“Detection of Azoospermic Factor (AZF) Microdeletions in Azoospermic and Severe Oligospermic Males.”

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FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY IN ANATOMY

UNDER THE FACULTY OF MEDICINE

BY

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UNDER THE GUIDANCE OF

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2020

DECLARATION

I hereby declare that the thesis entitled, **"Detection of Azoospermic Factor (AZF) Microdeletions in Azoospermic and Severe Oligospermic Males"** which is being submitted here with for the Degree of Doctor of Philosophy in Anatomy, under the faculty of medicine is completed and written by me and has not previously formed the basis for the Degree or Diploma at any other University or examining body.

Place : Kolhapur

Date : 27/11/2020

Research Student

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CERTIFICATE

This is to certify that the thesis entitled **"Detection of Azoospermic Factor (AZF) Microdeletions in Azoospermic and Severe Oligospermic Males"** which is being submitted here with for the award of The Degree of Doctor of Philosophy in Anatomy from D. Y. Patil Education Society, Kolhapur (Deemed to be University, Declared u/s 3 of the UGC Act 1956), under the faculty of Medicine, is the result of original Research work completed by **Dr. Anita Rahul Gune** under my supervision and guidance and to the best of our knowledge and belief, the work bound in this thesis has not formed earlier the basis for the award of any Degree at any other University or examining body.

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Dr. Anita Rahul Gune

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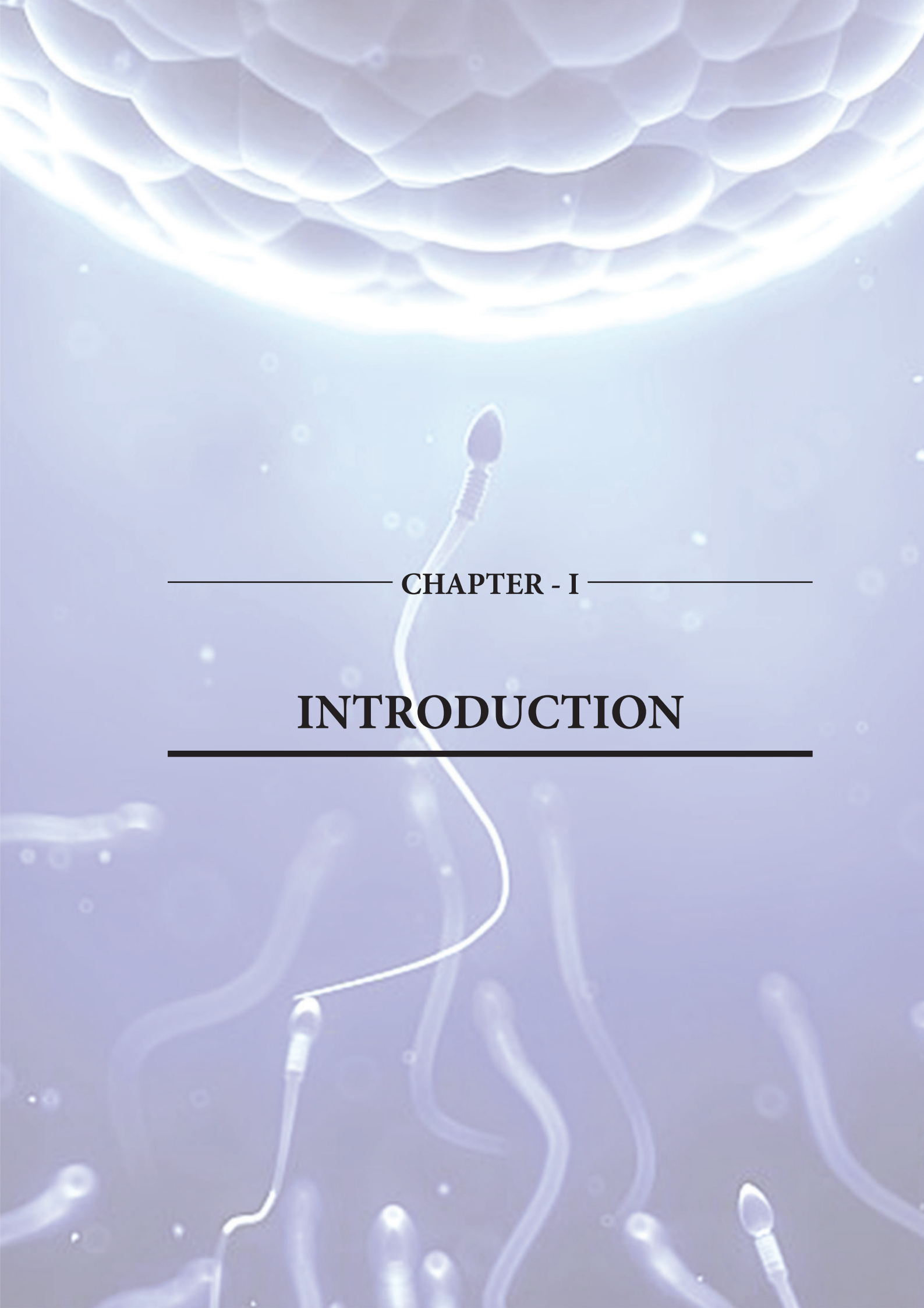
ABBREVIATIONS

ATP	Adenosine Triphosphate
AUA	Andro-urology Association
AZF	Azoospermic Factor
Bp	base pair
°C	Centigrade
CAH	Congenital Adrenal Hyperplasia
CFTR	Cystic fibrosis trans membrane regulator
Cm	Centimeter
CNVs	Gene duplicate range varieties
CBAVD	congenital bilateral absence of vas deferens
D/W	Distil water
DAZ	Deleted in Azoospermia
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphate
EAA	European Association of Andrology
EDTA	Ethylene diamine tetra acetic acid
F primer	Forward Primer
FSH	follicle stimulating hormone
GnRH	gonadotropin-releasing hormone
H and E	Haematoxylin and Eosin
HH	Hypogonadotropic hypogonadism
ICSI	intra-cytoplasmic sperm injection
INSL3	Insulin−like factor 3
IVF	in vitro fertilization
Kb	Kilo base
LH	luteinizing hormone
Mb	Mega bases
mIU	milli international units
MS-Excel 2010	Microsoft Excel 2010
MSY	Male Specific region of Y chromosome

ABBREVIATIONS

Ng	Nano gram
NOA	non obstructive azoospermia
NRY	Non-Recombining region of the Y chromosome
OA	obstructive azoospermia
OAT	oligo-asthenoteratozoospermia
PARs	pseudoautosomal regions
PCR	Polymerase chain reaction
pH	power of hydrogen
pMol	Pico moles
PR	progressively motile
PRL	Prolactin
R primer	Reverse Primer
RNase	Ribonuclease
Rpm	Rotation per minute
RT	Room temperature
SCOS	Sertoli cell only syndrome
SD	standard deviation of mean
SEM	standard error of mean
SNBPs	Sperm Nuclear Basic Proteins
SNP	single nucleotide polymorphism
SOAS	severe oligozoospermic
STS	Sequence Tagged site
T	testosterone
TESE	Testicular sperm extraction
WHO	World health organization
Yp	short arm of the Y chromosome
Yq	long arm of the Y chromosome



CHAPTER - I

INTRODUCTION

Infertility is the inability of a couple to reach a clinical pregnancy within one year of trying by regular intercourse.[1] Infertility amounts to about 8–12% world-wide reproductive couples.[2] It is classified as primary or secondary infertility. Primary infertility if no history of previous pregnancy and secondary if a positive history of pregnancy, whether or not it has resulted in live birth.[3] The most common etiology of infertility in women are ovulation disorders, blocked or damaged fallopian tubes and in males sperm count, motility and morphology disorders.[4] Genetic causes leading to infertility are chromosome abnormalities and gene mutations. Approximately 15% of male infertile cases and 10% of female infertile cases are to genetic etiology.[5]

Couple with infertility has a very painful struggle. Previously women of the infertile couples had to take the sole responsibility for the failure to conceive. Nowadays it is reported that males contribute about 50% of infertility.[6,7]

According to the World Health Organization,[8] male infertility refers to the inability of the male partner to procure a child. On an average about thirty million males are suffering infertility worldwide, reported highest percentage occurring in central Europe [8–12%] and Australia [8–9%].[9]

Etiology of male infertility may be Genetic or non-genetic. Non-Genetic causes attributed to infertility are many like abnormal condition [undescended or absence of testis within the scrotum], varicocele [abnormal enlargement of the pampiniform blood vessel], endocrinological disorders, obstruction / absence of seminal pathways, infections, alcohol consumption or chemotherapy.[10,11] Hereditary deformities regularly seen in infertile males incorporate karyotype anomalies, Gene duplicate range varieties [CNVs], single quality changes / polymorphisms and deletions on

the long arm of the Y chromosome [Yq microdeletions].[12] These genetic defects impede the development of the male gonads cause arrest of germ cell production and/ or maturation or produce non-functional spermatozoa. Amongst the various factors, karyotypic abnormalities and Yq microdeletions are the leading genetic causes of male sterility. However, causes are not known in about 40% of the cases.[13]

Intratesticular testosterone (TT) is vital for spermatogenesis maintenance. When indicated, the hormonal evaluation should include serum follicle stimulating hormone (FSH) and serum total testosterone (TT) levels. The AUA (Andro-urology Association) guidelines on infertility further state that if the TT level is low, a repeat measurement of TT along with free testosterone (FT) or bio available testosterone (BTT), serum luteinizing hormone (LH), and prolactin level should be obtained.[14]

Male infertility is a complicated complex condition that presents with extremely heterogeneous phenotypes. The Y chromosome plays a central role in regulation of gametogenesis as it harbors Y-linked genes that are expressed within the sperm and concerned in numerous processes throughout gametogenesis. The importance of those genes is clear from the observations that the removal of these genes causes distinct pathological testis phenotypes.

Out of genetic causes, Y chromosome microdeletions are common disorders in infertile men.[15] Y chromosome microdeletion analyses is necessary procedure at the diagnosis phase and also prior to the application of assisted reproductive techniques in infertile couples.[5,16] After twenty years from the initial molecular definition of the AZF, Yq deletion screening has now become a routine check for infertile males in several countries to establish the explanation for male infertility. This test is also

helpful in deciding the success rates of sperm cell retrieval and prediction of success of assisted reproduction. Further as the deletion has 100% transmission rate to male offspring's, the couple needs to remember that the males within the future generation will be infertile. Further as the age advances the Oligospermic males may advance to Azoospermic stage.[17,18]

Presently, there is no long-term follow-up data of men harboring Yq microdeletions and there is an urgent need for data on the health status of children born from AZF deletion carriers. As deleted Y chromosome is instable, there are risk of expansion of deletions leading to "genomic instability". Studies should be conducted to understand long term effects of deletion of Y chromosomes especially in conditions like testicular cancers and neurological dysfunctions.

