Materials & Methods
MATERIALS AND METHODS

I. STUDY POPULATION

The study was carried out at the Centre for Research in Assisted Reproduction and Foetal Therapy (CRAFT), Kodungallur, Trichur (Dt., Kerala during the period from 1999 to 2002. A total of 319 male factor patients were randomly selected for this purpose. The standard procedures for the collection and examination of semen, as outlined in the world Health Organization Manual for the examination of Human semen and sperm cervical mucus interaction (WHO 1992) were followed throughout the investigation. Hormonal estimations were made for LH, FSH and Testosterone was made in relevant cases. These hormones were analysed by chemiluminescence using reagents provided by CIBA Corning. Chromosome analysis was carried out for the patients with azoospermic and severe oligospermic cases. Pedigree analysis was carried out for the patients with family history of infertility.

II. QUESTIONNAIRES

Each subject, after providing his semen sample was given a self-administered questionnaire, which could be completed on the site. The questionnaire included age, religion, family history, infertility type, past history of diseases, past history of surgery, sex
habits, occupation and occupational exposures such as heat, chemicals, sitting posture.

III. SEMEN ANALYSIS

Routine semen analysis included both macroscopic and microscopic examination of the ejaculate following WHO (1992).

Initial macroscopic examination

The semen samples were collected by masturbation generally in the laboratory premises.

Liquefaction

The semen samples were allowed to liquefy at room temperature for 30 minutes before analysis. Those cases where complete liquefaction did not occur in 60min. were considered abnormal.

Appearance

The semen samples were examined immediately after liquefaction or within one hour of ejaculation. A normal semen sample appeared homogenous, and grey-opalescent. It appeared less opaque when the sperm concentration was very low.

Volume

The volume of the ejaculate was determined aspirating the liquefied sample into a graduated disposable pipette.
Viscosity

The viscosity (also referred as consistency) was estimated by gentle aspiration into a wide-bore 5 ml pipette and then allowed the semen to crop by gravity and observed the length of the thread. A normal sample leaves the pipette as small discrete drops. In cases of abnormal viscosity the drop produced a thread more than 2 cm long.

pH

The pH was measured at a fixed time within one hour of ejaculation. A drop of semen was spread evenly on to the pH paper (range of pH 6.1 to 10.0 or 6.4 to 8.0). After 30 seconds, the colour of the impregnated zone was compared with the calibration strip to read the pH.

Initial microscopic investigation

During the initial microscopic investigation the samples were examined through phase contrast microscope and estimates were made of the concentration, motility, agglutination of spermatozoa and presence of cellular elements other than spermatozoa.

Assessment of Sperm motility

Preparation for routine semen analysis

A fixed volume of 10 μl semen was delivered on to a clean glass slide with a positive displacement pipette and covered with
22mm x 22 mm coverslip. Under the weight of the coverslip the sample spreads for optimum viewing.

The freshly made wet preparation was left to stabilize for approximately one minute. The examination was carried out at room temperature, between 20 and 24°C. Initial evaluation at 100X total magnification i.e. (10X objective and 10X ocular) provides an overview for determining mucus strand formation, sperm aggregation and the evenness of the spread of spermatozoa on the slide. The preparation was then examined at a magnification of 400X total magnification.

Sperm motility was assessed by a simple grading system in which at least five microscopic fields were assessed in a systematic way to classify 200 spermatozoa. The motility of each spermatozoon was graded ‘a’, ‘b’, ‘c’ or ‘d’ according to WHO (1999) standard procedure.

a. rapid progressive motility.

b. slow or sluggish progressive motility

c. non progressive motility (<5 mm/s)

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

Within a defined area of the field, indicated by lines formed by a graticule in the focal plane of the microscope or in the whole field in the sperm concentration was low, all the spermatozoa with grade a and b motility were counted first. Subsequently spermatozoa with non progressive motility (grade c) and immotile spermatozoa (grade d) were counted in the same area. The number of spermatozoa in each category was tallied with the aid of a laboratory counts. The count of 200 spermatozoa was repeated on a separate 10μl specimen from the same semen sample and the percentages in each motility grade from the two independent counts were compared.
**Agglutination**

Agglutination of spermatozoa means that motile spermatozoa stick to each other head to head, tail to tail or in a mixed way. The presence of agglutination was assessed at the time of determining sperm motility. The type of agglutination was recorded, e.g. head to head, tail to tail or mixed. Semiquantitation grading from – (no agglutination) to +++ (Severe clumping in which all the motile spermatozoa were agglutinated) was used.

