"HISTOPATHOLOGICAL EVALUATION OF TESTICULAR BIOPSIES IN MALE INFERTILITY"

By

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A Dissertation submitted to the

BLDE University, Bijapur, Karnataka

In partial fulfillment of the requirements for the award of the degree of

DOCTOR OF MEDICINE

IN

PATHOLOGY

Under the Guidance of

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2015

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I hereby declare that this dissertation entitled **"HISTOPATHOLOGICAL EVALUATION OF TESTICULAR BIOPSIES IN MALE INFERTILITY"** is a bonafide and genuine research work carried out by me under the guidance of **Dr. R.M.POTEKAR, Professor Department of Pathology.**

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ABSTRACT

BACKGROUND

Testicular pathology is the cause of infertility in approximately 1% of the male populations and 10% of men who seek fertility evaluation. In men with testicular cause of infertility, the biopsy of the testis is must to know the histopathology of the testis & manage accordingly & to foresee the possibility of finding sperms in the testis for assisted reproductive therapy.

AIMS AND OBJECTIVES

To identify, categorize & compare histopathological changes in unilateral & bilateral testicular biopsies in men with infertility.

MATERIAL AND METHODS

The study consisted of 45 cases, of which 16 cases were prospective and 29 cases were retrospective. All were infertile male with azoospermia on semen analysis. Out of 45 cases, 30 cases had undergone bilateral testicular biopsies; while 15 cases had unilateral testicular biopsies. Testicular biopsies were processed & histological findings were recorded.

RESULTS

Age of the patients ranged from 22 years to 38 years. Maximum numbers of cases were seen in the age group of 26 to 30 years. 82.22% patients had primary infertility. The duration of infertility ranged from 2 years to 10 years. Maximum number of primary infertility cases presented with duration of infertility of less than 4 years; while none of the secondary infertility case presented with duration of infertility of less than 4 years. The most common testicular histopathological pattern observed was hypospermatogenesis & least common was maturation arrest. None of the secondary infertility cases showed maturation arrest. In bilateral testicular biopsy

cases, discordance in histology of right & left testes were seen in 26.67% cases. CIS in seminiferous tubules was not seen in any of the testicular biopsies.

CONCLUSION

In the evaluation of male infertility due to azoospermia due to testicular causes, bilateral testicular biopsy is recommended over unilateral testicular biopsy.

CIS should be carefully searched in testicular biopsy received for the evaluation of male infertility.

KEY WORDS

Male infertility evaluation, testis biopsy, testicular histopathology in male infertility.

LIST OF ABBREVIATIONS USED

ART	Assisted Reproductive Technology
CIS	Carcinoma In Situ
CD	Clusters of Differentiation
DNA	Deoxyribo Nucleic Acid
ERC	Excess residual cytoplasm
et al	And others
FNA	Fine Needle Aspiration
FNAC	Fine Needle Aspiration Cytology
FSH	Follicular Stimulating Hormone
GCA	Germ Cell Arrest
GCT	Germ Cell Tumor
GnRH	Gonadotropin Releasing Hormone
HCG	Human Chorionic Gonadotropin
HE	Haematoxylin–eosin
ICSI	Intracytoplasmic Sperm Injection
IHC	Immunohistochemistry
IUI	Intra-Uterine Insemination
IVF	In-vitro fertilization
IM	Immotility
LH	Leutinizing Hormone
MT	Masson Trichrome
NOA	Non Obstructive Azoospermia
NP	Non Progressive motility

OA	Obstructive Azoospermia
PAS	Per iodic Acid Schiff
PR	Progressive motility
Sd1	Spermatid d1
Sd2	Spermatid d2
STD	Sexually Transmitted Disease
SCO	Sertoli Cell Only
SCOS	Sertoli Cell Only Syndrome
TESE	Testicular Sperm Extraction
TRH	Thyrotropin Releasing Hormone
TSH	Thyroid Stimulating Hormone
viz.	Videlicet (namely)
WHO	World Health Organization

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INTRODUCTION

Out of every four couples in developing countries, one couple had been found to be affected by infertility, when an evaluation of responses from women in Demographic and Health Surveys from 1990 was completed in collaboration with WHO in 2004.[1]

Male infertility constitutes 30% of infertile couples, and both the female and the male account for another 20%.[2]

Male infertility causes are classified as pre-testicular, testicular & post-testicular.[3]

Testicular pathology is the cause of infertility in approximately 1% of the male population and 10% of men who seek fertility evaluation. In men with testicular cause of infertility, the biopsy of the testis is must to know the histopathology of the testis & manage accordingly & to foresee the possibility of finding sperms in the testis for assisted reproductive therapy.[2]

Obstructive azoospermia and non-obstructive azoospermia must be differentiated because their management differs. Endocrine and genetic tests cannot reliably distinguish OA from NOA or predict recovery of mature spermatids by testicular sperm extraction (TESE).[2,4]

The evolution of assisted reproductive technologies (ARTs) has seen new approaches in the assessment of semen quality and sperm function, and emphasis on identifying motile sperm in semen, or of elongated spermatids in testicular tissue is increasing, which could be used for ICSI.[4]

In recent studies of quantitative analysis of spermatogenesis, it is found that even men with germinal failure have minute foci of spermatogenesis which gives a ray of hope for them having a child of their own. This also requires testicular biopsy.[2]

Severity of testicular lesion cannot be assessed by semen analysis or clinical examination; hence many physicians believe that biopsy is required to assess the severity of testicular lesion for the prognosis & further treatment in cases of oligospermia and azoospermia.[5]

The rising incidence of testis cancer and carcinoma in situ (CIS) especially in at risk subgroups such as infertile men requires strategies for early detection, ideally at the pre-invasive stage.[6-8] Men at risk of CIS or testicular cancer are men with idiopathic infertility, history of prior cryptorchidism, history of testicular neoplasia or suggestive features on ultrasound, such as an identified lesion or microlithiasis.[8-13]

AIMS & OBJECTIVES

To identify, categorize & compare histopathological changes in unilateral & bilateral testicular biopsies in men with infertility.

REVIEW OF LITERATURE

Anatomy of Testis

The testes are a pair of gonads of male. They are ovoid organs, suspended in the scrotum by scrotal tissue including the dartos muscle spermatic cords.[14]

Normal testicular dimensions: [15]

Longest axis 3.6 to 5.5cm; average 4.6cm.

Shortest axis 2.1 to 3.2cm; average 2.6cm.

Weight: 15 to 19g .

The testis is covered by 3 coats, which from inside to outside are tunica vasculosa, tunica albuginea & tunica vaginalis.[14]

Histology of Testis

The testicular capsule proper, the tunica albuginea, is tough, collagenous & thickened posteriorly to form mediastinum of the testis. Septa from the mediastinum extend internally to partition the testis into approximately 250 lobules. These lobules differ in size, are largest & longest in the center. Each lobule contains 1 to 4 convoluted seminiferous tubules, whose both free ends open into channels within the mediastinum.[14]

There are 400-600 seminiferous tubules in each testis, each 70-80cm long & 0.12-0.3mm in diameter. Each tubule is surrounded by a basal lamina, on which rests a complex, stratified seminiferous epithelium containing spermatogenic cells & supportive sertoli cells. When active, the spermatogenic cells include basally situated spermatogonia & their progeny in the adluminal compartment, spermatocytes, spermatids & mature spermatozoa.[14]

Spermatogonia, the stem cells for all spermatozoa, are descended from primordial germ cells which migrate into the genital cords of the developing testis. The 3 basic groups of spermatogonia are

1. Dark type A (Ad) - these divide mitotically to maintain population of spermatogonia & to give rise to pale type A (Ap) cells.
2. Pale type A (Ap) – these divide mitotically but remain linked in clusters by fine cytoplasmic bridges. These are precursors of type B cells.
3. Type B – these are committed to the spermatogenic sequence. At about the time that type B cells enter a final round of DNA synthesis, they leave the basal compartment & cross the blood-testis barrier to enter meiosis as primary spermatocytes.[14]

Primary & Secondary Spermatocytes: Primary spermatocytes are larger cells with large round nuclei in which the nuclear chromatin is condensed into dark, threadlike, coiled chromatids. These give rise to secondary spermatocytes with a haploid chromosome complement. Few secondary spermatocytes are seen in tissue sections because they rapidly undergo the second meiotic division, where sister chromatids separate to form haploid spermatids. [14]

Spermatids: These do not divide again but gradually mature into spermatozoa by a process known as spermiogenesis.[14]

Spermatozoa that are released from the wall of the tubules into the lumen are non-motile but structurally mature. It has a head, a neck & a tail. The head has a maximum length of approximately 4 µm & a maximum diameter of 3 µm. The neck is approximately 0.3 µm long**.** The tail is divided from neck downwards into middle piece which is approximately 1 µm in diameter & 7 µm in length; principal piece, approximately 0.5 µm in diameter & 40 µm in length; & tail, 5-7 µm in length.[14]

Sertoli cells are the supporting, non-spermatogenic cells of the seminiferous tubules. Variable in overall shape, they all contact the basal lamina & their cytoplasm extends into the lumen. Their nucleus is euchromatic & irregular or pear shaped, contains 1 or 2 prominent nucleoli, & is usually aligned perpendicular to the basal lamina.[14]

Interstitium includes various connective tissue components, peritubular myoid cells, vessels, nerves & leydig cells. Leydig cells occur in singles or clusters, are polyhedral, 14-20 µm in size with an eccentric nuclei, 1-3 nucleoli, abundant acidophlic cytoplasm containing a considerable amount of smooth endoplasmic reticulum, lipid droplets & needle shaped crystals of Reinke upto 20 µm long.[14]

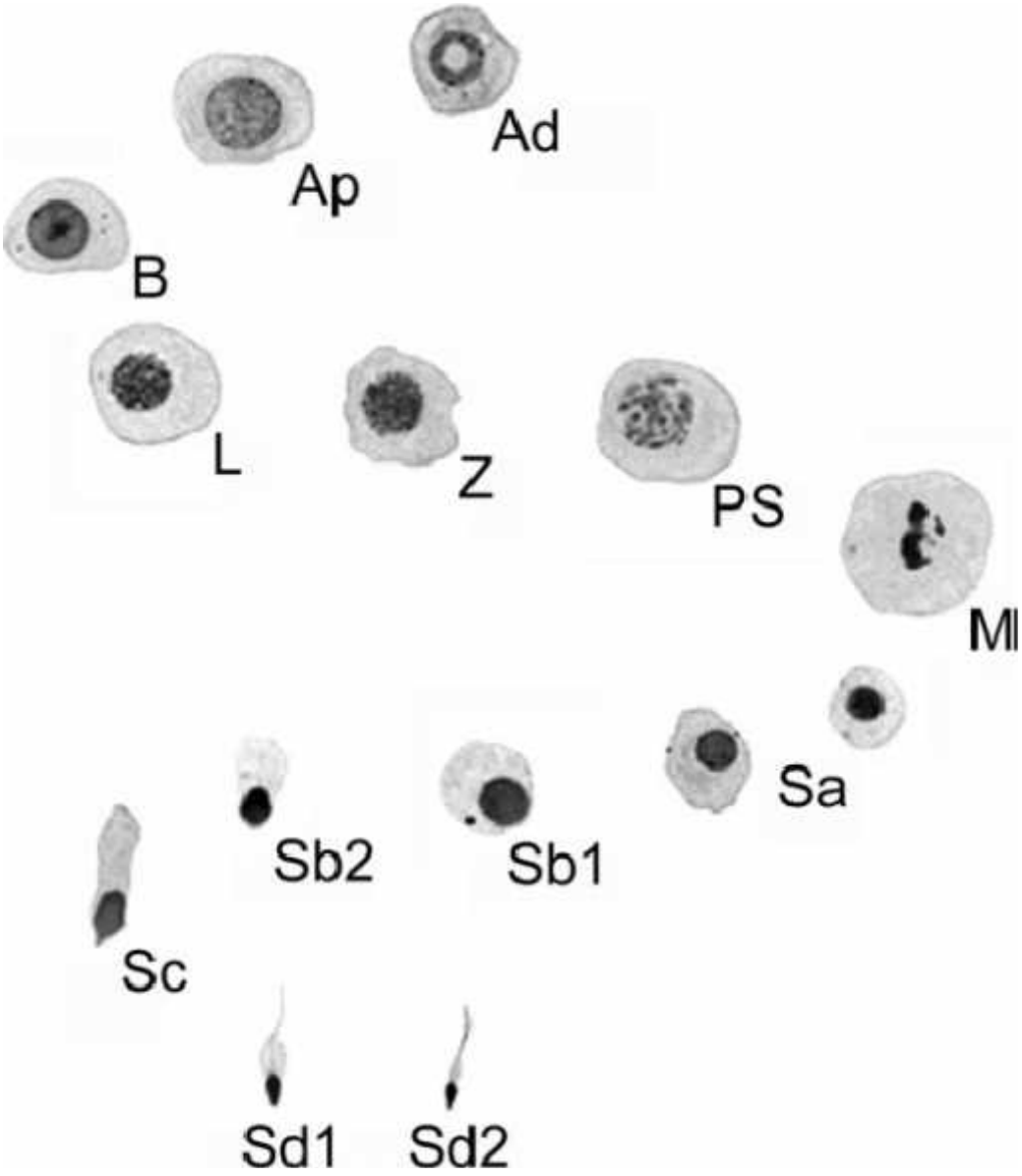


Figure 1: Normal spermatogenesis, the cell types. This diagram illustrates the cytological features of the major germ cell types within the seminiferous epithelium. Ad - spermatogonium A dark; Ap - spermatogonium A pale; B - spermatogonium B; L - leptotene spermatocyte; Z - zygotene spermatocyte; PS - pachytene spermatocyte; M - meiotic division; Sa to Sd2 - spermatid a to spermatid d2.[4]

Table 1: Testicular Features in Normal Adult Testes with Values per Cross-Section Tubule[16]

Seminiferous Tubules Feature	Value (Mean ± SD)
Mean tubular diameter	193.0 ± 8.0 µm
Number of Spermatogonia	21.0 ± 4.0
Number of primary spermatocytes	31.0 ± 6.0
Number of young (S_a + S_b) spermatids	37.0 ± 7.0
Number of mature (S_c + S_d) spermatids	25.0 ± 4.0
Number of sertoli cells	10.4 ± 2.0
Number of sertoli cell vacuoles	0.8 ± 0.3
Lamina propria thickness	5.3 ± 1.0 µm
Number of peritubular cells	21.0 ± 4.0
Testicular Interstitium Feature	**Value (Mean ± SD)**
Number of leydig cell clusters	1.2 ± 0.3
Number of leydig cells	5.0 ± 0.2

Prevalence of Infertility

An evaluation of responses from women in developing countries in Demographic and Health Surveys from 1990 was completed in collaboration with WHO in 2004 & showed that 1 in every 4 couples in had been found to be affected by infertility.[17]

In 2010, primary infertility rate among child-seeking women aged 20–44 years was 1.9% and secondary infertility rate was 10.5%. Worldwide 48.5 million couples were estimated to be infertile.[17]

Among the infertile couples, male infertility is the cause in 30%, and both the female and the male account for another 20%.[2]

Causes of Male Infertility

Male infertility causes are classified as pre-testicular, testicular & post-testicular.[3]

A) Pre-Testicular Causes of Male Infertility: These are extra-gonadal endocrine disorders, such as those originating in the hypothalamus, pituitary, or adrenals, which have an adverse effect on spermatogenesis.

1) Hypothalamic

A) Gonadotropin deficiency

Ex: Kallmann syndrome

B) Isolated LH deficiency

C) Isolated FSH deficiency

D) Congenital hypogonadotropic syndromes

2) Pituitary Disease

A) Pituitary insufficiency

B) Hyperprolactinemia

3) Exogenous or endogenous hormones

A) Estrogen excess

B) Androgen excess

C) Glucocorticoid excess

D) Hyperthyroidism and hypothyroidism

B) Testicular Causes of Male Infertility: These are primary defects of the testes

1) Chromosomal Causes

Ex: Klinefelter syndrome

2) Gonadotoxins

A) Radiation

B) Drugs: Ketoconazole, spironolactone, and alcohol inhibit testosterone synthesis; cimetidine is an androgen antagonist.

Recreational drugs such as marijuana, heroin are associated with lower testosterone levels.

Certain pesticides have estrogen-like activity.

Cancer chemotherapy.

3) Systemic Diseases

A) Renal Failure

B) Liver cirrhosis etc

4) Defective Androgen Activity:

Peripheral resistance to androgens occurs with 2 basic defects

A) Deficiency of androgen production through the absence of 5-alpha reductase.

B) Deficiency in the androgen receptor.

5) Testis Injury

A) Orchitis: Most commonly due to bacterial infection, termed epididymo orchitis. Viral infections also occur in the testis in the form of mumps orchitis.

B) Torsion: Ischemic injury to the testis secondary to twisting of the testis on the spermatic cord pedicle.