Such progressive loss of genetic material during spermatogenesis might lead to Y chromosome mosaicism in germ cells which might predispose them to testicular/germ cell tumors.

Microdeletions are common especially in second generation males born to fathers carrying the deletion. Secondly, recent reports have demonstrated a significantly deletion of the Y chromosome along with autosomes of infertile men[19,20] indicate more widespread effects of deletions on genomic stability. With the recent data on higher prevalence of neurological problems in infertile men with Yq deletions it is imperative that we carry out detailed analysis of men with Yq deletions with an outlook beyond infertility. We hope that careful clinical observations coupled with detailed genetic information will provide important insights into these unanswered basic questions and give a different perspective to the field of androgenetics.

Infertility is a burning problem in gynecological, Andrological, endocrine and genetic practice. Dramatic advances in diagnosis and treatment of male infertility allow conception for couples previously untreatable. Elucidation of the genetics of male infertility and new tests of sperm function have led to better understanding of the aetiology and prognosis for the treatment of the infertile male.

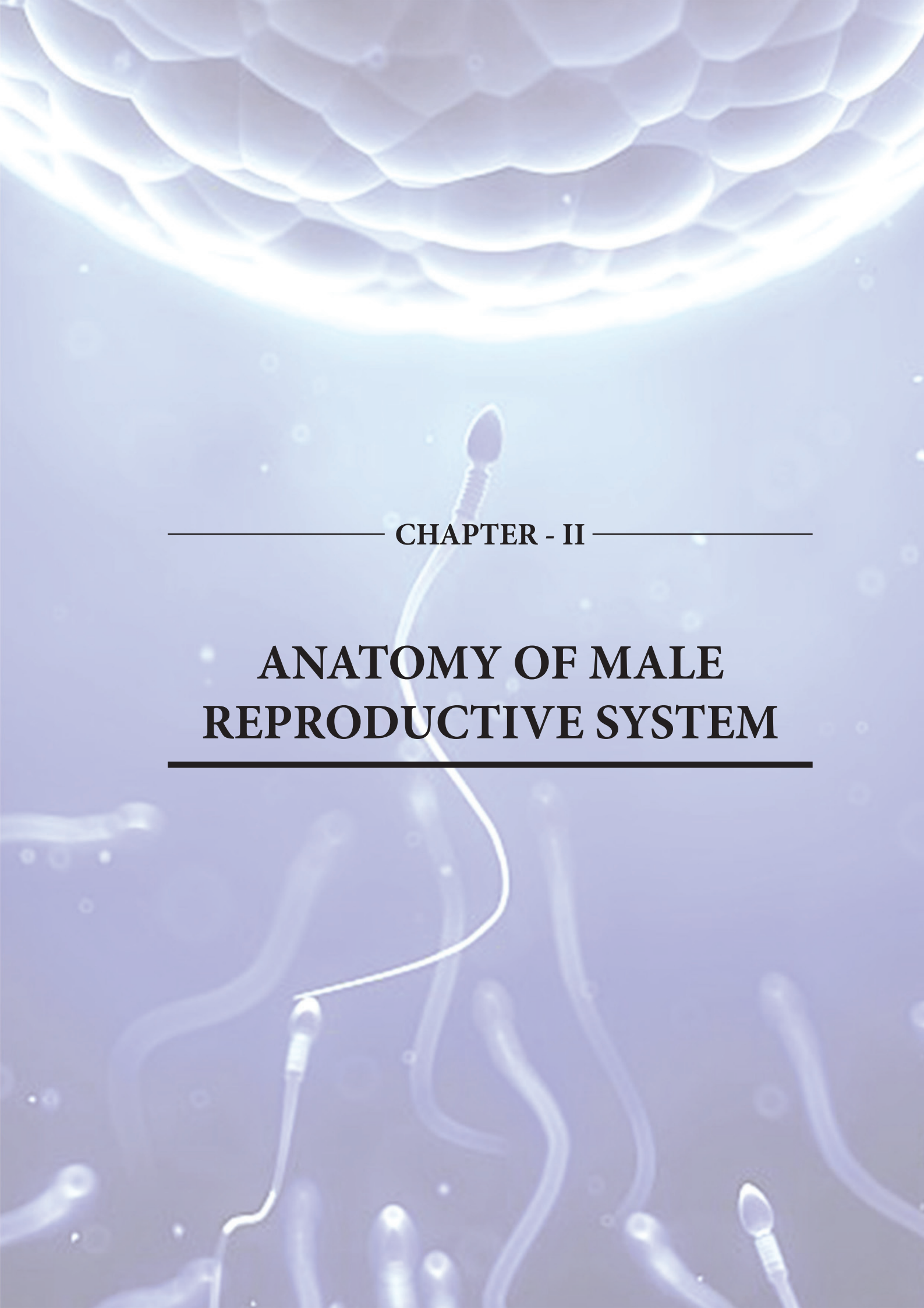
Men who have severe Oligospermia or Azoospermia should have genetic analysis (Karyotype and Y chromosomal micro-deletion assay) as Y chromosome micro-deletions constitute the second most common cause of male infertility. All the AZF microdeletions have no phenotypic or health consequences other than their effect on spermatogenesis. For detection of microdeletion of Y chromosomes 300 STS are generated.

Yq microdeletions, in the last decennia have emerged as one of the major genetic factors contributing to male infertility. Studies from India have reported a wide variation in the prevalence of Yq microdeletions in infertile males. The maximum frequency of Y chromosome microdeletions in the Indian population is reported as 36% [21].

European Association of Andrology suggested 6 markers very efficient for detection of microdeletion, but studies done using only these markers in Indian population have showed less percentage of microdeletion detection. So in this study we have attempted to use EAA and Non EAA markers and also compare deletion detected by these markers.

A semen analysis will remain a key component of a male infertility evaluation but a blood test performed in tandem with the semen analysis will be able to quickly scan the key genes with specific markers for region will prove helpful. Should a man be azoospermic, this assay would also provide information about the chances of finding sperm on testicular biopsy or micro TESE.

Therefore, testing for Yq micro deletion is just not clinically relevant in terms of establishing the diagnosis but is also required for appropriate genetic counselling and clinical management of the infertile cases.



CHAPTER - II

ANATOMY OF MALE REPRODUCTIVE SYSTEM

2 ANATOMY OF MALE REPRODUCTIVE SYSTEM

2.1 Development of Male Reproductive System

Unlike female reproductive system, male gonads lie extra abdominally to maintain temperature optimum for sperm production. From the development of testis till the maturation of sperm, various factors play an essential role to regulate mitotic, meiotic and post meiotic stages of germ cell development. Understanding the basic physiology of male reproductive system is vital to understand the pathology of male infertility.

2.2 Development of Testis

At 3rd week of development after fertilization, the germ cells migrate from the yolk sac to the genital ridge. From the 4th to the 8th week in male embryos, testis develops from the genital ridges and primordial germ cells migrate from wall of yolk

sacs to the gonads. Due to human chorionic gonadotropin, the Leydig cells of the developing testis start to release testosterone. At about ninth week, the labioscrotal swellings fuse to form the scrotum. Testosterone additionally induces development of the mesonephric (Wolfian) duct to develop the epididymis, vas deferens and seminal vesicles.[22]

Each testis is developed from medulla of the undifferentiated genital ridge, the cortex of which regresses. The genital ridge is formed by the proliferation of Coelomic mesothelium covering the medial surface of the mesonephric ridge. Primitive sex cells or gonocytes are developed from the proliferation of endoderm of the dorsal wall of hind gut and appear in the genital ridge by active dorsal and cephalic migration between the layers of the primitive dorsal mesentery of the gut. Numerous solid cellular testis cords arise from the surface of the genital ridge and project into its interior. Primitive sex cells are incorporated within the testis cords. Towards mesonephric blind end, inner ends of testicular cords form rete cord.

The mesenchymal cells invade the genital ridge and spread beneath the surface of the later disconnecting the peripheral ends of the testis cords from the surface. This part of the invaded cells forms the tunica albuginea. Some of the mesenchymal cells project inwards between the testis cords and persist as septa testis and the interstitial cells are derived from the detached mesenchymal cells. During the 7th month of intrauterine life, the testis cords and rete cords are canalized and form respectively the seminiferous tubules and rete testis. Secondly efferent ductules of the testis are developed to form the proximal 12-15 of the persistent mesonephric tubules which establish secondary connections with the rete testis. Thirdly canal of epididymis and vas deferens are developed from the mesonephric duct.

2.3 Histology of Testis

The histology structure of testis is made of two compartments, which morphologically and functionally different. Tubular compartment comprises of seminiferous tubules and interstitial compartment is present between seminiferous tubules as shown in Figure 2.3.1[23].The interstitium is responsible for blood supply and immunological responses[24]. Leydig cells are the most important cells of interstitial compartment, they are the source of testicular testosterone and of insulin−like factor 3 (INSL3). Cells in interstitial compartment are Leydig cells, immune cells and fibroblasts.