**Assessment of Sperm Concentration**

The Makler counting Chamber (Makler 1980) is a simple device for rapid and accurate sperm count. The chamber is composed of two parts.

1. The lower main part has a metal base (A) and two handles (H). In the centre of the base there is a flat
disc (D) on which the sample (S) is placed. On the periphery of the disc there are four quartz-coated pins (P), elevated by 10 microns above the surface of the central part.

2. The upper part is the cover glass (c). At the centre of its lower surface there is a 1 mm² grid, subdivided into 100 squares each 0.1 x 0.1 mm.

When the cover glass is placed on the four tips, the space bounded by the two surfaces and any row of 10 squares of the grid is exactly 0.001 mm³ or one millionth of ml.

Recommended combination of 20 X objective and 10 X eyepiece was used.

The sperms were immobilized by transferring a part of the sample to another test tube. The test tube was then inserted for 5 minutes into water at 50°C–60°C. A drop from well mixed preheated specimen is placed in the center of the lower disc. Grasp the cover glass opposite the 2 dark points and placed it on the four tips, to note the colour fringes. This ensures the automatic spread of the drop into a thickness of 10 microns. The sperm heads within the squares of the grid was counted in the same way as blood cells were counted in hemocytometer. The number of sperms in a strip of 10 squares was counted and this number represent, their
concentration in millions per ml. Repeated this count in another strip or two, to determine the average. 2 or 3 other drops of the sample was also taken to make the count more accurate. In the case of oligospermic specimen, sperm count was taken from whole grid area. Five zeros are then added to the number counted to get the concentration in millions per ml.

Assessment of sperm morphology.

Preparation of smears

The slides were thoroughly cleaned, washed in 70% ethanol and dried, before a small drop of semen (5 to 20µl) is applied to the slide. If the sperm concentration is over 20 x 10⁶/ml, then 5µl of semen was used; if the sperm concentration is less than 20x10⁶/ml, then 10 to 20 µl semen was used.

The feathering technique (where by the edge of a second slide is used to drag a drop of semen along the surface of the cleaned slide) was used to obtain a uniform thin smear of spermatozoa.

1. Staining method

Papanicolaou stain is most widely used in andrology laboratories. It gives good staining of spermatozoa and other cells. It permits staining of the acrosomal and post-acrosomal regions of the head, the cytoplasmic droplet, the midpiece, and the tail.
With these stains, the head is stained pale blue in the acrosomal region and dark blue in the post-acrosomal region. The midpiece may show some red staining. The tail is also stained blue or reddish. Cytoplasmic droplets, usually located behind the head and around the mid piece are stained green with the papanicolaou stain.

The smear was airdried and fixed in equal parts of 95% ethanol and ether for 5-15 minutes.

**Staining procedure**

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<tr>
<td>Ethanol 70%</td>
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<td>Ethanol 50%</td>
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<tr>
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<tr>
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<tr>
<td>Running water</td>
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<td>Xylene</td>
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**Assessment of sperm morphology**

At least 200 sperms were assessed in each observation. Each smear was assessed using a microscope with a total magnification 100X using a high qualitative 100X non phase contrast objective under oil immersion and correctly adjusted bright field.

2. **Testsimplets® Prestained slides**: (Schoenfeld et al., 1981)

Testsimplets application allows a differential for morphology evaluation of semen specimen. It is based on the presence of two dyes, new methylene blue N and cresylviolet acetate. The different staining of individual cell region with basic dyes is used for cell differentiation.
Procedure

1. About 3-5μl of thoroughly mixed liquified semen (room temperature) was pipetted to the center of stained portion of labelled slide.

2. Cover glass was placed over the specimen immediately.

3. Cover glass is then gently pressed by exerting mild pressure on the center.

4. Sealed slides were examined after 2hrs under oil immersion using 100X objective.

5. 100-200 spermatozoa were counted and classified them according to abnormalities of head, midpiece and tail (WHO 1992).

Immature germinal cells, white blood cells, epithelial cells were also noted.

Immature germinal cells and mature sperm.

The presence of immature forms were also noted which appeared as 'round cell'. Spermatids in the ejaculate have one to four distinct blue – reddish, round nuclei located peripherally in the cell. Spermatocytes have only one centrally located nucleus. In this way immature forms could be expressed per 100 sperm and their concentration in the original semen sample could be calculated from sperm concentration. The formula for this calculation is
concentration of immature germ cells = number of immature cells counted per 100 sperm (%) 100 sperm concentration.