C) Trauma: Because of the peculiar immunologic status of the testis in the body (that it is an immunologically privileged site), trauma to the testis can invoke an abnormal immune response in addition to atrophy resulting from injury. Both may contribute to infertility.

6) Cryptorchidism

7) Varicocele

C) Post-Testicular Causes of Male Infertility: These consist primarily of obstructions of the ducts leading away from the testes

1) Block of ducts leading away from testes

A) Congenital: Atresia of vas deferens or epididymis

B) Acquired: Infection

a) Gonorrheal epididymitis

b) Tubercular epididymitis

c) Others

C) Vas ligation

a) Voluntary

b) Iatrogenic

2) Impaired sperm motility, improper maturation of spermatozoa or biochemical, abnormalities of secretion from prostate and seminal vesicle.

Work Up of an Infertile Male

Clinical history, physical examination & semen analysis are the beginning of male infertility workup, which further guide hormonal assay, search for anti-sperm antibody, transrectal ultrasonography, vasography or testicular biopsy. Testicular biopsy is performed in cases of oligospermia or azoospermia when endocrine analyses are normal.[18]

The utility of clinical tests depends upon their appropriate application, technical performance and the provision of accurate and insightful reports. Continued re-evaluation of the service requirements of clinicians is necessary to avoid the adoption of procedures that does not best serve clinical decision-making. Examples of such phenomena have been the expedient adoption of multiplatform testosterone

assays that show significantly divergent normal ranges[19] and the use of inappropriately high serum FSH reference intervals that may lead to misdiagnosis of OA rather than NOA.[20, 21]

Work up of an infertile male includes the following.

A) History

B) Physical examination

C) Semen analysis

D) Hormonal studies

E) Testicular biopsy

A) History

- Cryptorchidism or retractive testes
- Pubertal onset – normal/delayed
- Previous surgeries in the genital area - hernial repair
- Meatal patency, ejaculatory competence
- Orchitis - mumps, STD
- Metabolic diseases
- Family history, drugs and toxin exposure history

B) Physical Examination

- Extent of masculinization
- Eunachoid features
- Penile curvature and meatus position abnormalities. Epididymis texture, mass, nodularity and Bayles sign.
- Hernia / hydrocele / vericocele
- Testes – ectopic / scrotal
- Size - volume, tenderness or reduced testicular sensation

C) Semen Analysis

Semen is analyzed for 2 aspects [22]

1. The total number of spermatozoa which reflects the sperm production by the testes and the patency of the post-testicular duct system.
2. The total fluid volume contributed by the various accessory glands which reflects the secretory activity of the glands.

Vitality, motility & morphology of the spermatozoa and seminal fluid composition are important for spermatozoa function. During sexual intercourse, the initial, sperm-rich prostatic fraction of the ejaculated semen may come into contact with cervical mucus extending into the vagina, with the rest of the fluid remaining as a pool in the vagina.[23] But in the laboratory setting, the entire ejaculate is collected in one container, where the coagulum developed from proteins of seminal vesicle secretions traps the spermatozoa. This coagulum is subsequently liquefied by the action of prostatic proteases, during which time its osmolality rises.[24,25]

The quality of ejaculate collected by using non-spermicidal condoms during intercourse at home is better than the ejaculate collected by masturbation in a container in a laboratory setting.[26] This difference may reflect a different form of sexual arousal, since the time spent producing a sample by masturbation also influences semen quality.[27]

Factors Affecting the Semen Quality[22]

- Completeness of The Sample Collection: During ejaculation, the initial fractions are rich in spermatozoa & prostatic fluids, whereas later fractions contain mainly seminal fluid.[24] Therefore; semen analysis is more adversely affected due to the loss of the initial fractions of the ejaculate compared to the loss of the last fractions.

- The Amount of Fluids Secreted By Accessory Sex Glands: The fluids secreted by accessory sex glands viz. seminal vesicles, prostate & bulbourethral glands dilute the concentrated epididymal spermatozoa during ejaculation.[28] Therefore total numbers of sperms per ejaculate gives correct measure of testicular sperm output than the sperm concentration, because sperm concentration is influenced by the functioning of other reproductive organs. For example, sperm concentrations in semen from young and old men may be the same, but total numbers of sperms per ejaculate is usually more in young men because both the volume of seminal fluid and total sperm output decrease with age, without affecting the sperm concentration.[29]
- Period of Abstinence: Sperm numbers and semen volume increases with duration of abstinence.[30] Sperm vitality and chromatin are not affected by increased length of abstinence unless there is dysfunction of epididymides.[31,32]
- The Size of Testis: In a study conducted by Andersen et al,[33] sperm counts were positively correlated with testis size.

The large biological variation in semen quality reflects the many factors listed above, and requires that all measurements on semen must be precise.[34]

Semen Analysis Involves the Following Steps[22]

- In the first 5 minutes: Placing the specimen container on the bench or in an incubator at 37 °C for liquefaction.
- Between 30 and 60 minutes:

  Assessing liquefaction and appearance of the semen.

  Measuring semen volume.

  Measuring semen pH if required.

Preparing a wet preparation for assessing microscopic appearance, sperm motility and the dilution required for assessing sperm number.

Assessing sperm vitality.

Making semen smears for assessing sperm morphology.

Making semen dilutions for assessing sperm concentration.

Assessing sperm number.

Performing the mixed antiglobulin reaction test if required.

Assessing peroxidase-positive cells if round cells are present.

Preparing spermatozoa for the immunobead test if required.

Centrifuging semen if biochemical markers are to be assayed.

- Within 3 hours:

  Sending samples to the microbiology laboratory if required.

- After 4 hours:

  Fixing, staining and assessing smears for sperm morphology.

- Later on the same day or on a subsequent day if samples are frozen: Assaying accessory gland markers if required & performing the indirect immunobead test if required.

A) Sample Collection[22]

1) Preparation

- The sample should be collected in a private room near the laboratory with few exceptions.
- Minimum of 2 days and a maximum of 7 days of sexual abstinence should be ensured. If additional samples are required, the number of days of sexual abstinence should be as constant as possible at each visit.

- Semen sample needs to be complete and any loss of any fraction of the sample should be reported.
- The following information should be recorded: the man's name, birth date, the period of abstinence, the date and time of collection, the completeness of the sample, any difficulties in producing the sample, and the interval between collection and the start of the semen analysis.

2) <u>Collection of Semen for Diagnostic or Research Purposes</u>

- The sample should be obtained by masturbation and ejaculated into a clean, wide-mouthed container made of glass or plastic, from a batch that has been confirmed to be non-toxic for spermatozoa.
- The specimen container should be kept at ambient temperature, between 20 °C and 37 °C, to avoid large changes in temperature that may affect the spermatozoa after they are ejaculated into it.
- The specimen container is placed on the bench or in an incubator at 37 °C while the semen liquefies.
- Note should be made in the report if the sample is incomplete, especially if the first, sperm-rich fraction is missing.
- If the sample is incomplete, a second sample should be collected, again after an abstinence period of 2–7 days.

3) <u>Collection of Semen at Home</u>

- A sample may be collected at home in exceptional circumstances, such as a demonstrated inability to produce a sample by masturbation in the clinic or the lack of adequate facilities near the laboratory.
- The man should be given clear written and spoken instructions concerning the collection and transport of the semen sample. These should emphasize that the

semen sample needs to be complete, i.e. all the ejaculate is collected, including the first, sperm-rich portion, and that the man should report any loss of any fraction of the sample. It should be noted in the report if the sample is incomplete.

- The man should be given a pre-weighed container, labeled with his name and identification number.
- The man should record the time of semen production and deliver the sample to the laboratory within 1 hour of collection.
- During transport to the laboratory, the sample should be kept between 20 °C and 37 °C.
- The report should note that the sample was collected at home or another location outside the laboratory.

4) <u>Collection of Semen by Condom</u>

- A sample may be collected in a condom during sexual intercourse only in exceptional circumstances, such as a demonstrated inability to produce a sample by masturbation.
- Only special non-toxic condoms designed for semen collection should be used; such condoms are available commercially.
- The man should be given information from the manufacturer on how to use the condom, close it, and send or transport it to the laboratory.
- The man should record the time of semen production and deliver the sample to the laboratory within 1 hour of collection.
- During transport to the laboratory, the sample should be kept between 20 °C and 37 °C.

- The report should note that the sample was collected by means of a special condom during sexual intercourse at home or another location outside the laboratory.

Note: Coitus interruptus is not a reliable method of semen collection, because the first portion of the ejaculate may be lost. Moreover, there may be cellular and bacteriological contamination of the sample and the low pH of the vaginal fluid could adversely affect sperm motility.

5) Safe Handling of Specimens

Semen samples may contain dangerous infectious agents such as human immunodeficiency virus (HIV), hepatitis viruses or herpes simplex virus etc and should therefore be handled as a biohazard material. If the sample is to be processed for bioassay, intra-uterine insemination (IUI), in-vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI), or if semen culture is to be performed, sterile materials and techniques must be used.

B) Initial Macroscopic Examination[22]

Semen analysis should begin with a simple inspection soon after liquefaction, preferably at 30 minutes, but no longer than 1 hour after ejaculation, to prevent dehydration or changes in temperature from affecting semen quality.

1) Liquefaction

Immediately after ejaculation into the collection vessel, semen is typically a semisolid coagulated mass. Within a few minutes at room temperature, the semen usually begins to liquefy, at which time a heterogeneous mixture of lumps will be seen in the fluid. As liquefaction continues, the semen becomes more homogeneous and quite watery, and in the final stages only small areas of coagulation remain. The complete sample usually liquefies within 15 minutes at room temperature, although

rarely it may take up to 60 minutes or more. If complete liquefaction does not occur within 60 minutes, this should be recorded. Semen samples collected at home or by condom will normally have liquefied by the time they arrive in the laboratory.

- Normal liquefied semen samples may contain jelly-like granules, which do not liquefy; these do not appear to have any clinical significance.
- The presence of mucus strands, however, may interfere with semen analysis.

Note: Liquefaction can be recognized both macroscopically, as described above, and microscopically. Immobilized spermatozoa gain the ability to move as the semen liquefies. If immobilized spermatozoa are observed on microscopic examination, more time must be allowed for the liquefaction process to be completed.

During liquefaction, continuous gentle mixing or rotation of the sample container on a two-dimensional shaker, either at room temperature or in an incubator set at 37 °C, can help to produce a homogeneous sample.

If the semen does not liquefy within 30 minutes, do not proceed with semen analysis but wait for another 30 minutes. If liquefaction has not occurred within 60 minutes, proceed as described below.

Delayed Liquefaction: Occasionally samples may not liquefy. In these cases, additional treatment, mechanical mixing or enzymatic digestion may be necessary.

1. Some samples can be induced to liquefy by the addition of an equal volume of physiological medium such as Dulbecco's phosphate-buffered saline, followed by repeated pipetting.
2. Inhomogeneity can be reduced by repeated (6–10 times) gentle passage through a blunt gauge 18 or gauge 19 needle attached to a syringe.

3. Digestion by bromelain, a broad-specificity proteolytic enzyme, may help to promote liquefaction.

These treatments may affect seminal plasma biochemistry, sperm motility and sperm morphology, and their use must be recorded. The 1 + 1 i.e. 1:2 dilution of semen with bromelain must be accounted for when calculating sperm concentration.

2) <u>Semen Viscosity</u>

After liquefaction, the viscosity of the sample can be estimated by gently aspirating it into a wide-bore, approximately 1.5 mm diameter plastic disposable pipette, allowing the semen to drop by gravity and observing the length of any thread. A normal sample leaves the pipette in small discrete drops. If viscosity is abnormal, the drop will form a thread more than 2 cm long.

Alternatively, the viscosity can be evaluated by introducing a glass rod into the sample and observing the length of the thread that forms upon withdrawal of the rod. The viscosity should be recorded as abnormal when the thread exceeds 2 cm.

In contrast to a partially unliquefied sample, a viscous semen specimen exhibits homogeneous stickiness and its consistency will not change with time. High viscosity can be recognized by the elastic properties of the sample, which adheres strongly to itself when attempts are made to pipette it. The methods to reduce viscosity are the same as those for delayed liquefaction.

High viscosity can interfere with determination of sperm motility, sperm concentration, detection of antibody-coated spermatozoa and measurement of biochemical markers.

3) <u>Appearance of the Ejaculate</u>

A normal liquefied semen sample has a homogeneous, grey-opalescent appearance. It may appear less opaque if the sperm concentration is very low; the

colour may also be different, i.e. red-brown when red blood cells are present, or yellow in a man with jaundice or taking certain vitamins or drugs.

4) <u>Semen Volume</u>

The volume of the ejaculate is contributed mainly by the seminal vesicles and prostate gland, with a small amount from the bulbourethral glands and epididymides. Precise measurement of volume is essential in any evaluation of semen, because it allows the total number of spermatozoa and non-sperm cells in the ejaculate to be calculated.

The volume is best measured by weighing the sample in the vessel in which it is collected.

- Collect the sample in a pre-weighed, clean, disposable container.
- Weigh the vessel with semen in it.
- Subtract the weight of the container.
- Calculate the volume from the sample weight, assuming the density of semen to be 1 g/ml.[34]

Note: Empty specimen containers may have different weights, so each container should be individually pre-weighed. The weight may be recorded on the container before it is given to the client. Use a permanent marker pen on the vessel itself or on a label. If a label is used for recording the weight, it should be attached before the empty container is weighed.

Alternatively, the volume can be measured directly:

- Collect the sample directly into a modified graduated glass measuring cylinder with a wide mouth. These can be obtained commercially.
- Read the volume directly from the graduations.

Note: Measuring volume by aspirating the sample from the specimen container into a pipette or syringe, or decanting it into a measuring cylinder, is not recommended, because not all the sample will be retrieved and the volume will therefore be underestimated. The volume lost can be between 0.3 and 0.9 ml. [36, 37]

5) <u>Semen pH</u>

The pH should be measured after liquefaction at a uniform time, preferably after 30 minutes, but in any case within 1 hour of ejaculation since it is influenced by the loss of CO_2 that occurs after production.

For normal samples, pH paper in the range 6.0 to 10.0 should be used. Mix the semen sample well.

- Spread a drop of semen evenly onto the pH paper.
- Wait for the colour of the impregnated zone to become uniform (<30 seconds).
- Compare the colour with the calibration strip to read the pH.

Note: The accuracy of the pH paper should be checked against known standards. For viscous samples, the pH of a small aliquot of the semen can be measured using a pH meter designed for measurement of viscous solutions.

C) <u>Initial Microscopic Examination</u>[22]

A phase-contrast microscope is recommended for all examinations of unstained preparations of fresh semen.

An initial microscopic examination of the sample involves scanning the preparation at a magnification of ×100.This provides an overview of the sample, to reveal:

- Mucus strand formation.
- Sperm aggregation or agglutination.

- Presence of cells other than spermatozoa, e.g. epithelial cells, “round cells” (leukocytes and immature germ cells) and isolated sperm heads or tails.

The preparation should then be observed at ×200 or ×400 magnification. This permits:

- Assessment of sperm motility.
- Determination of the dilution required for accurate assessment of sperm number.

Thorough Mixing and Representative Sampling of Semen: The nature of the liquefied ejaculate makes taking a representative sample problematic. If the sample is not well mixed, analysis of two separate aliquots may show marked differences in sperm motility, vitality, concentration and morphology. To be certain of obtaining reproducible data, the sample should be thoroughly mixed before aliquots are taken for assessment and the results of replicate aliquots should agree before the values are accepted.

Thorough Mixing of Semen: Before removing an aliquot of semen for assessment, mix the sample well in the original container, but not so vigorously that air bubbles are created. This can be achieved by aspirating the sample 10 times into a wide-bore, approximately 1.5 mm diameter disposable plastic pipette. Do not mix with a vortex mixer at high speed as this will damage spermatozoa.

Making a Wet Preparation

- Mix the semen sample well.
- Remove an aliquot of semen immediately after mixing, allowing no time for the spermatozoa to settle out of suspension.
- Remix the semen sample before removing replicate aliquots.

The volume of semen and the dimensions of the coverslip must be standardized, so that the analyses are carried out on a preparation of fixed depth of about 20 µm, which allows the spermatozoa to swim freely:

- Place a standard volume of semen, e.g. 10 µl, onto a clean glass slide.
- Cover it with a coverslip, e.g. 22 mm × 22 mm for 10 µl, to provide a chamber approximately 20 µm deep.
- Take care to avoid the formation and trapping of air bubbles between the coverslip and the slide.
- Assess the freshly made wet preparation as soon as the contents are no longer drifting.

Note: If the chamber is too deep, it will be difficult to assess spermatozoa as they move in and out of focus.

If the number of spermatozoa per visual field varies considerably, the sample is not homogeneous. In such cases, the semen sample should be mixed again thoroughly and a new slide prepared as above.

Lack of homogeneity may also result from abnormal consistency, abnormal liquefaction and aggregation or agglutination of spermatozoa.