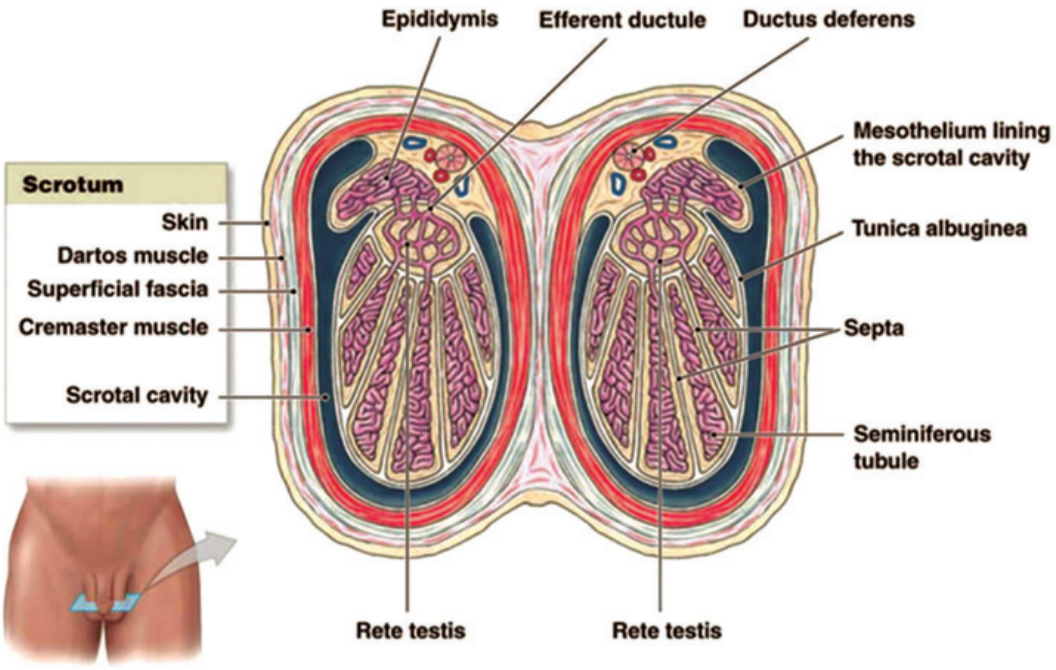


Figure 2.3.1 : Male Reproductive System a horizontal section through the scrotum showing the internal organization of the testis.

The seminiferous tubules are the functional units of the testis where spermatogenesis takes place; they represent 60−80% of total testicular volume. The seminiferous tubules are tightly coiled structures contained in 250–300 compartments within the

testes. The seminiferous tubules are composed of two cell types: the Sertoli cells and germs cells in various stages of development (Figure 2.3.2).[23] Sertoli cells are large cells that extend from the basement membrane (basal lamina) of the tubule to the lumen of seminiferous tubules. These cells are also referred to as nurse cells because they provide structural, functional and metabolic support to germ cells. The Sertoli cells are joined to each other by tight junctions at their lateral aspects and form blood-testis barrier and divide lumen of seminiferous tubules into a basal and adluminal compartment as shown in Figure 2.3.3.[25]

Sertoli cells proliferate in fetal life, in the immediate postnatal neonatal period and just prior to puberty. In the prepubertal period Sertoli cells are relatively quiescent and seminiferous tubules grows slowly. [26]

Sertoli cells secrete two hormones − inhibin and activin − which provide positive and negative feedback on FSH secretion from the pituitary. Additionally, Sertoli cells control release of mature spermatids into the tubular lumen, phagocytosis of the degenerating germ cells and the excess cytoplasm (residual body) that remains from released sperm.[27]

In fetal and neonatal, testosterone plays most important role, whereas during puberty follicle stimulating hormone (FSH) plays important role.[28] The peritubular cells are myoid and drive the peristalsis necessary to move the non−motile elongated testicular spermatozoa released from the nourishing Sertoli cells in the direction of the efferent ducts. [24]

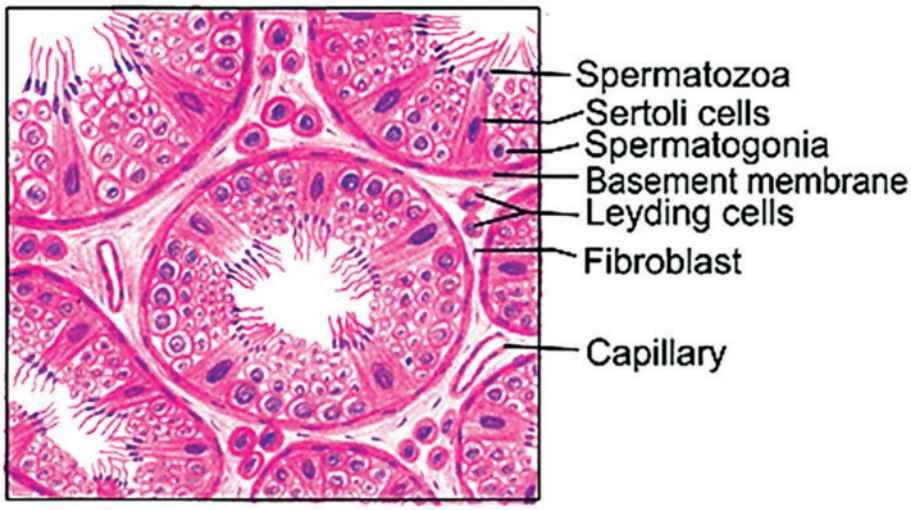


Figure 2.3.2 : A cross-section through a testicular tubule, showing the germ cells at different stages of maturation developing embedded in somatic Sertoli cells. Leydig cells (LC) - where testosterone is synthesized-are present in the interstitium.

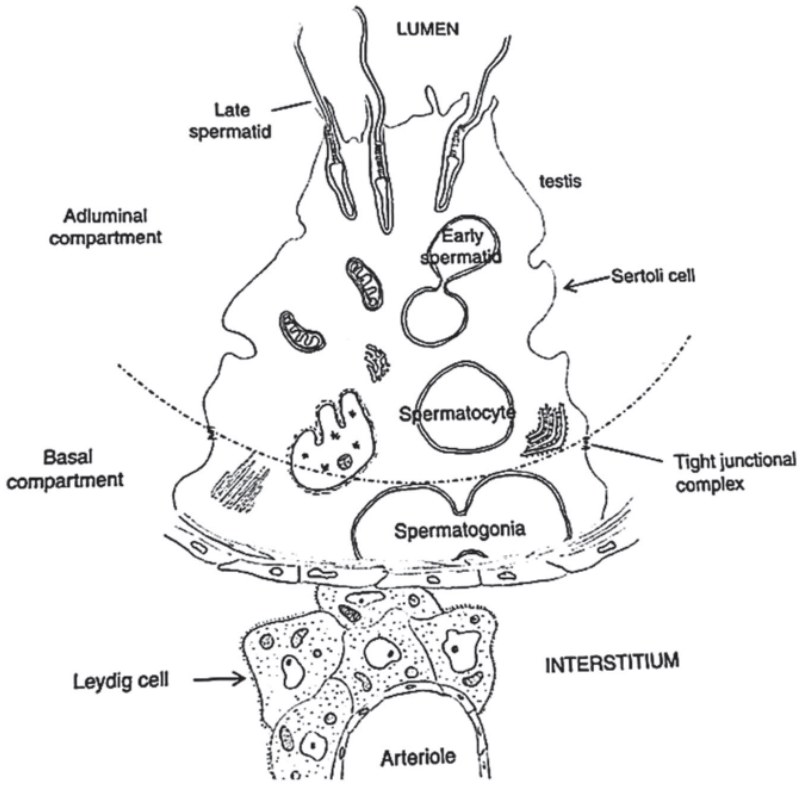


Figure 2.3.3 : A single Sertoli cell with its associated germ cells.

The spermatogenesis is regulated by the FSH of the anterior pituitary with a small amount of testosterone and is helped by Vitamin E and low scrotal temperature. Sperm production is initiated at puberty is continuous throughout the man's adult life. Spermatogonia (containing 46 chromosomes) are located just adjacent to the basement membrane of the seminiferous tubule between Sertoli cells. Near the basement membrane, some spermatogonia remain as reserve cells. These reserve cells via mitosis produce spermatogonia. In first step of sperm cell development, other spermatogonia become primary spermatocytes. The development and maturation of primary spermatocyte takes approximately days and represents the longest developmental period of the spermatogenic cycle. Under the microscope, majority of primary spermatocytes are visible as the period of development is lengthy. Secondary spermatocytes are produced at the end of the first meiotic division. These secondary spermatocytes immediately enter into the second meiotic division, they contain 23 diploid chromosomes. Because secondary spermatocytes begin the second meiotic division so rapidly, no DNA replication has occurred. The second meiotic division then results in the production of spermatids containing 23 chromosomes with only half the DNA.

2.4 Spermatogenesis

Spermatogenesis is the process of conversion of spermatogonia into spermatozoa and take place in the seminiferous tubules**.** Each seminiferous tubule is covered by basement membrane which supports internally two varieties of cells - a generation of spermatogenic cells and supporting cells of Sertoli. During sexual maturity, the spermatogenic cells proliferate cyclically until the spermatozoa are set free in the lumen of seminiferous tubules. There are three ill-defined outer, intermediate and inner zones, in these zones the spermatogenic cells are arranged (Figure 2.4.1).[29]

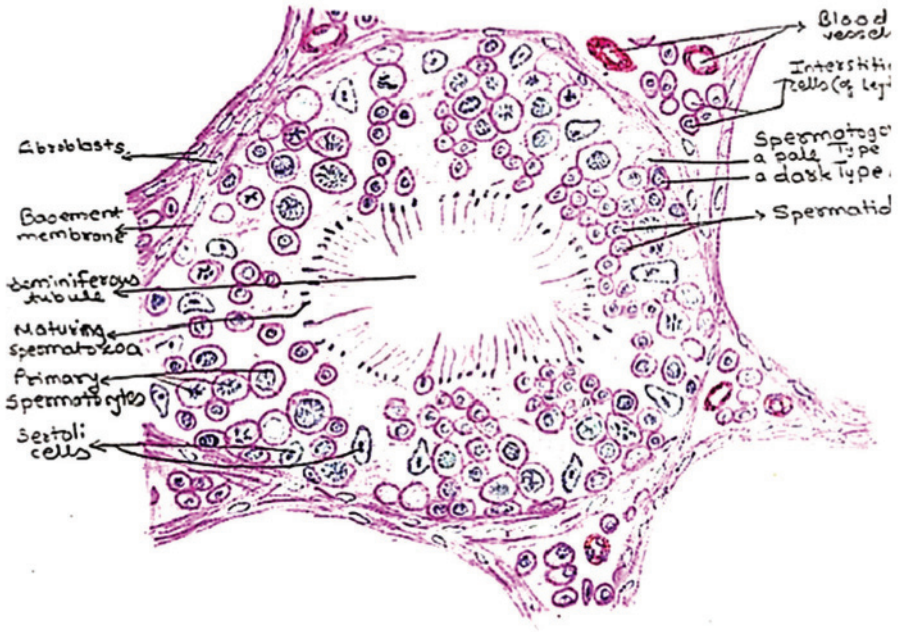


Figure 2.4.1 : Microscopic anatomy of the seminiferous tubules.

