfortable for the patient, the room was kept semi dark during assessment. The patients were explained the technique of the scanning. For transrectal ultrasound, the ultrasound transmissiongel was applied to the endorectal transducer and it was covered with a sterile probe cover or a sterile condom.

Although, in our study, the procedure was performed without any infiltrative anesthesia in the past it is a common practice to use lidocaine infiltration in the periprostatic area. Pareek et al., (2001) described a technique of periprostatic nerve blockade. Accordingly 2.5 mL of lidocaine was injected (using a 5-in 22-gauge spinal needle through the ultrasound probe) on each side at the prostate base at the junction of the prostate and the seminal vesicle. In a randomized, double-blind, placebo-controlled study, Pareek et al., (2001) showed significant pain control during and after biopsy. Alavi et al., (2001) compared the efficacy of intrarectallidocaine gel with that of periprostatic nerve block and concluded that the nerve block was superior for pain control. Using this technique, saturation biopsies, with up to 20 cores, could be performed. However, if only diagnostic TRUS is done, without taking biopsy, as in this study, no need of injection is required as it is almost a painless procedure.

Currently, the most widely used probe is a 7-MHz transducer within an endorectal probe, which can produce images in both the sagittal and axial planes.
Scanning begins in the axial plane, and the base of the prostate and seminal vesicles are visualized first. A small amount of urine in the bladder facilitates the examination. Seminal vesicles are identified bilaterally, with the ampullae of the vas on either side of the midline. The seminal vesicles are convoluted cystic structures that are darkly anechoic. Men who have abstained from ejaculation for a long period may have dilated seminal vesicles. Measurements were taken. Length of the seminal vesicle is taken using dotted lines along the curvature of the organ in its midline. Width is taken at the midpoint. Volume is calculated using inbuilt calculator in the scanning machine. Vas deferens is visualized and the diameter of the vas deferens was measured.

Next, the base of the prostate is visualized. Volume assessment of the prostate is an important and integral part of this procedure. Several formulas have been used, the most common of which is the ellipsoid formula, which requires measurement of three prostate dimensions. The length and breadth of the prostate was taken in the longitudinal axis of the prostate. Thickness was taken in the transverse axis. The ellipsoid volume formula is then applied, as follows:

Prostatic volumes were calculated by using the ellipsoid volume formula of: length × breadth × thickness × 0.52. This was available in inbuilt calculator of volume in the software of the scanning machine. Prostatic scanning was done in various slices.

The central zone comprises the posterior part of the gland and is often hyperechoic. The mid gland is the widest portion of the gland. The peripheral zone forms most of the gland volume. Echoes are described as isoechoic and closely packed.

The transition zone is the central part of the gland and is hypoechoic. The junction of the peripheral zone and the transition zone is distinct posteriorly and is
characterized by a hyperechoic region, which results from prostatic calculi or corpora amylacea. The transition zone is often filled with cystic spaces in patients with benign prostatic hyperplasia (BPH).

Scanning at the level of the verumontanum and observing the Eiffel tower sign (anterior shadowing) help to identify the urethra and the verumontanum. The prostate distal to the verumontanum is composed mainly of the peripheral zone. The capsule is a hyperechoic structure that can be identified all around the prostate gland. Several hypoechoic rounded structures can be identified around the prostate gland. These are the prostatic venous plexi. The position of the neurovascular bundles can often be identified by the vascular structures. Imaging in the sagittal plane allows visualization of the urethra. The median lobes of the prostate are often visualized.

**Scrotal scanning:**

**Preparation:** For scrotal ultrasound patient is instructed to shave the parts and come for scanning. An ultrasound examination of was done per cutaneous to investigate the testis. Testes and the epididymal tail were visualized in longitudinal plane scrotal wall, with the later present at the distal extremity of testes.

The scanning was done by using latest 4D color Doppler scanner manufactured by Shenzhen Mindray Biomedical Electronics Co., Ltd., Germany, with linear-array 10Mz transducer. The sector transducer was used whenever testis was larger than the size of the linear transducer. Scanning was performed with the patient in the supine position. The scrotum was supported by a towel between the thighs. Ultrasound gel was applied over the scrotum. Testicular scanning was done in both longitudinal and transverse axis. The length and breadth of the testis was taken in the longitudinal axis of the testis. Thickness was taken in the transverse axis. Testicular
volumes were calculated by using the empiric formula of Lambert: length × breadth × thickness × 0.71. This was available in inbuilt calculator of volume in the software of the scanning machine. Then, the testis and paratesticular area, mediastinum testis, epididymal head, epididymal body, and epididymal tail were examined sequentially.

Spermatic chord and vasculature were studied. Additional techniques such as Valsalva maneuver was used for venous evaluation. Enlargement of veins was assessed. Diameter of veins was measured both in resting posture as well as in Valselva maneuver. Presence of Varicose veins if any was noted. Gradations of varicosity was done.