Aggregation of Spermatozoa: The adherence either of immotile spermatozoa to each other or of motile spermatozoa to mucus strands, non-sperm cells or debris is considered to be nonspecific aggregation and should be recorded as such.

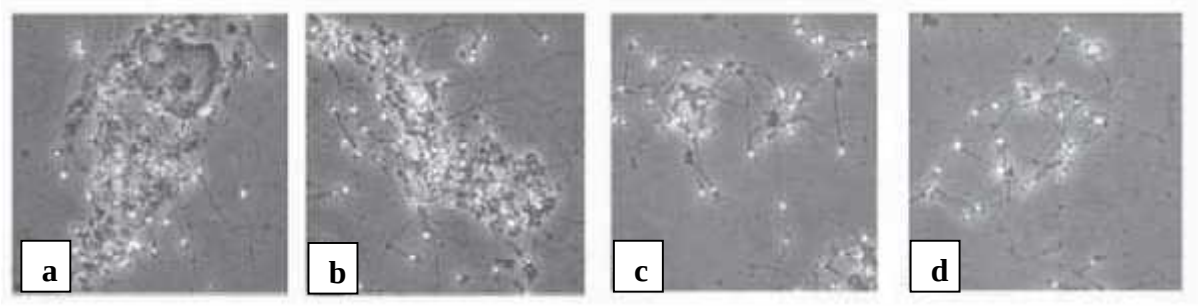


Figure 2: Aggregation of spermatozoa with (a) An epithelial cell, (b) Debris, (c) & (d) Other spermatozoa.[22]

Agglutination of Spermatozoa: Agglutination specifically refers to motile spermatozoa sticking to each other, head-to-head, tail-to-tail or in a mixed way. The motility is often vigorous with a frantic shaking motion, but sometimes the spermatozoa are so agglutinated that their motion is limited.

The major type of agglutination and the site of attachment should be recorded.

The presence of agglutination is not sufficient evidence to deduce an immunological cause of infertility, but is suggestive of the presence of anti-sperm antibodies for which further testing is required.

Severe agglutination can affect the assessment of sperm motility and concentration.

Cellular Elements other than Spermatozoa: The ejaculate contains cells other than spermatozoa, some of which may be clinically relevant. These include epithelial cells from the genitourinary tract, as well as leukocytes and immature germ cells, the latter two collectively referred to as "round cells".[38] They can be identified by examining a stained smear at ×1000 magnification. These cells can be more precisely identified and quantified by detecting peroxidase activity or the antigen CD45. Their concentration can be estimated as for spermatozoa, from wet preparations or from the ratio of these cells to the number of spermatozoa on the stained smear and the sperm concentration.

1) Sperm Motility

The extent of progressive sperm motility is related to pregnancy rates. [39,40]

Sperm motility within semen should be assessed as soon as possible after liquefaction of the sample, preferably at 30 minutes, but in any case within 1 hour, following ejaculation, to limit the deleterious effects of dehydration, pH change & temperature change on sperm motility.

- Mix the semen sample well.
- Remove an aliquot of semen immediately after mixing, allowing no time for the spermatozoa to settle out of suspension.
- Remix the semen sample before removing a replicate aliquot.
- For each replicate, prepare a wet preparation.
- Wait for the sample to stop drifting, usually within 60 seconds.
- Examine the slide with phase-contrast optics at ×200 or ×400 magnifications.
- Assess approximately 200 spermatozoa per replicate for the percentage of different motile categories.
- Compare the replicate values to check if they are acceptably close. If so, proceed with calculations; if not, prepare new samples.

Categories of Sperm Movement: A simple system for grading motility is recommended that distinguishes spermatozoa with progressive or non-progressive motility from those that are immotile.

- Progressive motility (PR): spermatozoa moving actively, either linearly or in a large circle, regardless of speed.
- Non-progressive motility (NP): all other patterns of motility with an absence of progression, e.g. swimming in small circles, the flagellar force hardly displacing the head, or when only a flagellar beat can be observed.
- Immotility (IM): no movement.

2) Sperm Vitality

Sperm vitality, as estimated by assessing the membrane integrity of the cells, may be determined routinely on all samples, but is especially important for samples with less than about 40% progressively motile spermatozoa. This test can provide a check on the motility evaluation, since the percentage of dead cells should not exceed (within sampling error) the percentage of immotile spermatozoa. The percentage of

viable cells normally exceeds that of motile cells. The percentage of live spermatozoa is assessed by identifying those with an intact cell membrane, from dye exclusion or by hypotonic swelling tests. The dye exclusion method is based on the principle that damaged plasma membranes, such as those found in non-vital (dead) cells, allow entry of membrane-impermeant stains. The hypo-osmotic swelling test presumes that only cells with intact membranes (live cells) will swell in hypotonic solutions.

Sperm vitality should be assessed as soon as possible after liquefaction of the semen sample, preferably at 30 minutes, but in any case within 1 hour of ejaculation, to prevent observation of deleterious effects of dehydration or of changes in temperature on vitality.

The presence of a large proportion of vital but immotile cells may be indicative of structural defects in the flagellum; a high percentage of immotile and non-viable cells may indicate epididymal pathology.[32,41]

Vitality Test Using Eosin–Nigrosin: This one-step staining technique uses nigrosin to increase the contrast between the background and the sperm heads, which makes them easier to discern. It also permits slides to be stored for re-evaluation and qualitycontrol purposes.[24]

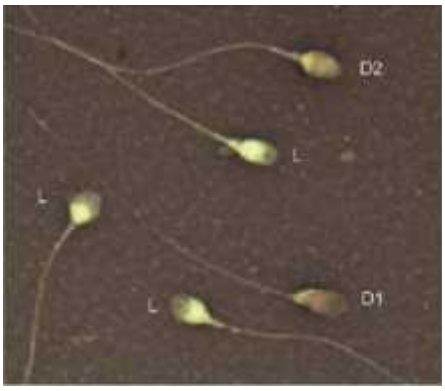


Figure 3: Eosin–nigrosin smear observed in brightfield optics.[22] Spermatozoa with red (D1) or dark pink (D2) heads are considered dead (membrane-damaged), whereas spermatozoa with white heads (L) or light pink heads are considered alive (membrane intact).

3) Sperm Numbers

The editorial committee of the 'WHO Laboratory Manual for the Examination and processing of human semen' 5th edition has considered that total sperm number per ejaculate provides a more accurate assessment of testicular function than does sperm concentration.[22]

Determination of sperm number comprises the following steps.

- Examining a well-mixed, undiluted preparation of liquefied semen on a glass slide under a coverslip, to determine the appropriate dilution and appropriate chambers to use. This is usually the wet preparation used for evaluation of motility.
- Mixing semen and preparing dilutions with fixative.
- Loading the haemocytometer chamber and allowing spermatozoa to settle in a humid chamber.
- Assessing the samples within 10–15 minutes, because after this time evaporation has noticeable effects on sperm position within the chamber.
- Counting at least 200 spermatozoa per replicate.
- Comparing replicate counts to see if they are acceptably close. If so, proceeding with calculations; if not, preparing new dilutions.
- Calculating the concentration in spermatozoa per ml.
- Calculating the total number of spermatozoa per ejaculate.

4) Sperm Morphology

Determination of sperm morphology comprises the following steps.

- Preparing a smear of semen on a slide.
- Air-drying, fixing and staining the slide.

- Mounting the slide with a coverslip if the slide is to be kept for a long time.
- Examining the slide with brightfield optics at ×1000 magnification with oil immersion.
- Assessing approximately 200 spermatozoa per replicate for the percentage of normal forms or of normal and abnormal forms.
- Comparing replicate values to see if they are acceptably close: if so, proceeding with calculations; if not, re-reading the slides.

Analysing a Sperm Morphology Smear

a) Assessment of Normal Sperm Morphology

A spermatozoon consist of a head, neck, midpiece, principal piece and endpiece. As the endpiece is difficult to see with a light microscope, the cell can be considered to comprise a head (and neck) and tail (midpiece and principal piece). For a spermatozoon to be considered normal, both its head and tail must be normal. All borderline forms should be considered abnormal.[22]

- The head should be smooth, regularly contoured and generally oval in shape. There should be a well-defined acrosomal region comprising 40–70% of the head area.[43]
- The acrosomal region should contain no large vacuoles, and not more than two small vacuoles, which should not occupy more than 20% of the sperm head. The post-acrosomal region should not contain any vacuoles.[22]
- The midpiece should be slender, regular and about the same length as the sperm head. The major axis of the midpiece should be aligned with the major axis of the sperm head. Residual cytoplasm is considered an anomaly only when in excess, i.e. when it exceeds one third of the sperm

head size.[44]

- The principal piece should have a uniform calibre along its length, be thinner than the midpiece, and be approximately 45 micro m long (about 10 times the head length). It may be looped back on itself , provided there is no sharp angle indicative of a flagellar break.[22]

Morphological evaluation should be performed on every assessable spermatozoon in several systematically selected areas of the slide, to prevent biased selection of particular spermatozoa.

- Examine the slide using brightfield optics at ×1000 magnification with oil immersion.
- Assess all spermatozoa in each field, going from one microscopic field to another.
- Evaluate at least 200 spermatozoa in each replicate, in order to achieve an acceptably low sampling error.
- Repeat the assessment of at least 200 spermatozoa, preferably on the replicate slide, but alternatively it can be done on the same slide also.
- Report the average percentage of normal forms to the nearest whole number.

Note: Assess only intact spermatozoa, defined as having a head and a tail, since only intact spermatozoa are counted for sperm concentration. Do not count immature germ cells.

Do not assess overlapping spermatozoa and those lying with the head on edge; these cannot be analysed adequately.

b) Assessment of Abnormal Sperm Morphology

Categorizing all abnormal forms of spermatozoa may be of diagnostic benefit.

If desired, note the nature of the defects and calculate the percentage of spermatozoa with defects of the head (%H), midpiece (%M) or principal piece (%P), and those with excess residual cytoplasm (%C).

A multi-key counter can be used, with one key for normal, one for abnormal, and one for each of the four abnormal categories (H, M, P, C). Such a counter allows each spermatozoon to be counted only once, and each of its abnormalities to be scored separately.

- From the final assessment of 400 spermatozoa, it is possible to obtain the percentage of normal and abnormal spermatozoa, as well as the percentage with each type of abnormality, i.e. %H, %M, %P and %C.
- The percentage of spermatozoa in these abnormality classes is obtained by dividing the total number of abnormal spermatozoa with a specific defect by the total number of normal and abnormal spermatozoa scored ×100.

Human semen samples contain spermatozoa with different kinds of malformations. Defective spermatogenesis and some epididymal pathologies are commonly associated with an increased percentage of spermatozoa with abnormal shapes. The morphological defects are usually mixed. Abnormal spermatozoa generally have a lower fertilizing potential, depending on the types of anomalies, and may also have abnormal DNA.

Morphological defects have been associated with

1. Increased DNA fragmentation.[45]
2. An increased incidence of structural chromosomal aberrations. [46]
3. Immature chromatin.[47]
4. Aneuploidy.[48,49]

Therefore emphasis is given to the form of the head, although the sperm tail is also considered.

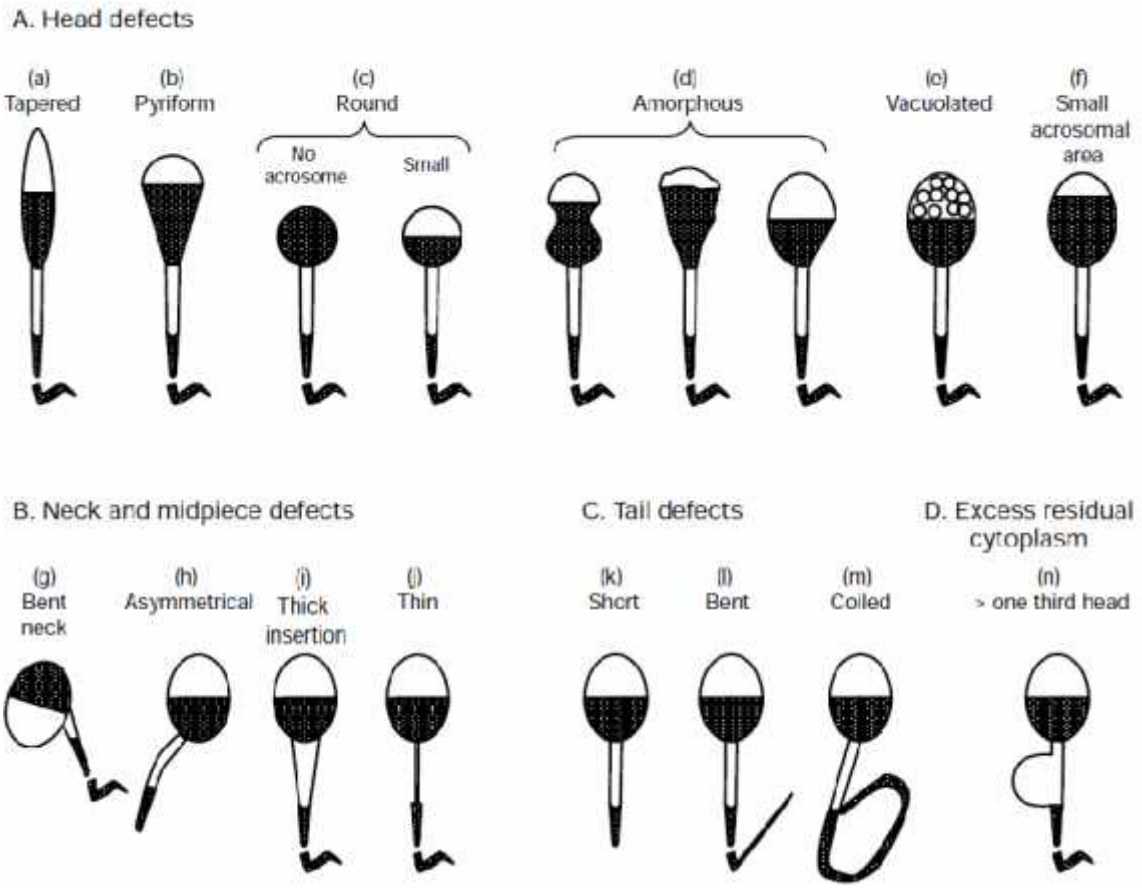
The following categories of defects should be noted.

1. Head defects: large or small, tapered, pyriform, round, amorphous, vacuolated (more than two vacuoles or >20% of the head area occupied by unstained vacuolar areas), vacuoles in the post-acrosomal region, small or large acrosomal areas (<40% or >70% of the head area), double heads, or any combination of these.
2. Neck and midpiece defects: asymmetrical insertion of the midpiece into the head, thick or irregular, sharply bent, abnormally thin, or any combination of these.
3. Principal piece defects: short, multiple, broken, smooth hairpin bends, sharply angulated bends, of irregular width, coiled, or any combination of these.
4. Excess residual cytoplasm (ERC): this is associated with abnormal spermatozoa produced from a defective spermatogenic process. Spermatozoa characterized by large amounts of irregular stained cytoplasm, one third or more of the sperm head size, often associated with defective midpieces are abnormal.[44]

The abnormal excess cytoplasm should not be called a cytoplasmic droplet.[50]

Note: Cytoplasmic droplets (membrane-bound vesicles on the midpiece at the head–neck junction) are normal components of physiologically functional human spermatozoa.[51]

Figure 4: Schematic drawings of some abnormal forms of human spermatozoa.[22]



Assessment of Leukocytes in Semen

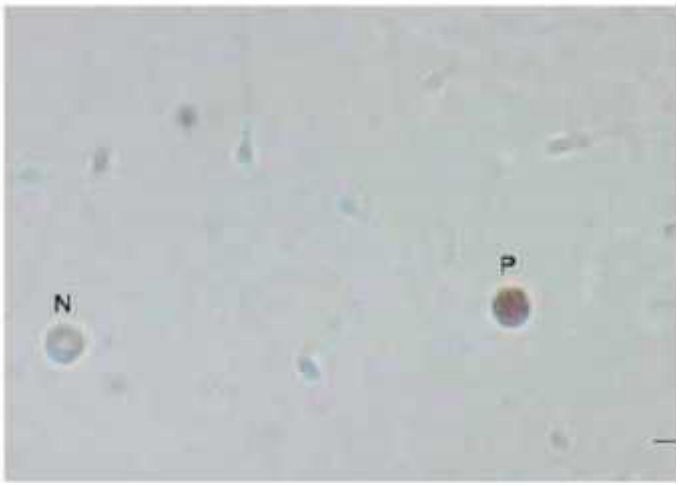
Leukocytes, predominantly neutrophils, are present in most human ejaculates & can be confused with multinucleated spermatids, but stain a bluish colour, in contrast to the more pinkish colour of spermatids. There are several techniques for quantifying the leukocyte population in semen. As peroxidase-positive granulocytes are the predominant form of leukocytes in semen, routine assay of peroxidase activity is useful as an initial screening technique.[38]

Leukocytes can be further differentiated with more time-consuming and expensive immunocytochemical assays against common leukocyte and sperm antigens.[52,53]

Cellular peroxidase staining using Ortho-Toluidine is a quick and inexpensive test useful for initial screening of granulocytes but it does not detect:

1. Activated polymorphs which have released their granules
2. Other types of leukocyte, such as lymphocytes, macrophages and monocytes, which do not contain peroxidase.