The outer zone consists of a population of spermatogonia which are the direct descendants of primitive male sex cells or gonocytes. Spermatogonia contain diploid chromosomes (46, XY), and consist of three types - dark A, pale A, and type B. Each gonocyte divides by mitosis into two dark A spermatogonia which act as stem cells. Dark A cell divides in turn into one dark A cell which acts as a reserve and one pale A cell which is more differentiated. Pale A cell undergoes mitosis into two pale A cells, out of which one is kept as reserve and the other cell divides into two type B cells which are more differentiated. Each type B cell undergoes repeated mitosis so that four generations of type B spermatogonia are found in man. Roughly speaking each pale A spermatogonia gives rise to 16 type B spermatogonia. The intermediate zone consists of outer layers of primary spermatocytes and inner layers of secondary spermatocytes. Primary spermatocytes are the daughter cells of type B spermatogonia and produced by mitotic division and each cell presenting diploid chromosomes.

Secondary spermatocytes are the daughter cells of primary spermatocytes and are produced by first meiotic division (reduction division) and each cell presenting haploid chromosomes (23, X) or (23, Y). Primary spermatocytes enter into prolonged prophase of first meiosis hence, are more easily seen than secondary spermatocytes.

The inner zone consists of two or more rows of spermatids and some residual bodies between them. Spermatids are the daughter cells of secondary spermatocytes and produced by second meiotic division and each cell presenting haploid chromosomes. New spermatids are elongated and plunge into the cytoplasm of the Sertoli cells until the spermatozoa are released into the lumen of the seminiferous tubules. Maturation of spermatozoa takes place with the extrusion of some part of cytoplasmic organelles of spermatids. The extruded mass forms residual bodies which are phagocytosed probably by the Sertoli cells. The two-phase meiotic process therefore results in the formation of spermatids that contain only half the number of chromosomes (haploid) of the original spermatogonia (Figure 2.4.2).[30]

Spermatids are metamorphosed into spermatozoa without further cell division and this process is known as spermiogenesis. The final phase of sperm production is spermiogenesis, which lasts approximately 24 days and initiates when spermatids begin to develop polarity in four stages, the Golgi phase, acrosome phase, tail formation, and the maturation phase. During these stages, spermatid DNA is packaged tightly to condense the nucleus for better mobility of the sperm. The Golgi apparatus then surrounds the nucleus and produces enzymes, to form the acrosomal cap around the head of the sperm (acrosomes contain enzymes essential for egg penetration). The tail is formed by elongation of a single centriole, while the midpiece forms as mitochondria wrap in a spiral form around the core of the tail. Figure 2.4.3

shows a diagram of a complete, mature sperm cell. Once the structures are completely developed, excess cytoplasm is phagocytosed by surrounding Sertoli cells, resulting in fully formed non- motile sperm. Sperm are then released from Sertoli cells into the lumen of seminiferous tubules to remove any traces of cytoplasm or residual organelles.

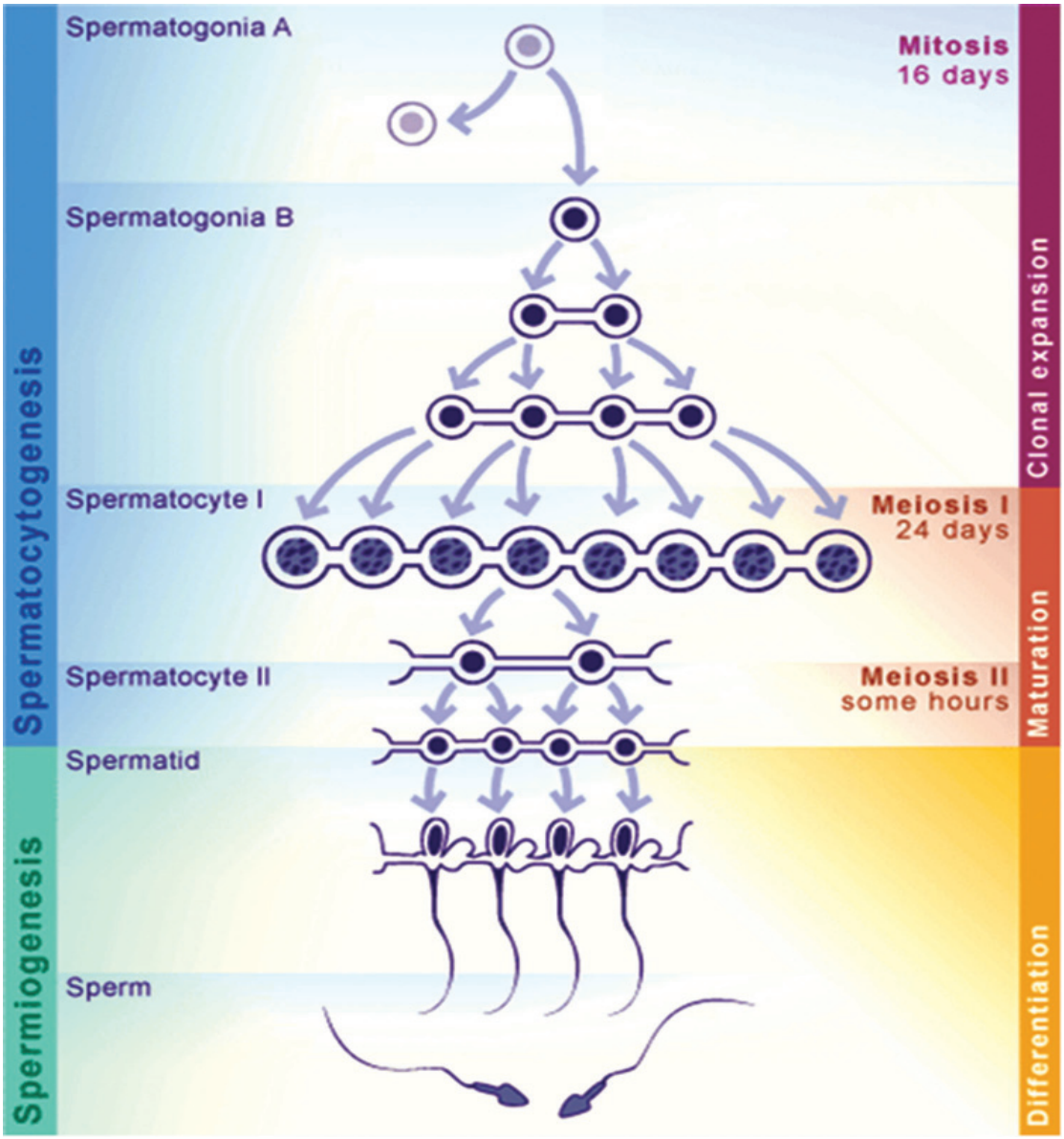


Figure 2.4.2 : An overview of spermatogenesis.

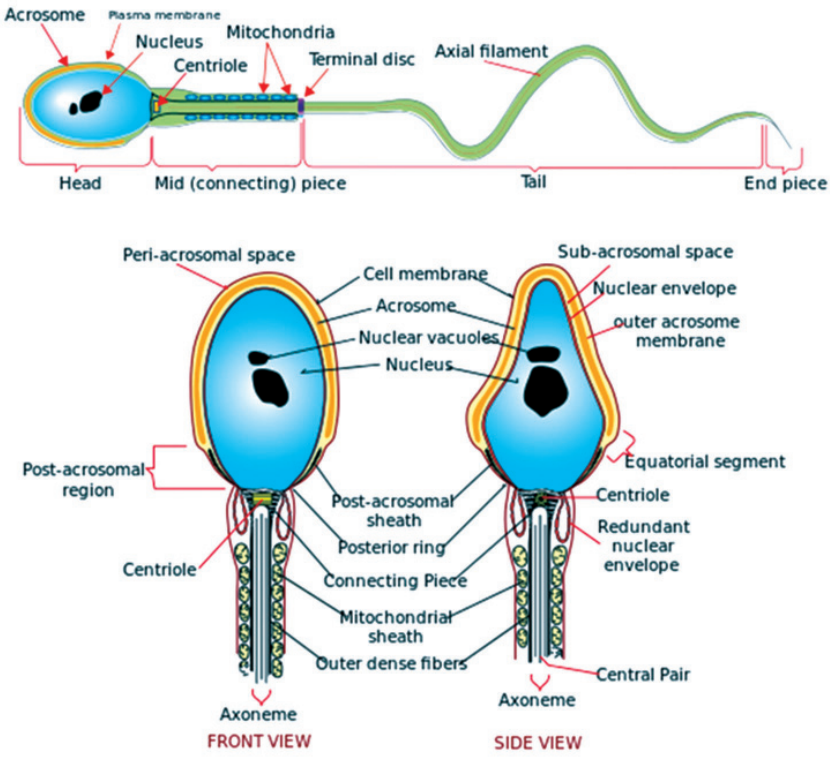


Figure 2.4.3 : The structures found in a completely functional spermatozoon.

The whole process of maturation from type A spermatogonia to spermatozoa is known as spermatogenesis. Non-motile sperm are transported to the epididymis by peristalsis, where they gain motility. The complex process of spermatogenesis takes between 64 and 72 days to completely form each functional sperm.[30]

2.5 Structure of spermatozoa

The spermatozoon consists of a head, a midpiece and a tail (Figure 2.4.3).[31] The head contains nucleus with densely packed chromatin surrounded by an acrosome. Mitochondria are spirally arranged in midpiece for ATP production, required for sperm motility. For penetrating the oocyte lots of enzymes are present in Acrosome.

The tail or flagellum helps in propulsion of the spermatozoa**.** The motile tail of a sperm is a long flagellum whose focal axoneme radiates from a basal body arranged only back to the core. The axoneme comprises of two focal singlet microtubules encompassed by nine equally separated microtubule doublets.[31,32] The flagellum of some sperm (including those of mammals) differs from other flagella in that the usual 9 + 2 pattern of the axoneme is further surrounded by nine outer dense fibers composed mainly of keratin (Figure 2.5.1). By sliding of microtubules active bending of the flagellum is seen. Stiff non-contractile dense fibres role is not known.

Flagellar movement is driven by dynein motor proteins, which use the energy of ATP hydrolysis generated by highly specialized mitochondria in the anterior part of the sperm tail (called the midpiece).