IV. SPERM PREPARATION FOR INTRA UTERINE INSEMINATION

(Dale et al., 1997; Brinsden et al., 1997).

The choice of sperm preparation method or combination of methods depends upon the assessment of

a. Motile count
b. Ratio between motile: immotile count
c. Volume
d. Presence of antibodies, agglutination per cells or debris.

1. The Swim – up technique

This technique is useful for viscous samples and when the total volume of semen is very low. This method is not recommended when motility is very poor or when there is a large degree of cellular contamination and debris.

Sperm preparation media

SAGE Biopharma sperm preparation media are designed for procedures involving sperm washing and incubation. The formulations are based on HEPES buffered human tubal fluid (HTF), first described by Quinn et al., (1985).
Components

- Sodium chloride
- Potassium chloride
- Magnesium sulphate
- Potassium phosphate, anhydrous
- Calcium chloride
- Sodium bicarbonate
- HEPES
- Glucose
- Sodium pyruvate
- Sodium lactate (DL)
- Taurine
- Alanyl-glutamine
- EDTA
- Gentamicin
- Phenol red
- Human serum albumin, 5 mg/ml

Procedure

1. The semen was diluted with twice its volume of Quinn’s sperm washing medium (cat No ART 1005, 1006) and spun at 350g for 10 minutes.
2. Discarded the supernatant, and resuspended the pellet with 1.0 ml sperm washing medium.

3. Repeated centrifugation at 1000 rpm for 5 minutes and decanted the supernatant completely.

4. The pellet was overlayed with 0.5 ml of sperm washing medium.

5. Incubated at 37°C at an angle of 60° in 5% CO₂ in air for 45 minutes.

6. Supernatent was removed carefully from pellet and placed in clean centrifuge tube to assess count and motility.

2. Two step discontinuous buoyant density gradient centrifugation

Sterile colloidal suspension of silica particles stabilized with covalently bound hydrophilic silane supplied with HEPES buffered human tubal fluid (HTF). Buoyant density apparently protects the sperm from trauma of centrifugation, and a high proportion of functional sperm could be recovered from the gradients. Discontinuous two step gradients are simple to prepare and highly effective in preparing motile sperm fractions from suboptimal semen samples.
Reagents

Pure Ception 40\% upper phase

[Catalog no. ART 2040 SAGE Biopharma]

Pure Ception 80\% lower phase

[ART 2080 SAGE Biopharma]

Quinn's sperm washing Medium

[Catalog No ART 1006 SAGE Biopharma]

Procedure

1. All components of the kit and the semen sample brought to room temperature before using, to avoid the cold 'shock' to the spermatozoa.

2. To prepare the single bilayered gradient is prepared.
   a. 3.0 ml of Pure Ception lower phase was transferred (Pure Ception 80\%) into the conical tube.
   b. Using a pipette or syringe, layer the 3.0 ml of upper phase (pureception 40\%) on top of the lower phase. This is done by carefully contacting the surface of the lower phase with the tip of the pipette or syringe needle.

3. 2.5ml of liquefied semen was gently placed onto the upper phase using a pipette or syringe.

4. Centrifuged the sample for 20 minutes at 350 to 400 g (or up to 750g for highly viscous samples)
5. Following centrifugation, all the layers except the lowest portion (approximately 0.3 ml) is removed.

6. Added 2 to 3 ml of sperm washing Medium and resuspended the pellet.

7. Centrifuged for 4 to 8 minutes at 250g. The higher number of sperms would require maximum 8 minute centrifugation time to ensure a complete and thorough sperm wash.

8. Removed the supernatant and resuspended the pellet in 0.5 ml of sperm washing medium.

9. Sample was ready to count and estimate motility.

V. CHROMOSOME ANALYSIS (Barch, 1991; Yunis, 1975)

2-3 ml of peripheral blood was collected in heparinized syringe under aseptic condition.

_Culture Requirements_

- RPMI 1640 with glutamine _{(Sigma. Cat No. R-6504)}_
- PHA _{(Sigma. Cat No. L-9132)}_
- Foetal Bovine serum _{(Sigma. Cat No. F4135)}_
- Heparin _{(Sigma. Cat No. H 3149)}_
- Penicillin – streptomycin solution _{(Sigma. Cat No. P4333)}_
- Demecolchicine _{(Sigma. Cat No. D1925)}_
- Trypsin 1:250 _{(Sigma. Cat No. T 4799)}_
- Giemsa _{(Sigma. Cat No. G 3032)}_
Procedure

To 5 ml of RPMI Media added 0.2 ml of PHA, 1.0 ml of FBS, 0.4 ml of heparinised blood and 100μl of Pencillin–streptomycin solution, incubated culture vial for 72hrs at 37°C.