Figure 5: A peroxidase-positive granulocyte (P) (brown colour) and a peroxidase-negative round cell (N).[22]



Assessment of Immature Germ Cells in Semen

Germ cells found in semen include round spermatids and spermatocytes, but rarely spermatogonia. They can be detected in stained semen smears, but may be difficult to distinguish from inflammatory cells when the cells are degenerating. Spermatids and spermatocytes can usually be differentiated from leukocytes in a semen smear stained by the Papanicolaou procedure.[38]

Identification can be based on staining coloration, nuclear size and shape, absence of intracellular peroxidase, and lack of leukocyte-specific antigens.

Round spermatids may be identified with stains specific for the developing acrosome, lectins or specific antibodies.[54]

Normal Range of Semen Parameters

Conventional statistical tradition is to take the 2.5th centile from a two-sided reference interval as the threshold, below which values may be considered to come

from a different population. However, in case of semen analysis, a one-sided reference interval was considered to be more appropriate for semen parameters, because high values are unlikely to be detrimental to fertility.[22]

Table 2

Parameter (units)	Centile								
	2.5	5	10	25	50	75	90	95	97.5
Semen volume (ml)	1.2	1.5	2	2.7	3.7	4.8	6	6.8	7.6
Total sperm number (10^6 per ejaculate)	23	39	69	142	255	422	647	802	928
Sperm concentration (10^6 per ml)	9	15	22	41	73	116	169	213	259
Total motility (PR + NP, %)	34	40	45	53	61	69	75	78	81
Progressive motility (PR, %)	28	32	39	47	55	62	69	72	75
Non-progressive motility (NP, %)	1	1	2	3	5	9	15	18	22
Immotile spermatozoa (IM, %)	19	22	25	31	39	46	54	59	65
Vitality (%)	53	58	64	72	79	84	88	91	92
Normal forms (%)	3	4	5.5	9	15	24.5	36	44	48

Above are given the distribution of values for semen parameters from men whose partners became pregnant within 12 months of discontinuing contraceptive use.[22]

However following points should be considered[22]

- A man's semen characteristics need to be interpreted in conjunction with clinical information.
- Semen parameters that lie within the 95% reference interval do not guarantee fertility.
- Men whose semen characteristics fall below the lower limits given here are not necessarily infertile; their semen characteristics are below the reference range for recent fathers—as are, by definition, those of 5% of the fertile men who provided data used in the calculation of the reference range.

D) Hormonal Studies

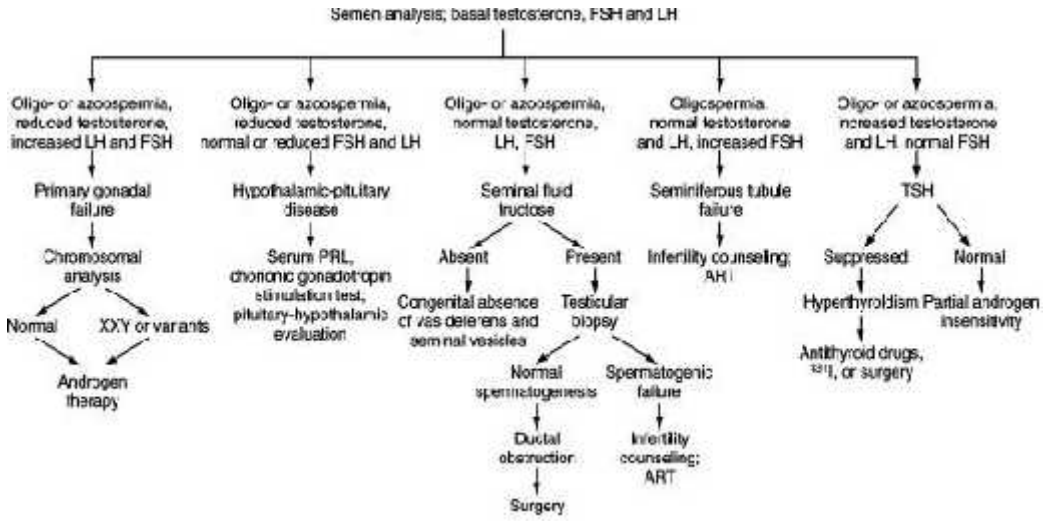
Hormone analyses are required to find the specific dysfunction, if multiple semen analyses demonstrate azoospermia, oligospermia or other abnormality.[55]

Endocrinopathies are a rare cause of male infertility. Endocrine screening of men with sperm counts of less than 10 million/ml with serum testosterone and FSH levels alone will detect vast majority of clinically significant endocrinopathies.[56]

Gonadotropins and testosterone are secreted in a pulsatile manner, and some authors suggest measurement in pooled specimens drawn at 15 minute intervals to increase accuracy, although most recommend screening with a single morning specimen. Morning specimens are preferred due to a normal physiologic decline in testosterone levels throughout the day.[57]

Normal FSH with bilaterally normal testicular volume indicates obstruction with reasonable predictive value. But normal FSH is also seen in approximately 29% of men defective spermatogenesis.[58]

A model diagnostic algorithm for hormonal analysis is given below.[55]



ART = assisted reproductive technology, FSH = follicle stimulating hormone, LH = leutinizing hormone, PRL = prolactin, TSH = Thyroid stimulating hormone.

Primary testicular failure is indicated by decreased testosterone with increased LH and FSH, which can be either a genetic or acquired disease. Secondary testicular failure due to hypothalamic–pituitary disease is reflected by decreased testosterone with decreased or inappropriately normal LH and FSH. HCG stimulation test can be used to confirm secondary disease if LH and FSH levels are normal. HCG interacts with LH receptors & stimulates normal Leydig cells to secrete testosterone. Therefore HCG administration causes an increase in serum testosterone in patients with secondary testicular failure.[55]

Direct measurement of serum inhibin levels may provide a more accurate assessment of spermatogenesis than FSH levels, but the cost of its assay and lack of widespread availability limit its clinical utility.[57]

In cases of suspected hypothalamic–pituitary disease, measurement of prolactin levels should be done. Regulation of FSH and LH secretion by GnRH is disrupted by hyperprolactinemia, the most common cause of which is prolactinoma, others being primary hypothyroidism etc. TSH & TRH are elevated in primary hypothyroidism. The function of TRH is to stimulate the synthesis and secretion of

TSH, but it also stimulates the synthesis and secretion of prolactin. Thus in cases of hyperprolactinemia, elevated TSH suggests primary hypothyroidism & normal TSH suggests prolactinoma.[55]

Seminal fluid fructose is evaluated in cases of normal testosterone, LH, and FSH. Congenital absence of vasa deferentia and seminal vesicles is suggested by absence of fructose in seminal fluid. Presence of fructose in seminal fluid suggests either ductal obstruction or spermatogenic failure, which can be distinguished by testicular biopsy. Oligospermia with normal testosterone and LH with increased FSH suggest seminiferous tubule failure. The raised FSH level is probably due to decrease in negative-feedback inhibition which in turn is due decreased inhibin secretion by sertoli cells. Oligospermia or azoospermia with increased testosterone and LH with normal FSH suggest either partial androgen insensitivity or primary hyperthyroidism, which can be distinguished TSH levels.[55]

D) Testicular Biopsy

History and Evolution:

The history of testicular biopsy in male infertility starts from the early 20th century when needle aspiration was discussed by Huhner in 1928. In those days, testis biopsy was considered as an essential component of the male infertility evaluation. Many descriptive papers were written regarding the description of normal testicular cells from immaturity to adulthood. Papers were also written describing the common patterns of testicular biopsy seen in male infertility. Nelson in 1950 used the terms such as ‘germ cell aplasia’, ‘arrest’ and ‘generalized fibrosis’. Correlations were made between testicular histomorphology & prognosis; ex: inevitable sterility in Klinefelter’s syndrome, or possible reconstructive surgery in ductal obstruction.[4]

Levin in 1979 described how the place of testicular biopsy had changed with

endocrinological analysis & genetic testings, so that in some cases diagnosis and management of male infertility was possible without testicular biopsy.[4]

Fertility practice in present days is impacted by the following aspects[4]

1. ICSI and the identification of mature spermatids in NOA.
2. Recognized linkage between developmental biology of testis, cancer predisposition and infertility.
3. The research effort aimed at understanding phenotype-genotype relationships in male infertility.

A scoring system for quantitatively describing spermatogenesis was proposed by Johnsen in 1970. This scoring system graded each tubule between 1 and 10 according to the most advanced germ cell. And then mean score of all the tubules was calculated. The scoring system made an assumption that the loss of the cells in seminiferous tubules occurs in orderly fashion beginning from the most mature cells i.e. spermatozoa, proceeding to the loss of least mature cells i.e. spermatogonia, then to sertoli cells.[4]

Fundamentally, the Johnsen score is flawed. First, assumption of a progressive loss of germ cells from the most mature to the most immature stages is incorrect. Quantitative data published in 1972 by de Kretser et al[59] clearly demonstrated that in biopsies where there were marked decreases in the number of late spermatids, there was also loss of immature cells such as round spermatids, primary spermatocytes and even spermatogonia. The second flaw is the use of the ‘mean tubule score’. To illustrate the problem, consider the following three settings.[4]

1. Complete germ cell arrest (GCA) at the primary spermatocyte stage in all tubules.
2. Combined spermatogenic failure and obstruction with 50% Sertoli cell-only

(SCO) profiles and 50% normal tubules.

3. Moderately severe hypospermatogenesis in a patient with NOA in which an even scatter of most advanced scores from 1 to 10 is present.

All provide a mean score of 5.0, yet from modern perspective, their outlook and management are starkly different, specifically

1. A poor outlook with TESE requiring at least consideration of donor sperm as an option.
2. Consideration of ductal obstruction and repair or TESE.
3. An excellent prognosis using TESE.

Despite its inherent flaws, the Johnsen score is still widely used.

Indications for Testicular Biopsy

According to the experience of McLachlan et al[4] who studied 1068 bilateral testicular biopsies of 534 consecutive patients undergoing fertility assessment at University Department of Growth and Reproduction, Rigshospitalet, Copenhagen, Denmark, the indications for testicular biopsy are as follows.

1) Determination of severity and type of spermatogenic failure

a) Prognostic indicator for subsequent TESE and ICSI

b) Phenotyping in clinical research context

2) Differentiation of obstructive from non-obstructive azoospermia

a) Equivocal endocrine (FSH, inhibin B) and/or clinical findings

b) Confirmation of spermatogenesis prior to:

i. ART/ICSI (e.g. vasectomy, cystic fibrosis)

ii. Reconstructive surgery for obstruction

3) Evaluation for testicular neoplasia/CIS

a) At time of orchidopexy (after childhood)

b) In ‘at risk’ populations, e.g. prior cryptorchidism, suggestive ultrasonography (microlithiasis)

c) Contralateral testis in setting of testis cancer

4) Cryopreservation of spermatids for ART

5) Failure of prolonged gonadotropin therapy in hypogonadotrophic hypogonadism

Evaluation of prospects with continued therapy.

Techniques of Testicular Biopsy

Considerations in the Number and Type of Biopsy

Testicular tissue can be obtained by one of the following 3 methods viz. fine needle aspiration cytology or open biopsy or percutaneous biopsy using Tru-Cut device. These can be done under local or general anesthesia. One or two samples can be taken; one is representative for most cases, using two biopsies increases CIS detection rate. Other factors should be considered on a case-by-case basis including the issues such as the cost and availability of services.[4]

1. FNAC

FNAC is a simple, low-cost and low-risk procedure that, despite some of artefacts, provides a histological picture that is quite sufficient for differentiating between OA and NOA and for assessing spermatogenic defects, as it shows good agreement with open biopsy data.[60] For sperm retrieval, FNA readily yields sperm for ICSI in OA with minimal cost and morbidity and may also provide sperm in NOA, particularly in the setting of hypospermatogenesis.[61,62] Sperm recovery rates are significantly lower than with multiple open biopsies.[63]

2. Open Testicular Biopsy

This procedure remains the gold standard because it provides optimal amount of tissue both for accurate diagnosis and for retrieval of sperms for IVF.[57]

Open testicular biopsy of at least 3 × 3 × 3 mm provides many more tubule profiles which provide more accurate regional representation and thus a higher probability of revealing a mixed phenotype that may then alter the clinical approach.[4]

McLachlan et al[4] recommend an open surgical biopsy of the contralateral testis at the time of surgery for the primary unilateral testicular cancer, in order to exclude the presence of CIS. Added benefits include the excellent assessment of the spermatogenic/fertility potential of the other testicle and the patient's peace of mind concerning the possibility of bilateral cancer. CIS is often generalized and identifiable even within the small piece of tissue provided by FNA, but existing detection strategies are based on open biopsies. McLachlan et al[4] advocate performing open biopsy in all cases with some suspicion of CIS (e.g. testicular atrophy, microlithiasis) with a single biopsy per testis whereas others like Kliesch et al[64] & Dieckmann et al[8] have argued for at least two biopsies, despite the increased risk of testicular damage.

3. <u>Percutaneous Testicular Biopsy</u>

Percutaneous testis biopsy using a Tru-Cut type of devise has been performed as an office procedure. It has been used for evaluation of both histology and cytology. This blind biopsy procedure could result in unintentional injury to either the epididymis or testicular artery coursing under the surface of the tunica albuginia. Specimens obtained by this method often contain only three to six tubules with poorly preserved architecture.[65]

Yadav et al[66] conducted a prospective study comparing the testicular FNAC & open biopsy. Of the 50 cases, 45 i.e. 90% cases showed agreement between FNAC & histopathology.

Rosenlund et al[67] in a prospective study concluded that percutaneous biopsy with a 19G butterfly needle is a quick and reliable method for demonstrating spermatozoa (Ex: for ICSI) but not for histopathological diagnosis.

Approach to Laboratory Processing of Testicular Biopsy

Based on the combined experience of their two centers and the previous suggestions and observations, McLachlan et al[4] recommend a systematic approach (Table 3) for making high-quality slides for histological analysis, which is as follows.[68,69]

Table 3: Approach to the Preparation of Testis Biopsy for Histological Evaluation

Procedure	Approach	Rationale
Surgical tissue sampling	Laterally at the cranial portion of the testis, away from Epididymis.	Minimization of damage to testicular vessels and a risk of sampling epididymis.
Size of biopsy	3×3×3 mm (a grain of pepper or rice).	Representative for most cases, not too invasive.
Number of biopsies	One, some centres advocate two.	One is representative for most cases, using two biopsies increases CIS detection rate.
Tissue handling	Drop the tissue directly from the scissors into a fixative receptacle: do not compress tissue. Alternatively, run a new scalpel blade horizontally across the tunica and drop the blade and tissue into fixative. For bilateral biopsies, use separate clearly marked vials (left or right testis)	Minimize artefacts, e.g. evulsion of tubule content into intertubular space. Essential to recognize possibility of discrepancy of histology between the 2 testes
Tissue processing (fixation and embedding)	Bouin's fluid or similar fixatives. Fixation time 3–24 h. Some centres prefer glutaraldehyde and plastic embedding (semithin section).	Formalin induces marked shrinkage and gives poor nuclear morphology. Deterioration with more prolonged exposure. Semithin sections give excellent morphology but are not suitable for IHC.

Sectioning and number of slides	Standard microtome, 4 µm thick sections. Cut 10 serial sections on each glass slide. Total of 12 slides. At the beginning and at the end of the series, cut additional double sections for MT, PAS and IHC staining.	Good two-dimensional images. A large number of sections minimize a risk of overlooking infrequent abnormalities or tubules showing completed spermatogenesis.
Staining	Haematoxylin–eosin (HE) staining of every second glass with 10 serial sections. Single slide stained by MT. PAS staining of preferably 2 slides (e.g. numbers 2 and 10). Placental-like alkaline phosphatase (PLAP) [or other CIS marker] immunostaining of 2 sections at the beginning and the end of the series.	HE provides excellent analysis of testicular morphology. MT assists defining fibrosis and spermatid stage. PAS helps to recognize microlithiasis and hyalinization & in some fixatives also CIS. Immunohistochemical staining assists in detection of CIS.
Evaluation and scoring	The observer must be well trained and experienced. It is preferable to have a second observer independently evaluate the biopsy, especially in cases with suspicion of neoplasia. A third opinion may be needed.	Minimizes the risk of an inadvertent overlooking of important findings and reporting errors.

These guidelines can be readily followed in any pathology department.