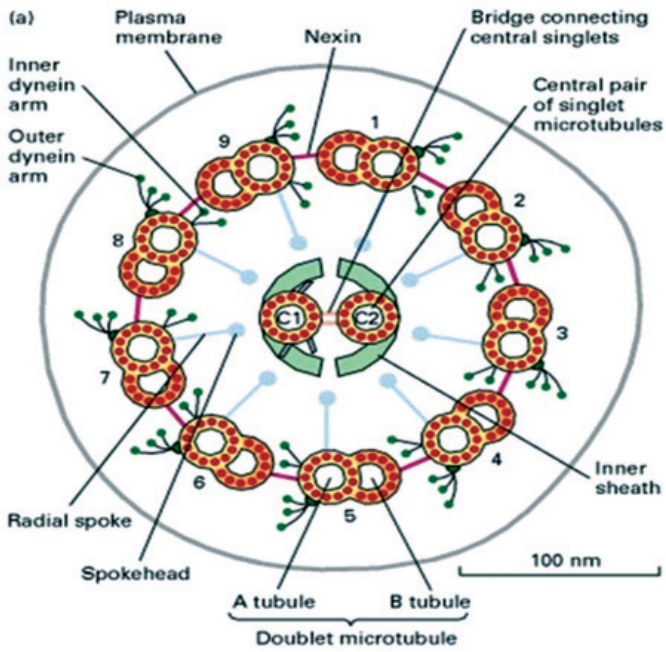


Figure 2.5.1 : Structure of ciliary and flagellar Axoneme.

Cross-sectional chart of a flagellum showing major structures. The dynein arms and spiral spokes with joined heads happen just at interims along the longitudinal pivot. The central microtubules, C1 and C2, are distinguished by fibers bound only to C1.

2.6 Hormones and Spermatogenesis

Sperm production is controlled by the hypothalamopituitary - gonadal axis (Figures 2.6.1).[33] Gonadotropin releasing hormone is secreted by the hypothalamus and stimulates the anterior pituitary gland to release luteinizing hormone (LH) and follicle stimulating hormone (FSH). LH and follicle-stimulating hormone stimulate Leydig cells to secrete testosterone and the germinal epithelium to produce sperm.

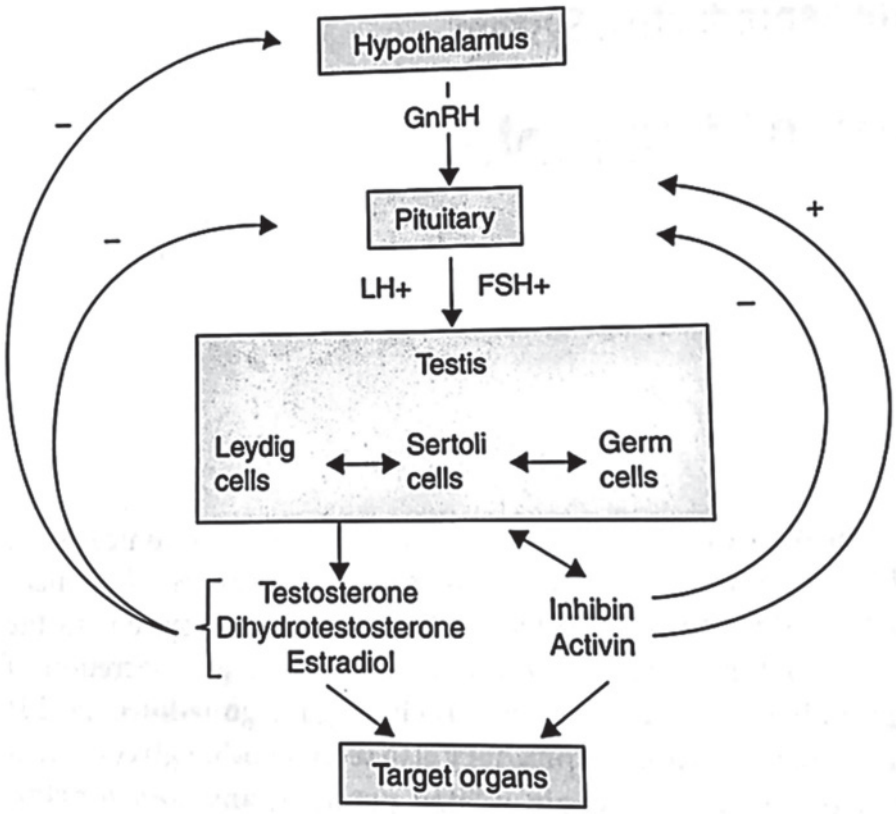


Figure 2.6.1 : Illustration of the hypothalmo - pituitary - gonadal axis.

Testosterone is needed for the completion of the cellular division and gamete development and so plays a vital role in the initiation and maintenance of gametogenesis. FSH additionally stimulates Sertoli cells, which produce inhibin B. LH and FSH are under negative feedback control by testosterone and inhibin B, respectively.[34]

2.7 Y CHROMOSOME

2.7.1 *Y chromosome Structure*

Mammalian sex chromosomes evolved from autosomes a minimum of one hundred eighty million years ago. The steps involved for differentiation, start with the acquisition of the testis-determining, followed by recombination between the sex chromosomes in a steps.[35-37] A detailed overview on the evolution of the human Y chromosome and its present day status has been a subject of recent reviews.[35] Y chromosome has a short arm (Yp) and the long arm (Yq) which are separated by a centromere. Structurally it is an acrocentric chromosome made of two pseudoautosomal regions (PARs) (Fig. 2.7.1).[38] While the PARs and the short arm are euchromatic, a large portion of the long arm is heterochromatic with the exception of the proximal portion close to the bodily structure that is euchromatic in nature. Traits that are familial via the Y chromosome are known as Y-linked, or holandric traits.

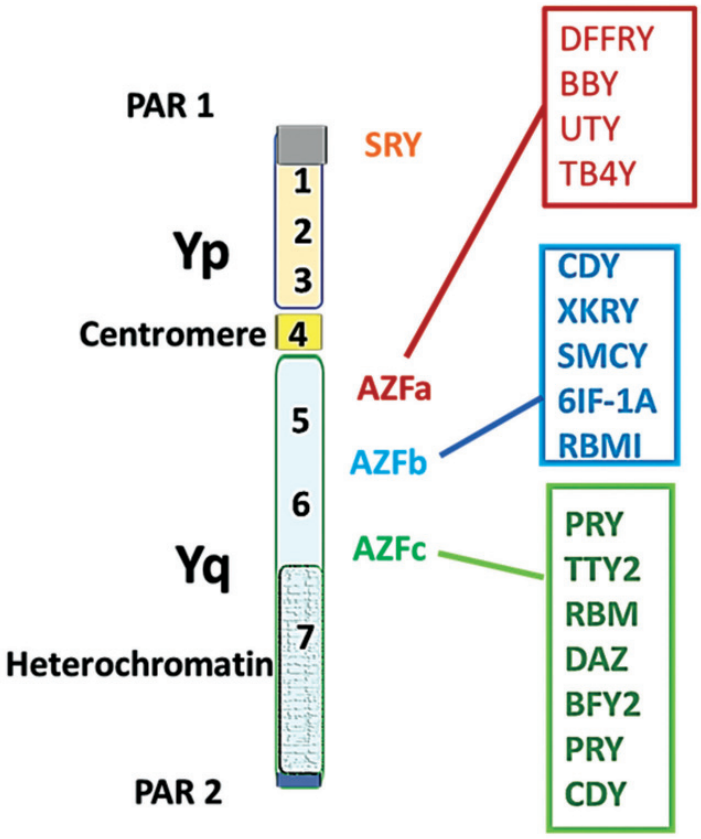


Figure 2.7.1 : Diagrammatic presentation of the Y chromosome, showing the seven deletion intervals. PAR 1, PAR 2 are Pseudoautosomal region 1 and 2 respectively. The Genes located in regions AZFa, AZFb and AZFc are illustrated.

Revolutionary changes have occurred in the knowledge of this chromosome [39] which is crucial for spermatogenesis. The Y chromosome is highly polymorphic.[40] Generally, of the 60 Mb length of the Y chromosome, 3 Mb belongs to pseudoautosomal regions and 57 Mb to a non-recombining region that contains heterochromatic and euchromatic regions. The male specific region of the Y chromosome comprises 95% of its length. The heterochromatin region harbors repeated genes, gene families, and palindromic motifs.[41] Total 23 Mb sequences of DNA are present the Y chromosome, including 14.5 Mb on the long arm and 8 Mb on the short arm.[39] There are three

classes of euchromatic sequences : X-transposed as they are transported from the X chromosome in the process of the evolution, X-degenerate are sequence similar to sequence on the X chromosome, and those repeated units across the proximal short arm of Yp and across most of Yq amplicons.[39]

In humans, the Y chromosome spans regarding fifty eight million base pairs (the building blocks of deoxyribonucleic acid) and represents around 1 percentage of the entire DNA in a male cell.[42] These are 122 genes and 110 pseudogenes identified in the Y chromosome. However, the exact role of these genes in spermatogenesis is not elucidated because microdeletions that cause impairment of spermatogenesis usually include more than one gene.[43]

The most important region of the Y chromosome is the unique ampliconic region in the euchromatin that is 10.5 Mb long.[41] They are located in 7 segments that are scattered across the euchromatin in the long arm and the proximal short arm of the Y chromosome (Fig. 2.7.2). Amplicons harbor the highest density of genes that are exclusively expressed in the testis. Ampliconic regions genes are present in the AZFb and AZFc.[40] The genes recognized in the AZFa are located in the X-degenerate regions.[40]

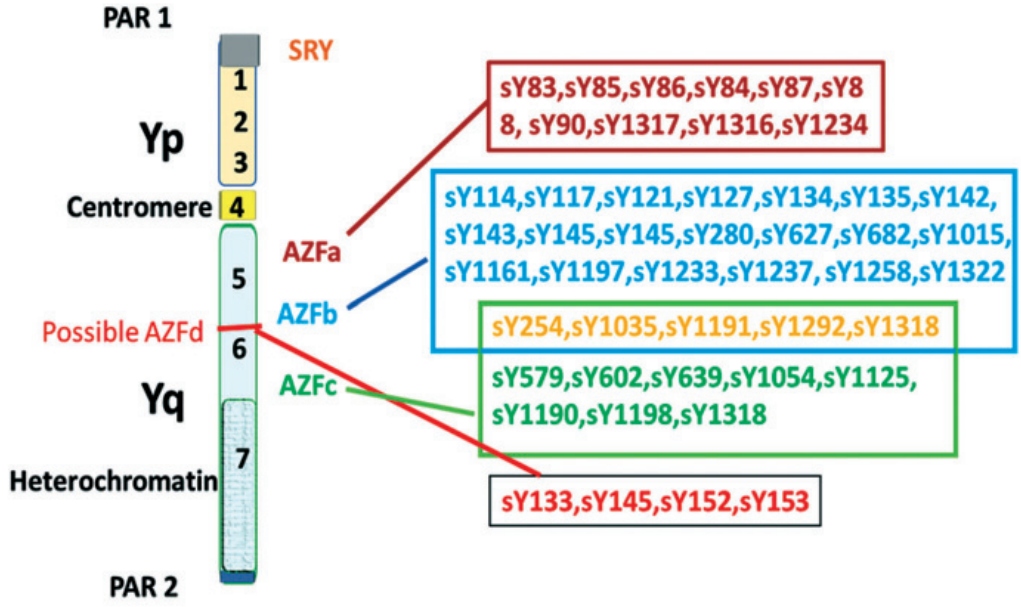


Figure 2.7.2 : Diagram of the human Y chromosome showing the AZF loci and STS markers. Along with STS markers associated with the AZF regions. STS markers for the genes in the AZFa, AZFb, AZFc, overlapping AZFb/AZFc and AZFd regions are presented by different colors.