Preparation of solutions for Harvesting

I. Hypotonic solution (0.56% or 0.075 M KCl)

Potassium chloride 560 mg
Distilled water 100 ml

II. Fixative

Methanol – absolute (three parts) 75 ml
Acetic Acid – glacial (one part) 25 ml

Harvesting and slide Preparation

1. After 70 hrs of incubation 0.1 ml of colcemid was added to each culture vial, mixed gently shaking the vial and incubated for 2 hours at 37°C

2. After colcemid treatment, culture was centrifuged at 1000 rpm for 10 minutes.

3. Discarded the supernatant by pipetting off media, leaving as little medium as possible over the cell button

4. Resuspended the cell button in 5 ml of hypotonic solution and incubated for 15 minutes in a water bath at 37°C.
5. 5-6 drops of freshly made chilled fixative was added drop by drop and mixed gently by pipette and centrifuged at 1000 rpm for 10 minutes.

6. Discarded the supernatant. Mixed the pellet and added 5 ml of fixative allowed to stand at room temperature for about 10 minutes.

7. Again centrifuged the tubes, discarded supernatant, and suspended the cells in fresh fixative. Repeated this step for two times.

8. After the final centrifugation, cells were suspended in a small volume of fixative (0.5 – 0.75 ml)

9. Pre-cleansed slides were refrigerated at 4°C in triple distilled water for 2-3 hours prior to use.

10. With a Pasteur pipette, 1-2 drops of cell suspension was dropped on the cold wet slides from a height of 1-2 feet to facilitate better chromosome spreading.

11. The slide was held at an angle and blown once vigorously on the suspension slide and then heat-dried on a slide. Warmed at 45°C-60°C for 1-2 minutes. Four to six slides were prepared for each case.
12. The test slide was checked under the microscope for the concentration of cell suspension spreading of chromosomes and mitotic index.

**G Banding**

The Giemsa bands (G bands) obtained by digesting the chromosomes with proteolytic enzyme trypsin are most widely used in clinical laboratories for routine chromosomal analysis.

**I. Phosphate buffer for trypsin**

- a. Sodium chloride (NaCl) 8.0 gm
- b. Potassium chloride (KCl) 0.2 gm
- c. Potassium dihydrogen orthophosphate $\text{KH}_2\text{PO}_4$ 0.2gm
- d. Di-sodium hydrogen orthophosphate $\text{Na}_2\text{HPO}_4$ 0.92gm
- e. Distilled water 1 litre

**II. Trypsin Solution**

- a. Trypsin powder 25mg
- b. Phosphate buffer (PBT) 50ml

**Procedure**

1. Aged the slides overnight at 50°C
2. Treated the slides in trypsin solution (37°C) for 6 to 10 seconds
3. The slides rinsed briefly in phosphate buffer saline
4. The treated slides were stained in Giemsa staining solution for 4 to 6 minutes.
5. The slides rinsed under running tap water and allowed to dry.
6. The slides were examined under microscope.

VI HORMONAL ASSAY

Hormone analysis was carried out using ACS 180 CIBA CORNING in a laboratory outside the hospital.

VII STATISTICAL ANALYSIS

Descriptive statistics like mean, median, standard deviation and range for all the semen parameters and hormone values were calculated for the fertile and infertile population.

An independent t-test was used for comparing the semen parameters and hormone values of fertile and infertile population. Semen parameters and hormone values of infertile men those having smoking habit and those having drinking habits were compared with control population using t-test. The same t-test was applied to compare the hormone values and semen parameters of different exposure group with that of no exposure group in the infertile population and also to the control group.
A non parametric test namely Mann-Whitney U test was applied to compare the semen parameters of smokers and non smokers of the infertile group. Same test was applied also to compare the semen parameters of drinkers and non drinkers of the infertile group. Hormone values and semen parameters of vegetarian and non vegetarians of the infertile population were compared using the above said Mann-Whitney U test.

Logistic regression with dichotomous variable infertility as dependent variable was applied to find out whether any of the parameters influence the infertility of men. Separate regression analysis was done for finding out whether each variable influence the fertility. (All the analysis was done using SPSS V.10.0). (Montgomery et al., 1982; Siegel 1956 ; Snedecor et al., 1980).