Evaluation of the Testicular Biopsy & the Importance of Uniform Definitions of the Pathological States

The observation must systematically proceed in a step-wise manner to evaluate and describe the following key features[4]:

i. The uniformity of the histological features, and, if heterogeneity is evident, an estimate of the proportion of tissue with a given histological pattern should be given.

ii. The diameter of the seminiferous tubules, the presence of a lumen and the presence or absence of fibrosis.

iii. Evaluation of the germ cells present within the seminiferous epithelium, specifically seeking to identify spermatogonia, primary spermatocytes, round spermatids and elongated, condensed spermatids representing stages Sd1 and Sd2. Evaluation and comment should be made as to whether any decrease in germ cells is mild, moderate or severe, and if spermatogenesis proceeds to the Sd2 stage, i.e. completed spermatid differentiation, then the term 'hypospermatogenesis' should be applied.

iv. If spermatogenesis ceases at a specific stage, the pathologist must identify at what stage of germ cell development the arrest occurs, and the term 'arrest' should not be used unless there is no progression beyond this stage in any tubule in the entire biopsy.

v. The presence of Sertoli cells in the epithelium should be sought and their features noted. Small Sertoli cell nuclei with small nucleoli are described as immature. The term 'Sertoli cell-only syndrome' (SCOS) should only be used if all the tubules in the biopsy show a total absence of germ cells, thus when tubules containing only Sertoli cells are found in a biopsy in which other

tubules contain advanced spermatids, the overall diagnosis is hypospermatogenesis.

vi. If both germ cells and Sertoli cells are absent from the tubules and there is marked fibrosis and the accumulation of hyaline amorphous basement membrane-like material, the term ‘seminiferous tubule hyalinization’ should be used.

vii. The spermatogonial compartment of the testis should be carefully examined to determine whether cells with the features of CIS cells are present and, if present, their distribution and whether there is any extratubular spread should be noted.

viii. The intertubular tissue should be examined to identify the presence of Leydig cells and their features. Accumulation of Leydig cells into large clumps should be noted. Additionally, the presence of any increase of macrophages or other inflammatory cells should be sought and described. The latter may vary from focal infiltration (e.g. around a tubule with CIS) to an extensive inflammatory process such as granulomatous orchitis.

ix. Finally, other abnormalities seen in association with disturbed spermatogenesis should be noted including the presence of microlithiasis (intra- or intertubular concretions, usually concentric and calcified to some degree), clumps of dysgenetic tubules with distorted shapes of tubule membranes, Leydig cells trapped within hyalinized basement membranes or unusual blood vessels thickened by proteinaceous deposits.

The use of the above methodology of evaluation of the testicular biopsy allows the classification of spermatogenesis as given in the following table. However, it is essential to recognize that these do not indicate a specific pathogenetic mechanism.

Table 4: Patterns of Human Spermatogenesis in Male Infertility[4]

Normal testicular biopsy	This term is used only when there is full spermatogenesis in the entire biopsy and the presence of a normal inter-tubular tissue.
Hypospermatogenesis	All stages of spermatogenesis are present but reduced to a varying degree. This definition also includes varying patterns that can result in some tubules showing an epithelium containing sertoli cells only.
Maturation arrest	Describes the total arrest at a particular stage, most often at the spermatogonial or primary spermatocyte stage. If even small numbers of spermatids are seen in a single tubule in a biopsy wherein the rest of the tubules contain only primary spermatocytes, the term maturation arrest should not be used, rather it should be classified as severe hypospermatogenesis.
Sertoli cell only syndrome (SCOS)	This term is only used when there are no tubules containing germ cells.
Seminiferous tubule hyalinization	Describes the appearance of tubules without either germ cells or sertoli cells and is usually accompanied by peritubular fibrosis and the accumulation of basement membrane-like material in a peritubular position.
Carcinoma in situ (CIS)	This term is used for pre-invasive malignant CIS cells, which are usually present in the place normally occupied by spermatogonia.
Immature testis (prepubertal)	A rare pattern in adult infertile men but typical of hypogonadotrophic hypogonadism. The seminiferous epithelium contains immature sertoli cells and germ cells, the tubules lack a lumen and the inter-tubular space shows no or very few identifiable leydig cells. A few immature tubules may be seen scattered frequently in association with low percentage mosaicism for sex chromosome aneuploidy, and with idiopathic forms of mild testicular dysgenesis.

Approach to Histopathological Reporting of Testicular Biopsy

McLachlan et al[4] suggested use of a descriptive system, which can be conveniently coded for laboratory reporting, which is specifically designed to be relevant to clinical planning. First: Describe the type of testis i.e. adult, immature or neoplastic.

Second: The main or only spermatogenic pattern.

Third: The second most prevalent pattern.

Fourth: Other features.

Significant additional features outside this classification system, such as the presence of multinucleated or degenerating germ cells, or vacuolation of sertoli cells, should be mentioned in the descriptive part of the report.

Table 5: Testicular Biopsy Diagnosis Code Proposed by McLachlan et al[4]

First digit—general pattern	Second digit—most prevalent component	Third digit—next important component	Fourth digit—other abnormalities
1 Adult testis (no neoplasia)	1 Homogeneous, normal spermatogenesis	1 Normal spermatogenesis	1 Lymphocytic infiltration
	2 Homogeneous, reduced spermatid numbers	2 Reduced spermatid number	2 Fibrosis
	3 Homogeneous, germ cell arrest at primary spermatocyte (GCA–spermatocyte)	3 GCA–spermatocyte	3 Leydig cell aggregations (micronodules)
	4 Homogeneous, only spermatogonia + Sertoli cells	4 Only spermatogonia + Sertoli cells	4 Leydig cell aplasia (or strongly reduced number)

	5 Homogeneous, Sertoli cell-only (SCOS)	5 SCO tubules	5 Hyaline bodies (microliths)
	6 Heterogeneous, predominantly 1 or 2	6 Hyalinized tubules	6 Granuloma (or 1+6)
	7 Heterogeneous, predominantly 3 (GCA–spermatocyte)	7 Undifferentiated tubules	7 1+3
	8 Heterogeneous, predominantly 4	8 TDS (undifferentiated tubules + hyaline bodies, microliths)	8 3+5 or 1+3+5
	9 Heterogeneous, predominantly 5 (SCO)	9 Klinefelter's phenotype (two types tubules + Leydig cell nodules)	9 Undifferentiated tubules
	0 Other	0 Other	0 No abnormalities
2 Prepubertal/fetal gonad (no neoplasia)	1 Homogeneous, normal numbers of gonocytes/ spermatogonia	1 Only infantile spermatogonia	1 Lymphocytic infiltration
	2 Homogeneous, reduced number gonocytes/ spermatogonia	2 Spermatocytes present (pubertal pattern)	2 Fibrosis
	3 Homogeneous, no germ cells	3 Elongated spermatids in single tubules	3 Leydig cell aggregations (micronodules)

	4 Heterogeneous, sporadic germ cells	4 Oocytes or primary follicles present	4 Leydig cell aplasia (or strongly reduced number)
	5 Ovarian tissue or ovary-like structures	5 1+5 or 2+5 (in ovotestis)	5 Hyaline bodies (microliths)
	6 Streak gonad	6 Hyalinized tubules	6 Granuloma
	7 Ovotestis	7 Gonocytes (PLAP+) present	7 1+3
	0 Other	8 Klinefelter's phenotype (two types tubules + Leydig cell nodules)	8 3+5
		0 Other	9 Other
			0 No other abnormalities
3 CIS or other neoplasia	1 Adult, only CIS (no spermatogenesis)	1 No invasion	1 Lymphocytic infiltration
	2 Adult, CIS and spermatogenesis	2 Microinvasion into interstitial tissue	2 Fibrosis
	3 CIS, prenatal pattern	3 Invasion into rete testis	3 Leydig cell aggregations (micronodules)
	4 CIS, prepubertal pattern	4 Atrophic tissue present	4 Leydig cell aplasia (or strongly reduced number)

	5 Gonadoblastoma (or gonadoblastoma and CIS)	5 Only undifferentiated tumour cells outside the tubules	5 Hyaline bodies (microliths)
	6 Microinvasive germ cell tumour (seminoma or non-seminoma)	6 Undifferentiated tubules (Sertoli cells)	6 Granuloma
	7 Leydig cell adenoma or tumour	7 Hyalinized tubules	7 1+3
	8 Sertoli cell tumour or sex-cord stromal tumour	8 Necrosis	8 3+5
	9 Lymphoma or leukemic infiltration	9 Ovarian-like structures	9 Undifferentiated tubules
	0 Other	0 Other	0 Other
Other tissue types	E-S Epididymis (spermatozoa visible)		
	E-0 Epididymis (no spermatozoa visible)		
	B Connective tissue		
	F Fat tissue		

Examples: A report coded ‘1222’ would be reported as ‘Moderate severe hypospermatogenesis with a general reduction in advanced elongated spermatids and interstitial fibrosis’ but with the added comment: ‘The recovery of elongated spermatids for ICSI is likely using FNA with open biopsy back up’.

Code 1500 - uniform SCO tubules i.e. SCOS

Code 1923 - a mixed pattern with predominantly SCO tubules but some tubules with spermatids present, and aggregations of leydig cells visible in the interstitial compartment.

Data from each testis are recorded separately.

McLachlan et al[4] studied 1068 bilateral testicular biopsies of 534 consecutive patients undergoing fertility assessment in Copenhagen, Denmark. They found bilateral concordance in 65.9% patients & discordance in 28.3% patients; therefore they support the use of bilateral testicular biopsy for the evaluation of male infertility. Most common testicular histopathology pattern was hypospermatogenesis, followed by sertoli cell-only syndrome, maturation arrest, normal histology & tubular hyalinization respectively. They have commented on the chances of sperm retrieval at TESE depending on the histopathology of testicular biopsy. It was observed that TESE is successful in about 25% of subjects with SCOS. They found the chances of sperm retrieval by TESE in maturation arrest appeared to carry a worse outlook than SCOS. The highest probability of finding sperms in TESE was in hypospermatogenesis cases. They have provided guidelines for a systematic approach to the histological classification of spermatogenic disorders and detection of CIS in adult patients. They proposed a new diagnosis code for testicular biopsies that addresses the needs of ART clinicians and allows data storage and retrieval of value in clinical practice and research.

Abdullah et al[18] retrospectively reviewed testicular biopsies of 100 patients. Patients' age ranged from 22 to 70 years & the mean age was 24.5 years. 33 patients had bilateral testicular biopsies & 5 i.e. 15.15% of these showed a discordant pattern & hence they recommend bilateral testicular biopsy over unilateral. Most common testicular histopathology pattern in their study was hypospermatogenesis, followed by sertoli cell-only syndrome & tubular hyalinization; normal histology; maturation arrest; mixed pattern & discordant pattern respectively. Similar findings between their study & previous studies from the same region of Saudi Arabia as well as other Middle Eastern countries such as Egypt suggested the influence of environmental

factors, socio-cultural habits and consanguineous marriages. The study showed normal histology pattern in 13% of cases, indicating the possibility of varicocele or obstruction at post-testicular site such as rete testes.

Jamali et al[5] reviewed 848 testicular biopsies of 664 infertile patients. The age of patients ranged from 18 to 67 years with mean age of 33 years. Bilateral biopsies were performed in 197 of 664 patients. Of the 197 bilateral biopsies, 76.1% cases showed concordance & 23.86% cases showed discordance. Therefore for the evaluation of male infertility, they were not in favor of unilateral testicular biopsy as compared to bilateral testicular biopsy. Leydig cell hyperplasia was seen in 20% of cases. Most common testicular histopathology pattern in their study was hypospermatogenesis, followed by complete maturation arrest, sertoli cell-only syndrome, peritubular-tubular hyalinization, incomplete maturation arrest, atrophic testis & orchitis respectively.

Venkatachala et al[70] studied testicular biopsies of 30 infertile men. Bilateral biopsies were done in 21 patients & unilateral biopsy was done in 9 patients. In bilateral biopsy cases, they compared the size & mean Johnsen's score of the 2 testes in each patient; they found that in 15 patients, the testicular size was same on either side & mean Johnsen's score was also same on either side; in the remaining 6 cases there was discrepancy in the size of right & left testis, which also showed discrepancy in mean Johnsen score on either side. Of the 21 bilateral biopsy cases, 16 cases showed concordance in histomorphology & 5 cases showed discordance. They opined that unilateral biopsy is sufficient unless there is a variation in the size of the testes. Most common testicular histopathology pattern in their study was maturation arrest, followed by hypospermatogenesis, sertoli cell-only syndrome, tubular hyalinization & normal histology. They mentioned that biopsies with mean Johnsen score 6 or more

respond better to hormonal therapy with human menopausal gonadotrophins & arginine. They also found that sertoli cell only syndrome & tubular hyalinization have worst prognosis and are indications for ART.

Plas et al[71] reviewed 100 bilateral testicular biopsies from 50 patients with azoospermia. 70% cases showed concordance & 28% cases showed discordance. An unsuspected burned out seminoma with maturation arrest in the contralateral testis was seen in 2% of cases. Bilateral biopsies increased the detection of focal spermatogenesis to 68%. If only unilateral diagnostic testicular biopsies had been performed, in 20% of patients focal spermatogenesis in the contralateral testis would have been missed. Due to the prognostic relevance of testicular biopsies for successful sperm retrieval before assisted reproduction, they recommend bilateral diagnostic testicular biopsies in the evaluation of patients with azoospermia.

MATERIALS AND METHODS

Source of Data

A 4 year 9 months retrospective study from January 2008 to September 2012 & 2 year 1 month prospective study from October 2012 to September 2014 was carried out on all testicular biopsy specimens received for the evaluation of male infertility in the Department of Pathology of BLDE University's Shri B.M. Patil Medical College, Hospital and Research centre, Bijapur.

Methods of Collection of Data

Biopsy samples were taken with small curved scissors through a small scrotal window and immediately placed into freshly prepared Bouins solution. Specimens were processed by the original methods as paraffin blocks. All paraffin blocks were cut at 3-5 micron sections on albuminized glass slides. For each case one slide was stained by conventional Haematoxylin and eosin and examined histologicaly by light microscopy.

The specimens were studied histopathologicaly for evaluations of the following

- General architecture
- Number of seminiferous tubules in specimen
- Seminiferous tubules Pattern
- Germ cell/ Sertoli cell ratio
- The basement membrane
- Interstitial tissue
- Leydig cells
- Tubular Hyalinization
- Tunica albuginea
- Epididymis (if present in biopsy)

According to the histopathology criteria, the testicular biopsy specimens were classified histologicaly as:

- Normal Histology
- Hypospermatogenesis
- Maturation arrest
- Sertoli cell only syndrome
- Tubular hyalinization

The seminiferous tubules were graded according to the Modified Johnsen Scoring System[1] as follows

- Full spermatogenesis : Score 10
- Slightly impaired spermatogenesis, many late spermatids, disorganized epithelium : Score 9
- Less than five spermatozoa per tubule, few late spermatids : Score 8
- No spermatozoa, no late spermatids, many early spermatids : Score 7
- No spermatozoa, no late spermatids, few early spermatids : Score 6
- No spermatozoa or spermatids, many spermatocytes : Score 5
- No spermatozoa or spermatids, few spermatocytes : Score 4
- Spermatogonia only : Score 3
- No germinal cells, Sertoli cells only : Score 2
- No seminiferous epithelium : Score 1

Inclusion Criteria:

Testicular biopsy specimens of patients with oligospermia or azoospermia with normal endocrine analysis (normal LH, FSH & Testosterone) were included in the study.

Exclusion Criteria:

Testicular biopsy specimens for evaluation other than infertility were excluded.

Statistical analysis:

Data is analysed by using percentage tables, bar charts & pie charts.

RESULTS & OBSERVATIONS

The present study consisted of 45 cases, of which 30 cases had undergone bilateral testicular biopsies; while 15 cases had unilateral testicular biopsies. Of the 30 cases of bilateral testicular biopsies, 12 were prospective & 18 were retrospective. Of the 15 cases of unilateral testicular biopsies, all were retrospective.

Table 6: Age Wise Distribution of Cases

Age Range in Years	Number of Cases	Percentage
20 – 25	13	28.89%
26 – 30	19	42.22%
31 – 35	10	22.22%
36 – 40	3	6.67%
Total	45	100%

Age of the patients ranged from 22 years to 38 years. Maximum numbers of cases were seen in the age group of 26 to 30 years, followed by 20 to 25 years, 31to 35 years & 36 to 40 years.