2.7.2 Genes and genetics of human

Initially there was belief that Genes on the long arm of the Y chromosome [Yq] are genetically inert. This was proven false when in 1997, 12 novel genes or gene families with ten full-length complementary DNA sequence were identified in the human testis that were localized to the Yq (Bellott DW et. al., 2014)[37]. After this study, the Y chromosome has been studied in many species and different functional genes identified.[44] These genes according to their expression pattern are categories as: 1st house keeping genes which have X homologous and escape X inactivation; 2nd the second group, consisting of the gene families expressed specifically in testes.

2.7.3 Male Specific Region of Y chromosome

In the Pseudoautosomal region (PAR) recombination is found which accounts only 5% of the total chromosome.[44] In the PARs meiotic recombination is not present so, is called as the Non-Recombining region of the Y chromosome (NRY) or as Male Specific region of Y chromosome (MSY), which accounts for 95% of the Y chromosome length.[45] In the Y chromosome of the male specific region both euchromatic and heterochromatic sequences are present. The heterochromatic sequences are present from the centromere region, but major part is confined to the distal Yq 12 which is 40 Mb in length.[45]

Three classes of sequences namely X transposed, X degenerate and ampliconic sequences are found in the euchromatic MSY. The X transposed sequences are the sequences which are acquired by transposition from the X chromosome around 3-4 million years ago during the emergence of human lineage.[46] X and Y chromosomes co-evolved from the X degenerate sequences which represent the ancestral sequences of the autosomes. The ampliconic sequences exist as seven blocks and nine families of protein coding genes are massive repeat units exhibiting 99.9% sequence identity.[45,47] The amplicon sequences harbors eight massive palindromes with highly symmetrical arms. Six palindromes out of the eight carry testis specific protein coding genes, these are almost identical gene copies which exist in the opposite arms. Out of the nine multicopy protein coding gene families in the MSY, eight have members in the palindromes.[45] In the palindromes the testis specific genes are present as two copies facing each other as mirror images. By means of molecular mechanisms between the two arms of the palindromes this unique organization enables to rectify the mutation occurring in one copy of the gene.[48] The X transposed sequences have

two genes while the X degenerate sequences have single copy gene or pseudogene homologs of 27 X linked genes and Y chromosome approximately has 78 protein coding genes of which 60 belong to nine different MSY gene families and the remaining 18 genes are present in single copy.[45]

2.7.4 Yq AZF Region on Y chromosome

The first demonstration of the capacity of the NRY of the human Y chromosome was way back in 1997, but importance of this locus with male infertility was understood later almost four decades ago. Italian researchers in 1976, identified deletions towards the distal end q11 of the Y chromosome only in six infertile males out of 1170.[49] Analysis indicated that the fathers of two of the same six males had undeleted Y chromosomes, indicating that the deletions arose de novo and could be the underlying etiology of their azoospermia. The authors proposed this spermatogenesis factor as the "azoospermia factor" [AZF] in the Yq locus. Due to the physical and molecular map of the human Y chromosome[38] lots of studies considering the AZF locus and male infertility were taken. Deletions of Yq were identified in series of subfertile males by using molecular markers.[38] Analysis of these deletion three sub-regions in proximal, middle, and distal Yq 11 were defined and named as "AZFa," "AZFb," and "AZFc," respectively.[50] While the DAZ (Deleted in Azoospermia) gene was considered as a strong candidate for male infertility[51] little was known about the existence of other genes within this locus. With the availability of the first complete sequence of AZFc locus[47] and later the detailed structure of the MSY including the AZFa, b and c regions [45] a large number of genes were identified.

Several genes (proteins) coded from the AZF region on the Y chromosome are referred as Azoospermia factors (AZF)[52] . Deletions in this region are associated with inability to produce sperms. Sub regions within the AZF region are AZFa, AZFb and AZFc.

AZFa

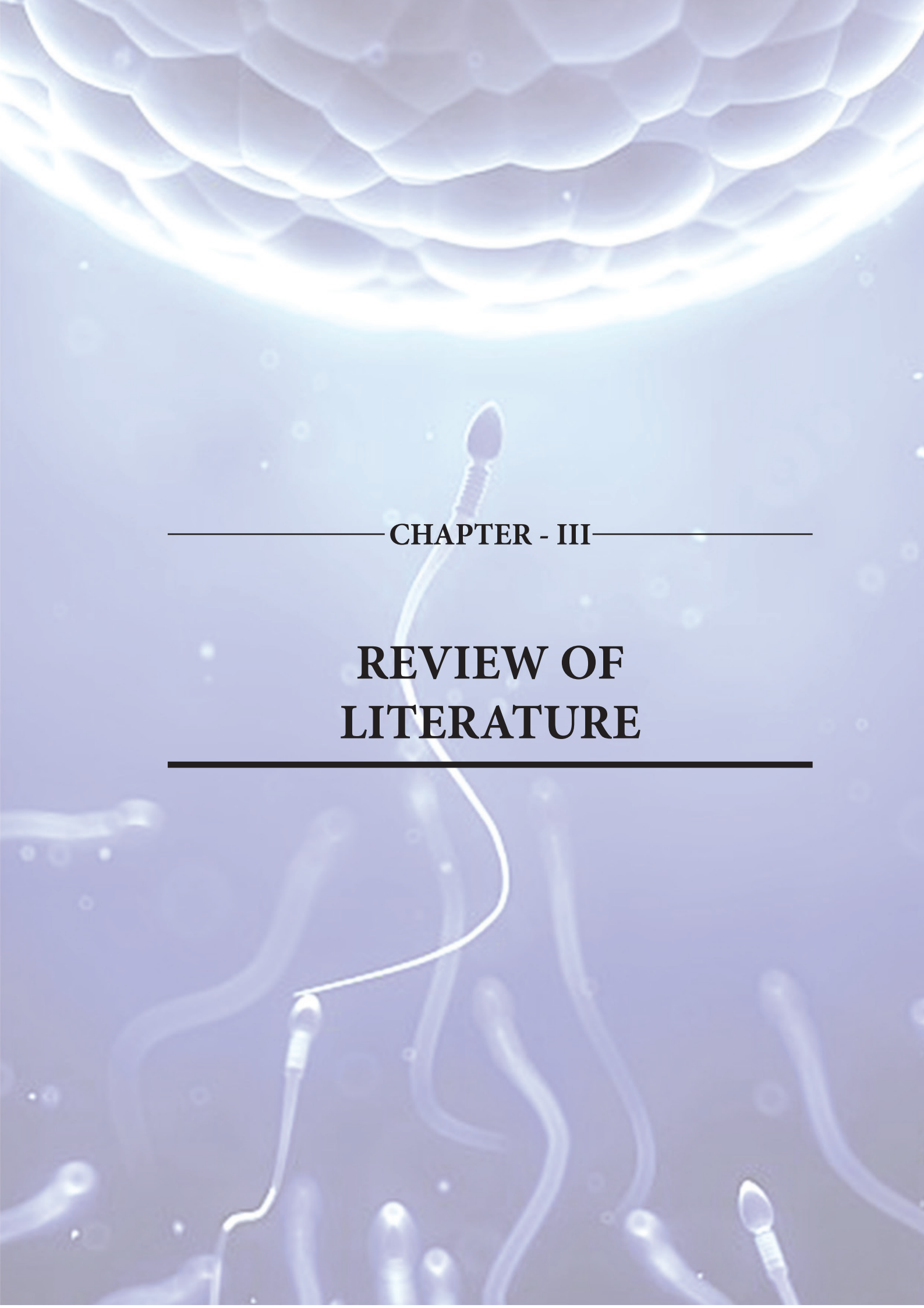
Studies have reported that in azoospermic males, deletion of AZFa region.[41] This region harbors two protein encoding genes USP9Y, and DBY. They are both located in the X-degenerate region of euchromatin and have homologous genes on the X chromosome.

AZFb

Complete deletion of AZFb is associated with azoospermia with no foci of testicular spermatozoa.[53] The known protein-encoding genes in this region that are associated with spermatogenesis are EIF1AY, RPS4Y2 and SMCY that are located in X-degenerate euchromatin and SMCY, PRY and RBMY that are in ampliconic region.

AZFc

Complete deletion of AZFc region is one of the most frequent causes of male infertility.[54] Spermatozoa can be found in the ejaculate or testicular tissue of about 50% of men with AZFc microdeletions (Georgiou et. al., 2006)[41]. AZFc region contains gene families including BPY2, CDY, DAZ, CSPG4LY and GOLGA2LY.



CHAPTER - III

REVIEW OF LITERATURE

3 REVIEW OF LITERATURE

3.1. Infertility

Infertility is the inability of a couple to reach a clinical pregnancy within one year of trying by regular intercourse (American Society for Reproductive Medicine, 2014)[1] Infertility affects millions of married couples around the world. Pregnancy if natural physiological process, though it seems to be simple it is quite complicated as it is regulated by many factors. The potential requirement for fertility therapeutic administrations, as demonstrated by the pervasiveness of current infertility in more and less very much developed, was 9% (Boivin J et. al., 2007).[55] Approximately15% of the married couples are affected.[1]

A male factor is recognized in about half of the couples who are childless (Samli H, et. al., 2006).[56] The international database on male infertility estimated that

there are about 7,831,401 infertile men in India (US Census Bureau, International Data Base, 2004).[57] It is categorized as primary or secondary, based on a couple's history of conception. Couples with primary infertility have never conceived, whereas those with secondary infertility were previously fertile with at least one child or abortion, but are unable to conceive again by natural means (Mascarenhas et al., 2012).[58] Male infertility affects at least 30 million males worldwide, 30% of which are azoospermic (Esteves et al., 2013).[59] The highest infertility rate, where the male factor is a sole or contributory factor, is 60-70% in the Middle East (Agarwal et al., 2015).[60] Despite enormous progress in understanding human reproductive anatomy & physiology, the under-lying cause of male infertility is still not known in majority of the cases (Gianotten J et al., 2004).[61] The known causes of male infertility include varicocele, hypogonadism, cryptorchidism and congenital bilateral absence of vas deferens (CBAVD). However, the number of infertile men with idiopathic cause is increasing and the basic semen analysis fails to identify the male factor infertility in all the cases. Much consideration has recently been given to genetic factors and oxidative stress which may constitute a major source of reproductive pathology in the infertile men (Dada et al., 2008, Agarwal et al., 2009).[62,63] For elucidating such mechanisms and understanding the basic anatomy & physiology of male reproduction and factors regulating spermatogenesis are essential. Genetic factors may lead to irreversible, partial or complete spermatogenic arrest and their knowledge is important in case such couples opt for assisted reproduction.