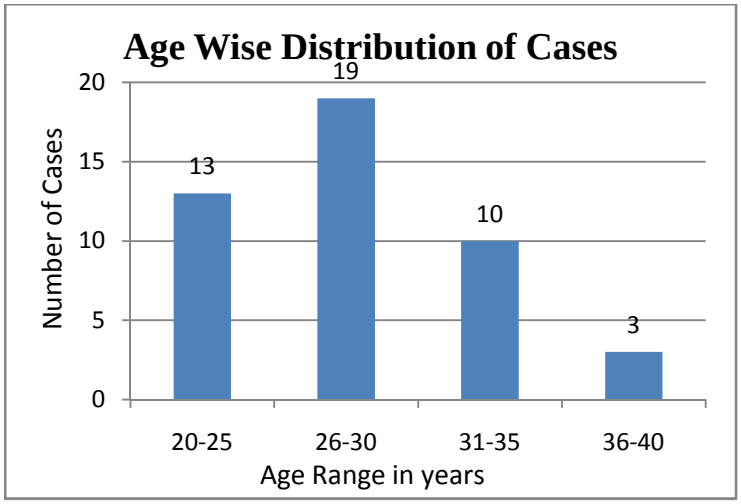


Table 7: Distribution of Cases According to Type of Infertility

Type of Infertility	Number of Cases	Percentage of Cases
Primary	37	82.22%
Secondary	8	17.78%
Total	45	100%

Of the 45 cases of infertility, 37 cases i.e. 82.22% presented with primary infertility. The rest 8 cases i.e. 17.78% patients had presented with history of secondary infertility.

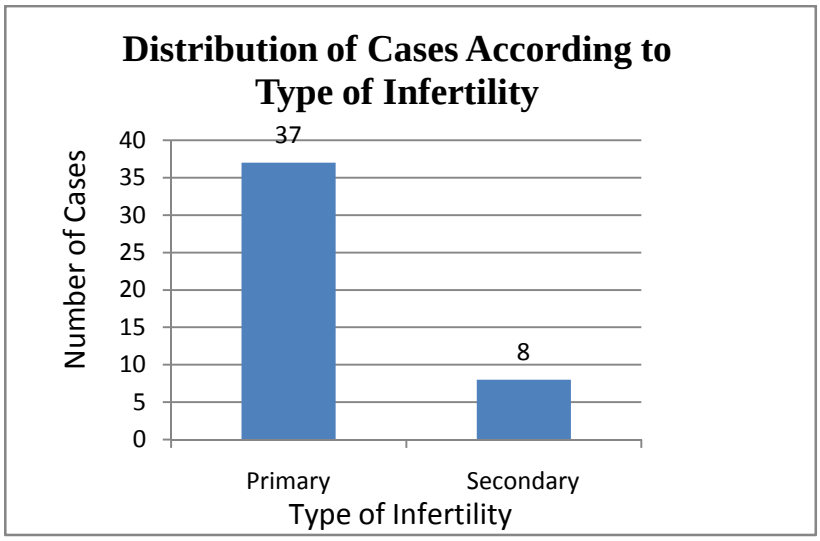


Table 8: Histological Patterns in Different Types of Infertility

Type of Infertility	Normal Spermatogenesis	Hypospermatogenesis	Maturation Arrest	SCOS	Discordant Pattern	Total
Primary	7	12	3	10	5	37
Secondary	1	4	0	0	3	8
Total	8	16	3	10	8	45

The most common histological pattern in both primary & secondary infertility is hypospermatogenesis.

Table 9: Histological Patterns in Right & Left Testes in Discordant Cases According to Types of Infertility

Type of Infertility	Sl. No.	Right	Left
Primary	1	Sertoli cell only syndrome	Hypospermatogenesis
	2	Maturation arrest	Hypospermatogenesis
	3	Sertoli cell only syndrome	Hypospermatogenesis
	4	Maturation arrest	Hypospermatogenesis
	5	Hypospermatogenesis	Maturation arrest
Secondary	1	Hypospermatogenesis	Sertoli cell only syndrome
	2	Sertoli cell only syndrome	Hypospermatogenesis
	3	Normal spermatogenesis	Hypospermatogenesis

None of the secondary infertility cases showed maturation arrest.

Table 10: Distribution of Cases According to Duration of Infertility

Duration of Infertility in years	Total Number of Cases (%)	Number of Cases with Primary Infertility (%)	Number of Cases with Secondary Infertility (%)
< 4	22 (48.89%)	22 (48.89%)	0 (0%)
4 – 6	17 (37.78%)	12 (26.67%)	5 (11.11%)
> 6	6 (13.33%)	3 (6.665%)	3 (6.665%)
Total	45 (100%)	37 (82.22%)	8 (17.78%)

The duration of infertility in the patients under study ranged from 2 years to 10 years. 48.89% cases presented with duration of infertility of less than 4 years. The percentage of cases which presented with duration of infertility of 4 to 6 years was

37.78%. 13.33% cases presented with duration of infertility of more than 6 years. Majority of primary infertility cases presented with duration of infertility less than 4 years, while none of the secondary infertility cases presented with duration of infertility of less than 4 years. The present study shows that secondary infertility cases present with longer duration of infertility compared to most of primary infertility cases.

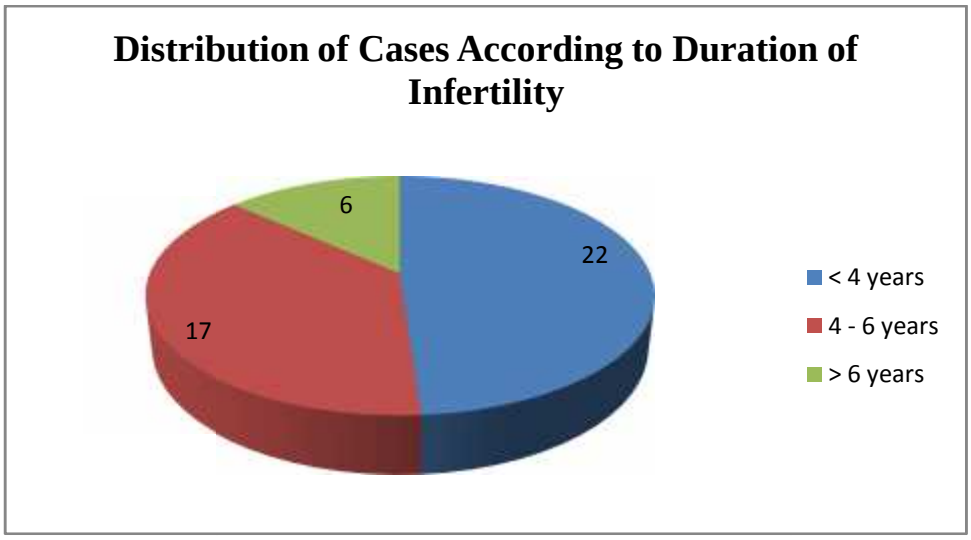


Table 11: Distribution of Cases in Bilateral Testicular Biopsies According to Histological Patterns

Histological Pattern	Number of cases	Percentage of Cases
Hypospermatogenesis	10	33.33%
Discordant pattern	8	26.67%
Sertoli cell only syndrome	7	23.33%
Normal spermatogenesis	4	13.34%
Maturation arrest	1	3.33%
Total	30	100%

Out of 30 bilateral testicular biopsies, the most common histological pattern observed was hypospermatogenesis, followed by discordant pattern, sertoli cell only syndrome, normal spermatogenesis & maturation arrest.

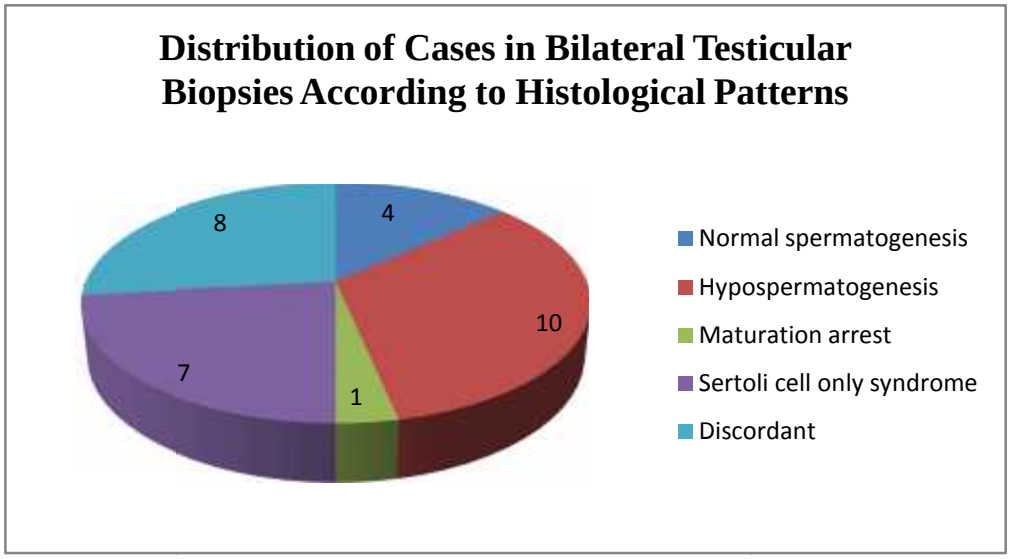
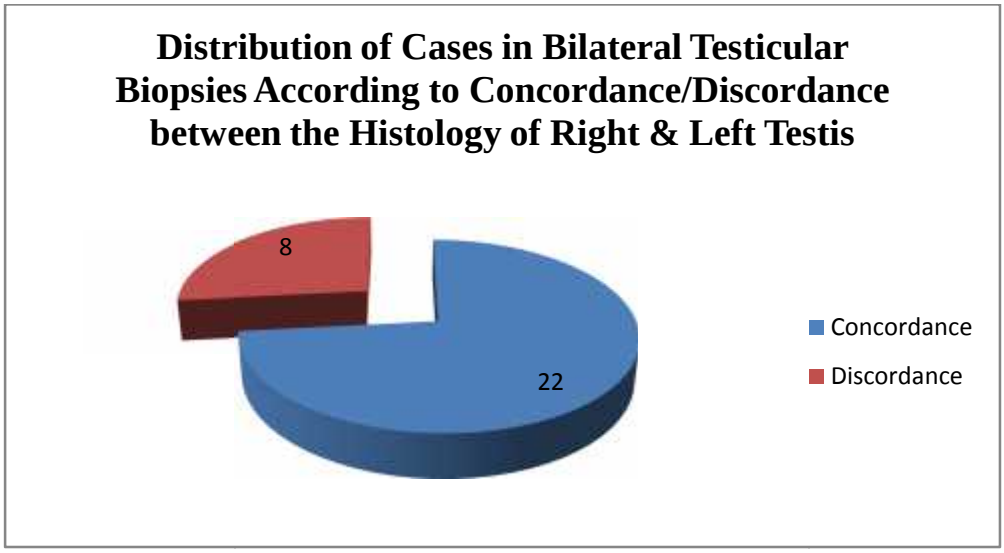


Table12: Distribution of Cases in Bilateral Testicular Biopsies According to Concordance/Discordance between the Histology of Right & Left Testis

Histological Concordance/Discordance	Number of Cases (%)
Concordance	22 (73.33%)
Discordance	8 (26.67%)
Total	30 (100%)

Out of 30 cases in which bilateral testicular biopsies were done, 22 i.e. 73.33% cases showed similar (concordant) histological pattern between right & left testicular biopsies; whereas 8 i.e. 26.67% cases showed different (discordant) histological pattern between right & left testicular biopsies.



The 8 cases which showed discordant histological pattern between right & left testis are as follows along with the comparison of their average modified Johnsen score.

Table 13

Serial Number	Right Testicular Biopsy Histology	Left Testicular Biopsy Histology	Right Average Modified Johnsen Score	Left Average Modified Johnsen Score
1)	Sertoli cell only syndrome	Hypospermatogenesis	1.6	8.5
2)	Hypospermatogenesis	Sertoli cell only syndrome	6.4	2
3)	Sertoli cell only syndrome	Hypospermatogenesis	2	3.4
4)	Maturation arrest	Hypospermatogenesis	4.3	4.1
5)	Sertoli cell only syndrome	Hypospermatogenesis	2	5.9
6)	Maturation arrest	Hypospermatogenesis	5	6.9
7)	Hypospermatogenesis	Maturation arrest	5.8	5
8)	Normal spermatogenesis	Hypospermatogenesis	9.5	5.4

The 8 cases which showed difference between the histological patterns of right & left testicular biopsies also showed difference between their average modified Johnsen score.

Table 14: Distribution of Cases in Unilateral Testicular Biopsies According to Histological Patterns

Histological Pattern	Number of cases	Percentage of Cases
Hypospermatogenesis	6	40.00%
Normal spermatogenesis	4	26.67%
Sertoli cell only syndrome	3	20.00%
Maturation arrest	2	13.33%
Total	15	100%

The most common histological pattern observed was hypospermatogenesis, followed by normal spermatogenesis, sertoli cell only syndrome & maturation arrest.

Table 15: Distribution of Cases According to CIS in Seminiferous Tubules

CIS cells	Number of Cases	Percentage
Present	0	0%
Absent	45	100%
Total	45	100%

Out of the 45 cases studied, none of the cases showed CIS cells in the seminiferous tubules.

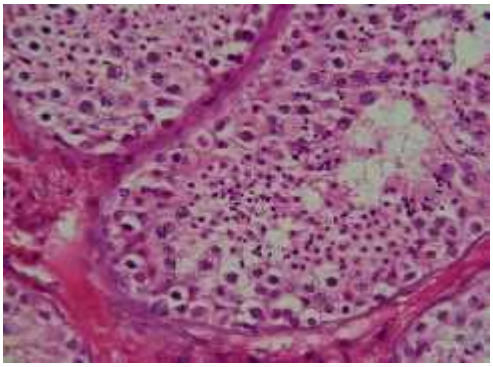


Figure 6: Photomicrograph of testis biopsy showing normal histology. (H&E staining, 400x magnification.)

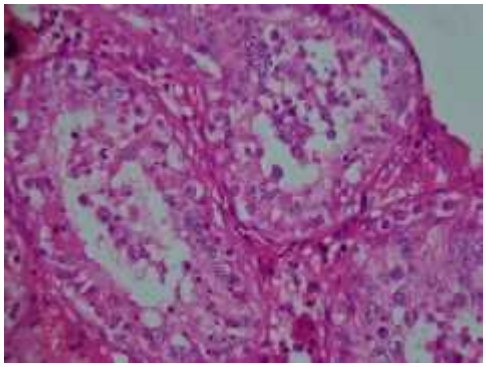


Figure 7: Photomicrograph of testis biopsy showing hypospermatogenesis. (H&E staining, 400x magnification.)

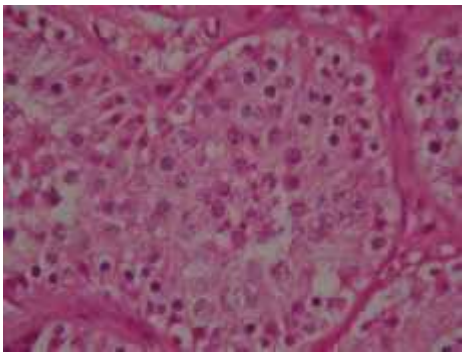


Figure 8: Photomicrograph of testis biopsy showing maturation arrest at primary spermatocyte. (H&E staining, 400x magnification.)

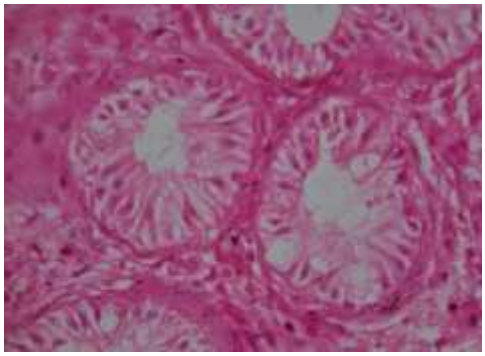


Figure 9: Photomicrograph of testis biopsy showing sertoli cell only syndrome. (H&E staining, 400x magnification.)

DISCUSSION

Testicular pathology is the cause of infertility in approximately 1% of the male populations and 10% of men who seek fertility evaluation. In men with testicular cause of infertility, the biopsy of the testis is must to know the histopathology of the testis & manage accordingly & to foresee the possibility of finding sperms in the testis for assisted reproductive therapy.[2]

Table 16: Comparison of Age Range of Cases Presented for the Evaluation Male Infertility

Studies	Number of Cases	Age Range in Years
Jamali et al[5]	664	18 to 67
Rashed et al[2]	50	23 to 44
Abdullah et al[18]	100	22 to 70
Present Study	45	22 to 38

The age of patients in our study ranged from 22 to 38 years. This is more or less similar to observations of Rashed et al[2] of 23 to 44 years. Age range of patients in the study by Abdullah et al[18] & Jamali et al[5] was 22 to 70 years & 18 to 67 years respectively; this wide age range of patients could be due to large number of cases in their study and could also be due to socio-cultural background.

Table 17: Comparison of Percentage of Cases in Different Age Ranges

Studies	Number of Cases Studied	20 To 25 years	26 To 30 years	31 To 35 years	36 To 40 years	> 40 years
Parikh et al[72]	80	25%	45%	25%	3.75%	1.25%
Present Study	45	28.89%	42.22%	22.22%	6.67%	0%

In the present study maximum numbers of cases were in the age group of 26 to 30 years, which is similar to the findings of Parikh et al[72] study. Minimum numbers of cases in the present study were in the age group of 36 to 40 years, which is more or less similar to the findings of Parikh et al[72] study.

Table 18: Comparison of Type of Infertility

Studies	Number of Cases	Primary Infertility	Secondary Infertility
Yadav et al[66]	25	88%	12%
Present Study	45	82.22%	17.78%

Majority of the patients had primary infertility & the rest had secondary infertility. Present study findings are more or less similar with Yadav et al[66] study findings.

Table 19: Comparison of Duration of Infertility

Studies	Number of Cases	< 4 years	4 – 6 years	> 6 years
Purohit et al[73]	50	24%	60%	16%
Present Study	45	48.89%	37.78%	13.33%

Maximum number of cases in the present study presented within 4 years, followed by 4 to 6 years & after 6 years of marriage. This is not similar to Purohit et al[73] study where maximum number of cases presented between 4 to 6 years, followed by within 4 years & after 6 years of marriage. This may indicate early seeking of medical care due to increased awareness, education, and availability of health care services.

Table 20: Comparison of Histological Patterns in Bilateral Testicular Biopsies

Study	Total (%)	Norm (%)	Hypo (%)	MA (%)	SCOS (%)	TH (%)	Discor (%)	Others (%)
McLachlan et al[4]	534 (100%)	17 (3.2%)	171 (32%)	76 (14.2%)	84 (15.7%)	4 (0.7%)	151 (28.3%)	31 (5.9%)
Present Study	30 (100%)	4 (13.34%)	10 (33.33%)	1 (3.33%)	7 (23.33%)	0 (0%)	8 (26.67%)	0 (0%)

Norm = Normal spermatogenesis, Hypo = Hypospermatogenesis, MA = Maturation Arrrest, SCOS = Sertoli Cell Only Syndrome, TH = Tubular Hyalinization, Discor = Discordance.

In the present study, the most common histological pattern among bilateral testicular biopsy was hypospermatogenesis which is similar to the findings of McLachlan et al[4] study.

The present study showed discordance in 26.67% of bilateral cases, which is comparable to the findings of McLachlan et al[4] study.

The other histological patterns in testicular biopsies in McLachlan et al[4] study included CIS, suspected Klinefelter's syndrome, immature testis, non-conclusive findings, granuloma, inflammation etc. These were not found in the present study which may probably be due to low sample size.

Table 21: Comparison of Distribution of Cases According to Concordance/Discordance between the Histology of Right & Left Testes

Study	Total Number of Cases with Bilateral Testicular Biopsies	Number of Discordant Cases (%)
Jamali et al[5]	197	47 (23.86%)
Plas et al[71]	50	14 (28%)
McLachlan et al[4]	534	151 (28.3%)
Venkatachala et al[70]	21	5 (23.81%)
Abdullah et al[18]	33	5 (15.15%)
Present Study	30	8 (26.67%)

In the present study 26.67% cases showed discordance which is more or less similar to the findings of following studies: Jamali et al,[5] Plas et al,[71] McLachlan et al[4] and Venkatachala et al.[70] All of these studies, except Venkatachala et al[70] recommend bilateral testicular biopsy over unilateral testicular biopsy in the evaluation of male infertility. However Venkatachala et al,[70] also observed variation in size of right & left testes of the discordant cases, therefore opined that unilateral biopsy is sufficient unless there is a variation in the size of the right & left testes. Abdullah et al[18] study reported discordance in 15.15% cases which is less compared the present study & above discussed other studies; however Abdullah et al[18] also suggested bilateral testicular biopsy over unilateral testicular biopsy in the evaluation of male infertility.

In a study conducted at Cornell Urology Center, it was found that for men with hypospermatogenesis on diagnostic testicular biopsy, spermatozoa were retrieved in 81% of attempts, whereas for men with maturation arrest, spermatozoa

were retrieved in only 42% of attempts. If the entire diagnostic biopsy had a Sertoli cell-only pattern, then sperm were retrieved in 24% of TESE attempts. Therefore diagnostic biopsy helps to predict the chance that a TESE procedure will obtain sperm.[74]

Table 22: Comparison of Histological Patterns Wise Distribution of Cases in Unilateral Testicular Biopsies

Study	Total (%)	Norm (%)	Hypo (%)	MA (%)	SCOS (%)	Others (%)
Nagpal et al[75]	100 (100%)	16 (16%)	42 (42%)	18 (18%)	17 (17%)	7 (7%)
Purohit et al[73]	50 (100%)	8 (16%)	13 (26%)	4 (8%)	13 (26%)	12 (24%)
Rashed et al[2]	50 (100%)	12 (24%)	4 (8%)	14 (28%)	17 (34%)	3 (6%)
Present Study	15 (100%)	4 (26.67%)	6 (40.00%)	2 (13.33%)	3 (20%)	0 (0%)

Norm = Normal spermatogenesis, Hypo = Hypospermatogenesis, MA = Maturation Arrrest, SCOS = Sertoli Cell Only Syndrome, TH = Tubular Hyalinization.

The most common histological pattern in unilateral testicular biopsies in the present study was hypospermatogenesis which is correlating with the studies of Nagpal et al[75] & Purohit et al.[73] The most common histological pattern in Rashed et al[2] study was SCOS. The least common histological pattern in unilateral testicular biopsies in the present study was maturation arrest which is correlating with the study of Purohit et al.[73] Others category in the above discussed studies comprised of Klinefelter's syndrome, interstitial orchitis, tubulosclerosis with fibrosis, atrophy & inflammatory lesions are not seen in our studies which could be due to low sample size of the present study & divergent histological reporting systems and the use of imprecise terminology.

Table 23: Comparison of Distribution of Cases According to CIS in Seminiferous Tubules

Study	Total Number of Cases	% CIS Positive Cases
McLachlan et al[4]	534	2.4%
Present Study	45	0%

In the present study none of cases showed CIS in the seminiferous tubules. McLachlan et al[4] reported CIS in 2.4% of cases. Comparatively low sample size in the present study than that of McLachlan et al[4] study explains not finding CIS cases in the present study.

In a study conducted by Raman et al,[76] it was found that the incidence of testicular cancer in infertile men with abnormal semen analyses is 20-fold greater compared to the general population. One of the causes of infertility could be cancer, particularly testicular cancer.[76]

The true prevalence of CIS among the infertile men is almost certainly greater as (i) men presenting with subfertility are not aware of this condition usually until their early thirties, while testicular cancer develops frequently in a younger age and (ii) testicular biopsies are performed after eliminating the possibility of a testicular mass by palpation and scrotal ultrasonographic examination.[4]

CONCLUSION

- Primary infertility is more common than secondary infertility.
- Secondary infertility cases present with longer duration of infertility compared to most of the primary infertility cases.
- Hypospermatogenesis is the most common testicular histological pattern in azoospermia due to testicular causes.
- In bilateral testicular biopsy cases, discordance in histology of right & left testes were seen in 26.67% cases.
- In the evaluation of male infertility due to azoospermia due to testicular causes, bilateral testicular biopsy is recommended over unilateral testicular biopsy.
- CIS should be carefully searched in testicular biopsy received for the evaluation of male infertility.

SUMMARY

- The present study was conducted in the Department of Pathology of BLDE University's Shri B.M. Patil Medical College, Hospital and Research centre, Bijapur.
- The study included 45 patients with azoospermia undergoing infertility evaluation, of which 30 had bilateral & 15 had unilateral testicular biopsy.
- Age of patients in the present study ranged from 22 to 38 years.
- Most common age range of presentation in the present study was 26 to 30 years.
- Primary infertility is more common than secondary infertility in the present study.
- Testicular biopsies were collected in Bouins fixative; H&E stained sections were prepared, examined & reported.
- Of the 30 patients in whom bilateral testicular biopsies were done, 8 i.e. 26.67% patients showed discordance between the right & left testes.
- Hypospermatogenesis was the most common histological pattern observed.
- As management & prognosis of azoospermia due to testicular causes depends on testicular histology, bilateral testicular biopsy is recommended over unilateral in the evaluation of male infertility.

BIBLIOGRAPHY

1) Global prevalence of infertility, infecundity and childlessness [Internet]. 2014 [cited 2014 Oct 17]. Available from: http://www.who.int/reproductivehealth/topics/infertility/burden/en/
2) Rashed MM, Ragab NM, Shalaby AR, Ragab WK. Patterns of Testicular Histopathology in Men with Primary Infertility. The Internet Journal of Urology. 2008;5:44-56.
3) Wong TW, Straus FH, Jones TM, Warner NE. Pathological aspects of the infertile testis. Urol Clin North Am. 1978;5:503-30.
4) McLachlan RI, Rajpert-De Meyts E, Hoei-Hansen CE, de Kretser DM and Skakkebaek NE. Histological evaluation of the human testis - approaches to optimizing the clinical value of the assessment: Mini Review. Hum Reprod. 2007;22:2–16.
5) Jamali M, Haeri H. Histopathologic Findings in 848 Testicular Biopsies of Infertile Males. Acta Medica Iranica. 1999;37:176-180.
6) Richiardi L, Bellocco R, Adami HO, Torrang A, Barlow L, Hakulinen T et al. Testicular cancer incidence in eight northern European countries: secular and recent trends. Cancer Epidemiol Biomarkers Prev. 2004;13:2157–66.
7) Skakkebæk NE, Rajpert-De Meyts E and Main KM. Testicular dysgenesis syndrome: an increasingly common developmental disorder with environmental aspects. Hum Reprod. 2001; 16:972–8.
8) Dieckmann KP, Pichlmeier U. Clinical epidemiology of testicular germ cell tumors. World J Urol 2004;22:2–14.
9) Møller H and Skakkebæk NE. Risk of testicular cancer in subfertile men: case-control study. BMJ. 1999;318:559–62.

10) Giwercman A, Bruun E, Frimodt-Møller C and Skakkebæk NE. Prevalence of carcinoma in situ and other histopathological abnormalities in testes of men with a history of cryptorchidism. J Urol 1989;142:998–1002.

11) Dieckmann KP and Loy V. Prevalence of contralateral testicular intraepithelial neoplasia in patients with testicular germ cell neoplasms. J Clin Oncol. 1996;14:3121–5.

12) Holm M, Lenz S, Rajpert-De Meyts E and Skakkebæk NE. Microcalcifications and carcinoma in situ of the testis. BJU Int. 2001;87:144–9.

13) Holm M, Hoei-Hansen CE, Rajpert-De Meyts E and Skakkebæk NE. Increased risk of carcinoma in situ in patients with testicular germ cell cancer with ultrasonic microlithiasis in the contralateral testicle. J Urol. 2003;170:1163–7.

14) Healy JC. Male reproductive system. In: Standring S, Borley NR, Collins P, Crossman AR, Gatzoulis MA, Healy JC et al, editors. Gray's anatomy. The antomic basis of clinical practice. 40th ed. London: Churchill Livingstone; 2008. p. 1262-7.

15) Levin HS, Schoenfield LR. Non neoplastic diseases of the testis. In: Mills SE, Carter D, Greenson JK, Reuter VE, Stoler MH, editors. Sternberg's diagnostic surgical pathology. 5th ed. Philadelphia: Lippincott Williams & Wilkins; 2010. p. 1914.

16) Nistal M, Paniagua R. Testicular biopsy: Contemporary Interpretation. Urol Clin North Am. 1999;26:555-93.

17) Mascarenhas MN, Flaxman SR, Boerma T, Vanderpoel S, Stevens GA. National, Regional, and Global Trends in Infertility Prevalence Since 1990: A Systematic Analysis of 277 Health Surveys. PLoS Med 2012;9:e1001356.

18) Abdullah L, Bondagji N. Histopathological patterns of testicular biopsy in male infertility: A retrospective study from a tertiary care center in the western part of Saudi Arabia. Urology Annals. 2011;3:19-23.

19) Wang C, Catlin DH, Demers LM, Starcevic B, Swerdloff RS. Measurement of total serum testosterone in adult men: comparison of current laboratory methods versus liquid chromatography-tandem mass spectrometry. J Clin Endocrinol Metab. 2004;89:534–43.

20) Schoor RA, Elhanbly S, Niederberger CS, Ross LS. The role of testicular biopsy in the modern management of male infertility. J Urol. 2002;167:197–200.

21) Sikaris K, McLachlan RI, Kazlauskas R, de Kretser D, Holden CA, Handelsman DJ. Reproductive hormone reference intervals for healthy fertile young men: Evaluation of automated platform assays. J Clin Endocrinol Metab. 2005;90:5928–36.

22) Cooper TG, Aitken J, Auger J, Gordon Baker HW, Barratt CL, Behre HM, et al, editors. WHO laboratory manual for the examination and processing of human semen. 5th ed. Geneva, Switzerland: WHO Press; 2010.

23) Sobrero AJ, MacLeod J. The immediate postcoital test. Fertil Steril. 1962;13:184-189.

24) Björndahl L, Kvist U. Sequence of ejaculation affects the spermatozoon as a carrier and its message. Reproductive BioMedicine Online. 2003;7:440-448.

25) Cooper TG. Changes in osmolality during liquefaction of human semen. Inte J Androl 2005;28:58-60.

26) Zavos PM, Goodpasture JC. Clinical improvements of specific seminal deficiencies via intercourse with a seminal collection device versus masturbation. Fertil Steril 1989;51;190-3.

27) Pound N, Javed MH, Ruberto C, Shaikh MA, Del Valle AP. Duration of sexual arousal predicts semen parameters for masturbatory ejaculates. Physiology and Behavior 2002;76:685-9.

28) Eliasson R. Basic semen analysis. In: Matson P, editor. Current topics in andrology. Perth, Ladybrook Publishing: 2003;35-89.

29) Ng KK, Donat R, Chan L, Lalak A, Di Pierro I, Handelsman DJ. Sperm output of older men. Hum Reprod 2004;19:1811-5.

30) De Jonge C, LaFromboise M, Bosmans E, Ombelet W, Cox A, Nijs M. Influence of the abstinence period on human sperm quality. Fertil Steril 2004;82:57-65.

31) Tyler JP, Crockett NG, Driscoll GL. Studies of human seminal parameters with frequent ejaculation II. Spermatozoal vitality and storage. Clin Reprod Fertil1982;4:287-93.

32) Correa-Perez JR, Fernandez-Pelegrina R, Aslanis P, Zavos PM. Clinical management of men producing ejaculates characterized by high levels of dead sperm and altered seminal plasma factors consistent with epididymal necrospermia. Fertil Steril 2004;81:1148-50.

33) Andersen AG, Jensen TK, Carlsen E, Jorgensen N, Andersson AM, Krarup T et al. High frequency of sub-optimal semen quality in an unselected population of young men. Hum Reprod 2000;15:366-72.

34) Castilla JA, Álvarez C, Aguilar J, González-Varea C, Gonzalvo MC, Martínez1 L. Influence of analytical and biological variation on the clinical interpretation of seminal parameters. Hum Reprod 2006;21:847-51.

35) Auger J, Kunstmann JM, Czyglik F, Jouannet P. Decline in semen quality among fertile men in Paris during the past 20 years. N Engl J Med 1995;332:281-5.

36) Cooper TG, Brazil C, Swan SH, Overstreet JW. Ejaculate volume is seriously underestimated when semen is pipetted or decanted into cylinders from the collection vessel. J Androl 2007;28:1-4.

37) Iwamoto T, Nozawa S, Yoshiike M, Hoshino T, Baba K, Matsushita T *et al*. Semen quality of 324 fertile Japanese men. Hum Reprod 2006;21:760-5.

38) Johanisson E, Campana A, Luthi R, de Agostini A. Evaluation of "round cells" in semen analysis: a comparative study. Hum Reprod Update 2000;6:404-12.

39) Larsen L, Sheike T, JensenTK, Bonde JP, Ernst E, Hjollund NH *et al*. Computer-assisted semen analysis parameters as predictors for fertility of men from the general population. The Danish First Pregnancy Planner Study Team. Hum Reprod 2000;15:1562-7.

40) Zinaman MJ, Brown CC, Selevan SG, Clegg ED. Semen quality and human fertility: a prospective study with healthy couples. J Androl 2000;21:145-53.

41) Chemes HE, Rawe YV. Sperm pathology: a step beyond descriptive morphology. Origin, characterization and fertility potential of abnormal sperm phenotypes in infertile men. Hum Reprod Update 2003;9:405-28.

42) Björndahl L, Kvist U. Sequence of ejaculation affects the spermatozoon as a carrier and its message. Reproductive BioMedicine Online 2003;7:440-448.

43) Menkveld R, Wong WY, Lombard CJ, Wetzels AM, Thomas CMG, Merkus HM *et al*. Semen parameters, including WHO and strict criteria morphology, in a fertile and subfertile population: an effort towards standardization of in-vivo thresholds. Hum Reprod 2001;16:1165-71.

44) Mortimer D, Menkveld R. Sperm morphology assessment—historical perspectives and current opinions. J Androl 2001;22:192-205.