3.2 Epidemiology

Infertility is the major concern among the married couples where male factor contributes approximately 50% of these cases. Infertility is stigma leading to severe

psychological stress in the couples. Since, male infertility is a multi-factorial disease, understanding mechanisms underlying is found to be difficult. During the past few decades an abrupt decline in semen quality was observed (Adiga SK et. al., 2008, Feki et. al., 2009). [64,65]

Indian population is growing with an average growth rate of 2% with equal reproductive concern also showed decline in semen quality (Adiga SK et. al., 2008, Mishra et. al., 2018).[64,66] Therefore, it is important to understand the basic mechanisms and factors that regulate spermatogenesis whereas, understanding role of genetic factors is also of critical importance.

3.3 Male Infertility

Infertility due to male factor may be associated with a wide range of anomalies in the semen, such as sperm number, motility, and morphology. The normal values for these parameters as defined by WHO (2010)[8] are given in Table 3.3.1.

Table 3.3.1 : Reference limits of various characteristics of semen.

Volume	>1.5 ml
pH	>7.2
Sperm concentration	>15X10^6 spermatozoa/ml
Total sperm count	>39X10^6 spermatozoa/ejaculate
Motility	>40% with forward progression or 25 % with rapid progression at 60 min after Ejaculation
Morphology	>40% with normal forms

As per WHO (2010)[8]

Azoospermia, oligozoospermic, asthenozoospermia, teratozoospermia are observed in at least 90% of infertile males. In about 10% of cases, no abnormalities are detected

on routine semen examination; and in these cases, specific metabolic (John AR et. al., 2004) [67] and membrane defects (Sha YW et. al., 2013)[68] of the sperm may lead to reduced ability for fertilization. The nomenclature for pathological findings in semen are depicted in Table 3.3.2.

Table 3.3.2 : Nomenclature for pathological findings in semen analysis.

Type	
Azoospermia	No spermatozoa in the ejaculate
Oligospermia	Total number of spermatozoa below the reference limit
Severe oligospermia	Sperm count less 5 million/ml
Teratospermia	Percentage of normal spermatozoa below the normal reference limit
Asthenozoospermia	Percentages of both progressively motile (PR) and morphologically normal spermatozoa below the reference limits
Oligoasthenoteratozoospermia	Total number of spermatozoa, and percentages of both progressively motile (PR) and morphologically normal spermatozoa, below the reference limits

Reference limits mentioned in Table 3.3.1 are as per WHO (2010)[8]

3.4 Etiology of Male Infertility

Causes of male infertility can be genetic or non-genetic factors. Hormonal imbalance, Infections, drugs, gonadotoxins, obstruction of spermatic ducts, agglutination of sperms, varicocele, cryptorchidism, vascular torsion and mumps orchitis are non-genetic factors. Genetic factors comprise chromosomal abnormalities, deletions of specific area of chromosomes involved in spermatogenesis, or specific mutations within genes (Table 3.4.1). A thorough and systematic evaluation is necessary to establish a precise diagnosis. Semen analysis is a crucial first step but it is by no means sufficient, to determine the cause or to dictate therapy.

3.4.1 Infertility due to Non-Genetic Factors

1) Drugs

Sulfasalazine, which is used to treat inflammatory bowel diseases, can lead to oligozoospermic. Cytotoxic drugs; especially the alkylating agents that are used to treat cancer and auto immune diseases cause gonadal failure. Anabolic steroid abuse has severe side effects on the testis, leading to oligo/azoospermia (Table 3.4.1).

2) *Exposure to Gonadotoxins*

Numerous substances and occupational exposures have been suspected to decrease semen quality. Medicines can impair sperm production and function (Table 3.4.1). Lifestyle choices that can have negative impact on semen quality include cigarettes, smoking, excessive alcohol consumption, marijuana use, and prolonged and repetitive use of hot tubs. Occupational exposure to heat, ionizing radiations, heavy metals, pesticides, and exposure to organic solvents have been reported to lead to a decline in semen quality (Table 3.4.1). Radiation and chemotherapy can permanently damage the germinal epithelium (Table 3.4.1).

3) *Infertility due to Endocrine Disorders*

The etiology is either congenital or acquired. The deficit of LH and FSH in the peripheral blood may be due to a primary lesion in the pituitary gland or secondary due to insufficient hypothalamic GnRH production. Acquired causes include tumors (prolactinoma, Craniopharyngioma), infection, infiltrative disease, empty Sella, radiation therapy and autoimmune hypophistis (Table 3.4.1) (Flynn et al., 2010). [69]

4) *Infertility due to Impairment of Sperm Transport*

Obstruction in the path of transportation of semen in epididymis, vas deferens or

accessory gland leads to azoospermia in the presence of normal testicular function. Acquired inflammatory diseases may lead to impaired sperm transport. Table 3.4.1.

5) *Infertility due to Autoimmune Factors*

In 5% infertile males, an auto immune reaction against the spermatozoa as an isolated abnormality. Circulating sperm antibodies are also present in association with epididymitis, varicocele, unilateral or bilateral obstruction of the genital tract and after reversal of vasectomy (Table 3.4.1).

6) *Infertility and Varicocele*

In about 25-40% infertile males, strong association between severe and varicocele and spermatogenetic failure are noted. However, the pathogenesis by which varicocele leads to this condition is poorly understood. Among the potential pathogenic factors are increased temperature and accumulation of carbon dioxide, another toxic product brought by venous reflux (Table 3.4.1) (Strom et al., 2010). [70]

7) *Infertility and Cryptorchidism*

In new born males undescended testis is a reported in 2-3%. Its prevalence after the first year of life is about 0.8 to 1.6%. Cryptorchidism, in the majority of cases, is associated with impaired spermatogenesis although the extent of impairment may vary from a Sertoli cell-only pattern to a slight form of hypospermatogenesis (Ferlin et. al., 2008) .[71] Cryptorchidism may be unilateral or bilateral; and the testis may be located intra-abdominally or in the inguinal canal (Table 3.4.1).

8) *Testicular Torsion*

Testicular torsion has been reported with an incidence of 1 in 4000 males having age less than 25 years (Daniel Da Justa et. al.2013).[72] Peaks of incidence occur in neonates

and adolescents entering puberty. Within the scrotum, on either side of the testicles lies spermatic cord. But, sometimes spermatic cord gets twisted around a testicle thus stopping the blood supply to that testicle (Table 3.4.1).

9) *Viral and bacterial infections*

Infections of the urogenital tract affecting the penis, bladder and urethra can compromise a man's fertility. Prostatitis (an infection of the prostate), orchitis (an inflammation of the testicles), epididymitis (an inflammation or infection of the Epididymis), and sexually transmitted diseases such as gonorrhea, and chlamydia infection can all lead to male infertility. Infections may lead to an excessive generation of reactive oxygen species with potential impairment of sperm functional capacity. Orchitis does not always accompany mumps, but is common if infection occur after puberty. It can cause major swelling and pain in the testis and can destroy the sperm-producing tubes (seminiferous tubules) and permanently stop sperm production (Table 3.4.1).

Table 3.4.1 : Etiology of male infertility.

Sr. No.		Effect on reproductive Function	Mechanism	References
	NON GENETIC FACTORS			
1	**Drug**			
	Sulfasalazine	Decreased spermatogenesis	Decreased testosterone	Sigman., 2007[73]
	Cyclosporine	Decreased spermatogenesis	Decreased testosterone	Sigman., 2007
	Thiazide	Decrease erectile function	Decreased vascular resistance	Sigman., 2007
	Tetracycline	Decrease motility	Binds sperm	Sigman., 2007
2	**Other Drugs**			
	Alcohol	Decreased spermatogenesis	Direct toxicity	Sigman., 2007
	Tobacco	Decreased spermatogenesis	Direct toxicity	Sigman., 2007
	Cocaine	Decreased spermatogenesis	Direct toxicity	Sigman., 2007
	Marijuna	Decreased spermatogenesis	Direct toxicity	Sigman., 2007
3	**Gonadotoxins**			
	Organic solvents	Decreased spermatogenesis	Direct toxicity	Sigman., 2007
	Pesticides	Decreased spermatogenesis	Direct toxicity	Sigman., 2007
	Heavy metal	Decreased spermatogenesis	Direct toxicity	Sigman., 2007
	Radiation	Decreased spermatogenesis	Increased DNA double strand Breakage	Sigman., 2007
	Heat	Decreased spermatogenesis	Unknown	Sigman., 2007
4	**Varicocele**	Spermatogenic failure	Increased temperature and accumulation of carbon dioxide	Strom et al., 2010[70]
5	**Cryptorchidism**	Impaired spermatogenesis	Undescended testis	Ferlin et al., 2008[71]
6	**Testicular torsion**	Spermatogenic failure	The blood vessels that feed the testes shut which causes testicular damage	Little., 2010[74]
7	**Endocrine Disorders**			
	Acquired	Spermatogenic failure	Deficit of LH and FSH due to primary lesion in pituitary gland due to tumor, infection, radiation etc.	Flynn et al., 2010[69]
8	**Impairment of Sperm Transport due to Obstruction**			
	Acquired	Impaired or absent of sperm Transport	Post-infective, Inflammatory or post vasectomy	Flynn et al., 2010
9	**Mumps orchitis**	Spermatogenic Failure	Testicular atrophy	Nordlander. 2011[75]
10	**Other Viral and Bacterial Infections**	Impaired or absent of sperm transport	An infection of the prostate, inflammation of the testicles, inflammation or infection of the Epididymis	Nordlander., 2011
11	**Autoimmune**	Inhibitory effect on Fertilization	Sperm antibodies	Brugh et.al., 2004[76]