45) Gandini L, Lombardo F, Paoli D, Caponecchia L, Familiari G, Verlengia C *et al*. Study of apoptotic DNA fragmentation in human spermatozoa. Hum Reprod 2000;15:830-9.

46) Lee JD, Kamiguchi Y, Yanagimachi R. Analysis of chromosome constitution of human spermatozoa with normal and aberrant head morphologies after injection into mouse oocytes. Hum Reprod 1996;11:1942-6.

47) Dadoune JP, Mayaux MJ, Guihard-Moscato ML. Correlation between defects in chromatin condensation of human spermatozoa stained by aniline blue and semen characteristics. Andrologia 1988;20:211-7.

48) Devillard F, Metzler-Guillemain C, Pelletier R, DeRobertis C, Bergues U, Hennebeciq S *et al*. Polyploidy in large-headed sperm: FISH study of three cases. Hum Reprod 2002;17:1292-8.

49) Martin RH, Rademaker AW, Greene C, Ko E, Hoang T, Barclay L *et al*. A comparison of the frequency of sperm chromosome abnormalities in men with mild, moderate, and severe oligozoospermia. Biol Reprod 2003;69:535-9.

50) Cooper TG. Cytoplasmic droplets: the good, the bad or just confusing? Hum Reprod 2005;20:9-11.

51) Fetic S, Yeung C, Sonntag B, Nieschlag E, Cooper TG. Relationship of cytoplasmic droplets to motility, migration in mucus, and volume regulation of human spermatozoa. J Androl 2006;27:294-301.

52) Homyk M, Anderson DJ, Wolff H, Herr JC. Differential diagnosis of immature germ cells in semen utilizing monoclonal antibody MHS-10 to the intra-acrosomal antigen SP-10. Fertil Steril 1990;53:323-30.

53) Eggert-Kruse W, Bellmann A, Rohr G, Tilgen W, Runnebaum B. Differentiation of round cells in semen by means of monoclonal antibodies and relationship with male fertility. Fertil Steril 1992;58:1046-55.

54) Ezeh UI, Martin M, Cooke ID, Moore HD. Correlation of testicular pathology and sperm extraction in azoospermic men with ejaculated spermatids detected by immunofl uorescent localization. Hum Reprod 1998;13:3061-5.

55) Borawski D, Bluth MH. Reproductive function and pregnancy. In: McPherson RA, Pincus MR. editors. Henry's clinical diagnosis and management by laboratory methods. 22nd ed. Philadelphia: Saunders; 2011. p. 402-16.

56) Sigman M, Jarow JP. Endocrine evaluation of infertile men. Urol 1997;50:659–64.

57) Sabanegh Jr E, Agarwal A. Male Infertility. In: Wein AJ, Kavoussi LR, Novick AC, Partin AW, Peters CA. editors. Campbell-Walsh Urology. 10th ed. Philadelphia: Saunders; 2012. p. 616-647.

58) Dohle GR. Clinical investigation of the infertile male. In: Bjorndahl L, Giwercman A, Tournaye H, Weidner W. editors. Clinical andrology EAU/ESAU course guidelines. London: Informa Healthcare; 2010. p. 9-17.

59) De Kretser DM, Burger HG, Fortune D, Hudson B, Long AR, Paulsen CA et al. Hormonal, histological and chromosomal studies in adult males with testicular disorders. J Clin Endocrinol Metab 1972;35:392-401.

60) Mallidis C, Baker HW. Fine needle tissue aspiration biopsy of the testis. Fertil Steril 1994;61,367–75.

61) Tuuri T, Moilanen J, Kaukoranta S, Makinen S, Kotola S, Hovatta O. Testicular biopsy gun needle biopsy in collecting spermatozoa for intracytoplasmic injection, cryopreservation and histology. Hum Reprod 1999;14,1274–8.

62) Mercan R, Urman B, Alatas C, Aksoy S, Nuhoglu A, Isiklar A et al. Outcome of testicular sperm retrieval procedures in non-obstructive azoospermia: percutaneous aspiration versus open biopsy. Hum Reprod 2000;15,1548–51.

63) Friedler S, Raziel A, Strassburger D, Soffer Y, Komarovsky D, Ron-el R. Testicular sperm retrieval by percutaneous fine needle sperm aspiration compared with testicular sperm extraction by open biopsy in men with non-obstructive azoospermia. Hum Reprod 1997;12:1488–93.

64) Kliesch S, Thomaidis T, Schütte B, Pühse G, Kater B, Roth S et al. Update on the diagnostic safety for detection of testicular intraepithelial neoplasia (TIN). APMIS 2003;111:70–5.

65) What's New in Male Infertility Treatment at Cornell. [Internet]. 2014 [cited 2014 Oct 17]. Available from: http://www.maleinfertility.org/new-biopsy.html#technique1

66) Yadav SP, Gopal M, Chadha I, Gulati P, Sharma R, Marya SK et al. Comparative evaluation of fine needle aspiration cytology & biopsy of testis in diagnosis of male infertility. J Indian Med Assoc 1997;95:129-30,134.

67) Rosenlund B, Kvist U, Ploen L, Lundh Rozell B, Sjo¨blom P, Hillensjo T. A comparison between open and percutaneous needle biopsies in men with azoospermia. Hum Reprod 1998;13:1266–71.

68) Bruun E, Frimodt-Møller C, Giwercman A, Lenz S and Skakkebæk NE. Testicular biopsy as an outpatient procedure in screening for carcinoma in situ: complications and the patient's acceptance. Int J Androl 1987;10:199–202.

69) Dieckmann KP, Classen J and Loy V. Diagnosis and management of testicular intraepithelial neoplasia (carcinoma in situ)—surgical aspects. APMIS 2003;111:64–8.

70) Venkatachala S, Malur PR, Nerli RB, Desai BR, Dhorigol V. Testicular biopsies – histomorphological patterns in male infertility. Indian J Pathol Microbiol 2007;50:726-9.

71) Plas E, Riedl CR, Engelhardt PF, Mühlbauer H, Pflüger H. Unilateral or bilateral testicular biopsy in the era of intracytoplasmic sperm injection. J Urol 1999;162:2010-3.

72) Parikh UR, Goswami HM, Deliwala KJ, Shah AM, Barot HP. Testicular Biopsy in Male Infertility: Study of 80 Cases. JIMSA 2012;25:75-7.

73) Purohit TM, Purohit MB, Dabhi BJ. Study of semen analysis and testicular in infertile male. Indian J Pathol Microbiol 2004;47:486-490.

74) Management of men with non-obstructive azoospermia. [Internet]. 2014 [cited 2014 Oct 17]. Available from: https://www.cornellurology.com/clinical-conditions/male-infertility/sperm-retrieval-techniques/management-of-men-with-non-obstructive-azoospermia/

75) Nagpal BL, Manjari M, Kapoor K, Dhaliwal US. Testicular biopsy in cases of male infertility: A retrospective study. J Indian Med Assoc 1993;91:171-4.

76) Raman JD, Nobert CF, Goldstein M. Increased incidence of testicular cancer in men presenting with infertility and abnormal semen analysis. J Urol 2005;174:1819–22.

ANNEXURE-I

ETHICAL CLEARANCE CERTIFICATE

B.L.D.E. UNIVERSITY'S
SHRI.B.M.PATIL MEDICAL COLLEGE, BIJAPUR-586 103
INSTITUTIONAL ETHICAL COMMITTEE

INSTITUTIONAL ETHICAL CLEARANCE CERTIFICATE

The Ethical Committee of this college met on 18-10-2012 at 3-30pm to scrutinize the Synopsis of Postgraduate Students of this college from Ethical Clearance point of view. After scrutiny the following original/corrected & revised version synopsis of the Thesis has been accorded Ethical Clearance.

Title Histopathological evaluation of testicular biopsies in male infertility

—x—x—

Name of P.G. student Dr. Jyotirling. S. Savle
pathology

Name of Guide/Co-investigator Dr R.M. Patekar
Prof. pathology

DR.TEJASWINI. VALLABHA
CHAIRMAN
INSTITUTIONAL ETHICAL COMMITTEE
BLDEU'S, SHRI.B.M.PATIL
MEDICAL COLLEGE, BIJAPUR.

Following documents were placed before E.C. for Scrutinization
1) Copy of Synopsis/Research project.
2) Copy of informed consent form
3) Any other relevant documents.

ANNEXURE-II

PROFORMA

NAME: **CASE NO:**

AGE: **OCCUPATION:**

SEX: **RESIDENCE:**

History of infertility for how many years?

Past history: Tesicular injury, chronic debilitating illness, H/O Mumps etc.

Family history:

Personal history: H/O smoking.

General physical examination: Built & Nourishment

Vitals:

Pulse Rate:

Blood Pressure:

Respiratory Rate:

Temperature:

Systemic examination:

- **Cardiovascular system**
- **Respiratory system**
- **Central Nervous System**
- **Per Abdomen Examination**
- **External Genital Examination**

	Right	**Left**
Testicular Size		

Clinical diagnosis:

Basic Hematological Investigations:

Parameters	
Hemoglobin	
TLC	
DLC	
Platelet Count	
ESR	

Urine Routine Examination:

Physical Examination	
Colour	
pH	
Volume	
Appearance	
Odour	
Specific gravity	

Chemical Examination	
Sugar	
Protein	
Microscopic Examination: Cells: Crystals & Casts:	

Semen Analysis Report:

Physical Examination	
Colour	
Volume	
Liquifaction Time	
pH	
Microscopic Examination	
Sperm Count	
Motility	
Morphology	

<u>Endocrine Evaluation</u>:

LH:

FSH:

Testosterone:

Histopathology Report of Testicular Biopsy:

- General architecture:
- Number of seminiferous tubules in specimen:
- Seminiferous tubules Pattern:
- Germ cell/ Sertoli cell ratio:
- The basement membrane:
- Interstitial tissue:
- Leydig cells:
- Tubular Hyalinization:
- Tunica albuginea:
- Epididymis (if present in biopsy):

Impression:

Modified Johnsen Score:

ANNEXURE-III

B.L.D.E.A'S SHRI B.M.PATIL MEDICAL COLLEGE HOSPITAL AND RESEARCH CENTER ,BIJAPUR-586103

RESEARCH INFORMED CONSENT FORM

TITLE OF THE PROJECT **:** PATTERN ANALYSIS OF TESTICULAR HISTOPATHOLOGY IN MEN WITH INFERTILITY

PRINCIPAL INVESTIGATOR **:** Dr. JYOTIRLING SIDDAPPA SAVLE
P.G. DEPARTMENT OF PATHOLOGY

P.G.GUIDE **:** Dr. R.M.POTEKAR M.D. PROFESSOR,
DEPARTMENT OF PATHOLOGY

PURPOSE OF RESEARCH:

I have been informed that this study is done to identify, categorize & compare histopathological changes in unilateral & bilateral testicular biopsies in men with infertility.

PROCEDURE:

I understand that a small sample of my testicular tissue will be taken under short general anesthesia & processed for histopathological analysis.

RISK AND DISCOMFORTS:

I understand the risks involved in the procedures performed.

BENEFITS:

I understand that my participation in the study will help to know the patterns of testicular histopathology in men with infertility.

CONFIDENTIALITY:

I understand that the medical information produced by the study will become a part of hospital record and will be subjected to confidentiality and privacy regulations of the hospital. If the data is used for publications the identity of patient will not be revealed

REQEUST FOR MORE INFORMATION:

I understand that I may be asked more information about the study at any time.

REFUSAL FOR WITHDRAWAL OF PARTICIPATION:

I understand that my participation is voluntary and that I may refuse to participate or may withdraw from the study at any time.

INJURY STATEMENT:

I understand that in the unlikely event of injury to me during the study I will get medical treatment but no further compensations.

I have read and fully understood this consent form. Therefore I agree to participate in the present study.

_____ _____

Participant / Guardian Date:

_____ _____

Signature of Witness Date:

I have explained the patient the purpose of the study, the procedure required and possible risk and benefit to the best of my ability in the vernacular language.

_____ _____

Investigator /P.G. Date:

_____ _____

Witness to Signature Date

KEY TO MASTER CHART

Ab	-	Absent
Avg. Mod. JS	-	Average Modified Johnsen Score
CIS	-	Carcinoma In Situ
Conco	-	Concordance
Discor	-	Discordance
Histo.	-	Histomorphological pattern
Hypo	-	Hypospermatogenesis
MA at Pr. Sp.	-	Maturation Arrest at Primary Spermatocyte Stage
N	-	Normal
Norm	-	Normal spermatogenesis
SCOS	-	Sertoli Cell Only Syndrome
Yrs	-	Years

MASTER CHART

Bilateral Testicular Biopsies

Sl. No.	Age in Yrs	Type of Infertility: Primary or Secondary	Duration of infertility in years	CIS in Right Testis	CIS in Left Testis	Histo. Right Testis	Histo. Left Testis	Conco Or Discor	Avg. Mod. JS Right Testis	Avg. Mod. JS Left Testis
1)	25	Primary	2	Ab	Ab	Norm	Norm	Conco	9.8	9.7
2)	28	Primary	4	Ab	Ab	SCOS	SCOS	Conco	2	2
3)	26	Primary	3	Ab	Ab	Hypo	Hypo	Conco	4.3	4.8
4)	31	Primary	6	Ab	Ab	SCOS	Hypo	Discor	1.6	8.5
5)	31	Primary	4	Ab	Ab	Hypo	Hypo	Conco	5.2	7.2
6)	25	Primary	3	Ab	Ab	Hypo	Hypo	Conco	7.1	7.4
7)	28	Secondary	7	Ab	Ab	Hypo	Hypo	Conco	3.8	2.9
8)	31	Secondary	6	Ab	Ab	Hypo	Hypo	Conco	6.3	6.8
9)	35	Primary	8	Ab	Ab	Hypo	Hypo	Conco	6.9	6.3
10)	34	Secondary	10	Ab	Ab	Hypo	Hypo	Conco	5.1	5.2
11)	30	Primary	3	Ab	Ab	Hypo	Hypo	Conco	3.6	3.8
12)	24	Primary	4	Ab	Ab	SCOS	SCOS	Conco	2	1.9
13)	26	Primary	3	Ab	Ab	SCOS	SCOS	Conco	2	2
14)	30	Secondary	6	Ab	Ab	Hypo	SCOS	Discor	6.4	2

15)	25	Primary	3	Ab	Ab	Norm	Norm	Conco	9.8	9.9
16)	30	Secondary	6	Ab	Ab	SCOS	Hypo	Discor	2	3.4
17)	30	Primary	6	Ab	Ab	SCOS	SCOS	Conco	2	1.5
18)	31	Primary	3	Ab	Ab	SCOS	SCOS	Conco	2	2
19)	36	Primary	4	Ab	Ab	Hypo	Hypo	Conco	7.2	6.7
20)	25	Primary	4	Ab	Ab	MA at Pr. Sp.	Hypo	Discor	4.3	4.1
21)	36	Primary	6	Ab	Ab	Hypo	Hypo	Conco	3.9	6.7
22)	26	Primary	3	Ab	Ab	SCOS	Hypo	Discor	2	5.9
23)	29	Primary	3	Ab	Ab	SCOS	SCOS	Conco	2	2
24)	25	Primary	3	Ab	Ab	MA at Pr. Sp.	Hypo	Discor	5	6.9
25)	31	Primary	5	Ab	Ab	SCOS	SCOS	Conco	1.6	2
26)	24	Primary	4	Ab	Ab	Norm	Norm	Conco	9.3	9.2
27)	28	Primary	3	Ab	Ab	Hypo	MA at Pr. Sp.	Discor	5.8	5
28)	28	Secondary	6	Ab	Ab	Norm	Hypo	Discor	9.5	5.4
29)	31	Primary	7	Ab	Ab	MA at Pr. Sp.	MA at Pr. Sp.	Conco	4.7	4.3
30)	30	Primary	4	Ab	Ab	Norm	Norm	Conco	9.4	9.6

Unilateral Testicular Biopsies

Sl. No.	Age in Yrs	Type of Infertility: Primary or Secondary	Duration of infertility in years	CIS in the Testis	Histo.	Avg. Mod. JS
1)	25	Primary	2	Ab	SCOS	1.5
2)	27	Primary	3	Ab	SCOS	1.9
3)	26	Primary	3	Ab	Hypo	6.3
4)	23	Primary	2	Ab	Norm	9.8
5)	27	Primary	3	Ab	Hypo	4.2
6)	24	Primary	3	Ab	MA at Pr. Sp.	4.1
7)	24	Primary	3	Ab	Hypo	3.6
8)	38	Primary	8	Ab	Hypo	4.8
9)	30	Primary	4	Ab	SCOS	2
10)	33	Secondary	7	Ab	Norm	9.6
11)	22	Primary	2	Ab	Hypo	6.1
12)	26	Primary	3	Ab	Norm	9.8
13)	25	Primary	2	Ab	Norm	9.8
14)	33	Secondary	6	Ab	Hypo	3.9
15)	28	Primary	3	Ab	MA at Pr. Sp.	5