3.4.2 Endocrine cause of Male Infertility

The complete male germ cell development is mainly dependent upon the balanced endocrine interplay of pituitary, hypothalamus and the testis (O'Donnell L et. al, 2000).[77] The presence of Follicle stimulating hormone (FSH), Luteinizing hormone (LH) and Testosterone is very much essential for the proper production and maintenance of spermatogenesis. The two main functions of adult testis are namely, the production of spermatozoa and the secretion of testosterone, both dependent on stimulation by the pituitary gonadotropins, FSH and LH. The Pituitary gonadotropins are secreted in response to hypothalamic gonadotropin releasing hormone (GnRH). FSH stimulates spermatogenesis by binding with receptors in the Sertoli cells, while LH stimulates the production of testosterone in Leydig cells, which in turn may act on the Sertoli and peritubular cells of the seminiferous tubules and stimulates spermatogenesis (O'Donnell L et. al, 2000).[77] Infertility may be due to the failure of the pituitary to secrete FSH and LH which result in disruption of testicular function. Through feedback mechanism Testosterone, estradiol and inhibin control the secretion of gonadotropins (Zeqiraj A et. al, 2017).[78]

Endocrine abnormalities are caused due to disturbance in these hormone levels which impairs the normal spermatozoa production. Hypogonadism is defined by impaired testicular function, which potentially affects spermatogenesis and/or testosterone synthesis. Sussman et. al.,[79] reported that the respective incidences of hypogonadism in males who visited an infertility clinic were 35.3%, 45% and 6.7% for those with normal sperm analysis, NOA and OA. Hypogonadism can be caused by primary testicular failure (hypergonadotropic hypogonadism) or secondary testicular failure resulting from a hypothalmo-pituitary deficiency (hypogonadotropic hypogonadism).

Rarely, hypogonadism can occur in complete (testicular feminization) or partial (Reifenstein's syndrome) androgen insensitivity syndrome.

Due to hypogonadism infertility, muscular hypotrophy, decreased acne, alterations in body hair distributions, low bone mineral density, anemia, and decreased libido are reported (Sussman EM et. al., 2008, Jungwirth A et. al., 2012).[79,80]

Primary hypogonadism can be observed in patients who exhibit testicular failure caused by congenital (anorchia, undescended testes and genetic abnormalities, such as Klinefelter syndrome or Y chromosome defects), acquired (trauma, tumor, torsion, orchitis or varicocele) or idiopathic causes.

Genetic hypothalamic disorders, such as Kallmann syndrome, and congenital or acquired pituitary deficiencies, such as empty sell a syndrome or pituitary tumors (functional or non-functional), can cause hypogonadotropic hypogonadism. Hypogonadotropic hypogonadism should be suspected in azoospermic men who also have complains of with anosmia, decreased libido, gynecomastia, headaches or visual field deficits. In such cases a complete endocrine work-up, including cranial imaging, should be performed.

Azoospermia or oligozoospermia may be caused due to Hypogonadotropic hypogonadism (HH), a condition in which there is a low serum gonadotropin accompanied by low serum testosterone.[81]

Congenital Adrenal Hyperplasia (CAH) is another important endocrine cause of male infertility (Speiser PW et. al, 2010)[82] in which there is a deficiency of an adrenal enzyme 21 hydroxylase (21-OHCAH) and it is evident that, in all its forms involved

in the bio synthesis of cortisol. The failure of normal testicular maturation which leads to infertility in men may be due to abnormal adrenal steroid production which inhibits the release of gonadotropins by the hypothalamic-hypophyseal axis (Whirledge S et. al. 2010). [83]

The main causes of male infertility are pituitary or suprasellar tumors, hypothyroidism, and intake of certain medications. These factors block the action of dopamine on the pituitary or may deplete the dopamine levels in hypothalamus (Fitzgerald, P et. al., 2008).[84]

3.5 Genetic basis of Male Infertility

Genetic disorders may alter spermatogenesis; impair development of genital tract, obstruct passage of semen along the seminal pathway. Genetic disorders comprise karyotype abnormalities, deletions of specific area of chromosomes involved in spermatogenesis, or specific mutations within genes for e.g. CFTR gene mutation in cases of CBAVD, immotile cilia syndrome, androgen insensitivity syndrome, Kallman Syndrome, Infertile male syndrome, Kennedy disease, persistent mullerian duct syndrome and inactivating FSH receptor mutations etc. (Table 3.5.1).

3.5.1 Chromosomal Abnormalities

From the large surveys on karyotypes in sub fertile males (6982), it became evident that compared with newborns, infertile males have higher prevalence of chromosomal abnormalities (Harton GL et. al., 2011).[85] Now a days due to newer procedures like IVF and ICSI, concern that carriers of chromosomal abnormalities, pose a genetic risk to their offspring has been voiced. Several chromosomal studies were undertaken in

the late 1980s and 1990s in order to establish the frequency and type of chromosomal abnormalities in patients affected either by severe oligozoospermia or azoospermia or those undergoing IVF/ ICSI procedures (Banu Değirmencia et. al., 2019).[15] The 47, XXY chromosome complement makes up the bulk of sex chromosome abnormalities and occurs more frequently than among newborns (frequency of 1 in 500). Among the structural abnormalities among infertile males, reciprocal translocations, Robertsonian translocations, paracentric inversions and marker chromosomes are the most common (Lin et. al., 2010).[86]

Table 3.5.1 : Genetic factors for Male Infertility

	GENETICS FACTORS			
S. No.		Effect on reproductive function	Mechanism	References
1	Klinefelter Syndrome	Spermatogenic failure	Causes all active testicular structures to atrophy	Flynn et. al., 2010[69]
2	Y chromosome microdeletions	Impaired spermatogenesis, Spermatogenic failure	Deletion of regions (AZFa, b and c) involved in spermatogenesis.	Flynn et. al., 2010
3	Kallman Syndrome	Spermatogenic failure	Abnormality of secretion of GnRH due to mutation in *KAL1* Gene	Flynn et. al., 2010
4	Obstructive azoospermia	Impaired or absent of sperm transport	Congenital bilateral absence of vas deferens	Flynn et. al., 2010

3.5.2 Y chromosome microdeletions and male infertility

The human Y chromosome is genetically dynamic and is also prone to significant variation owing to the high proportion of segmental duplications which form the basis of the wide variety of deletions and duplications seen in various loci of this chromosome. Genes having function in spermatogenesis are mainly represent in the Yq locus, loss of these regions would cause infertility**.**

Sub microscopic deletions of Y chromosome in proximal Yq that remove the entire or parts of AZF region are Microdeletions. Studies done on deletion on Yq in infertile men, reported five different patterns (Repping S et. al., 2002).[87] These in clinical practice are together termed as AZFa, AZFb, and AZFc deletions (Fig. 3.5.1). Spermatogenic failure mainly is caused by deletions of AZF, so the screening for AZF deletions is become a part of the routine diagnostic tool in infertile men (Krauz et. al., 2014, Sen S et. al., 2013, Simoni M et. al., 2004).[53,88,89]

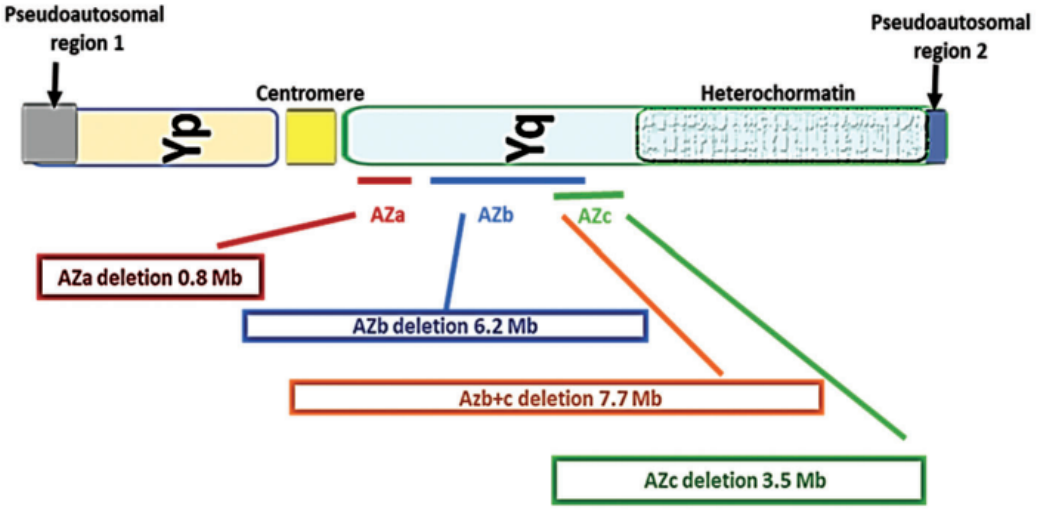


Figure 3.5.1 : Schematic of the Y chromosome indicating the approximate position of the previously defined regions AZFa, AZFb, and AZFc. The short arm (Yp), the centromere, the long arm (Yq) (including the polymorphic heterochromatic band Yq 12 of variable length), and the pseudoautosomal regions 1 and 2 are labelled.

Early studies have reported microdeletions of Y chromosome are detected in infertile males with abnormal sperm count, microdeletions are not observed in fertile men (Krauz et. al., 2014, Sen S et. al., 2013, Simoni M et. al., 2004)[53,88,89] suggesting that these deletions are the cause of failure of spermatogenesis and hence infertility. Yet there are studies that man with microdeletion have transmitted this Yq deletions to their off springs (Chang PL et. al, 1999, Gatta V et. al., 2002). [90,91]

Global data suggest that 1 in 4000 men have Yq microdeletions, whereas it is observed that 1 in 12 infertile males have Yq microdeletion. Of the > 30,000 Y chromosomes analyzed for AZF microdeletions (by STS-PCR method), the global prevalence of AZF microdeletions in infertile men is estimated to be 7%. As evident there is a wide variation in the frequency of Yq microdeletions in different parts of the world, this could reflect underlying differences in sample size, methodology used and the population screened (Colaco S et. al., 2018).[38]

Published data of 40,127 infertile males reported the lowest prevalence of Yq microdeletions is in Europe (3%) and Australia (5.8%); while the rest of the world has an average of 8–9%.Data from different studies from same continent were pooled and the average estimated as shown in Figure 3.5.2 (Colaco S et. al., 2018).[38]

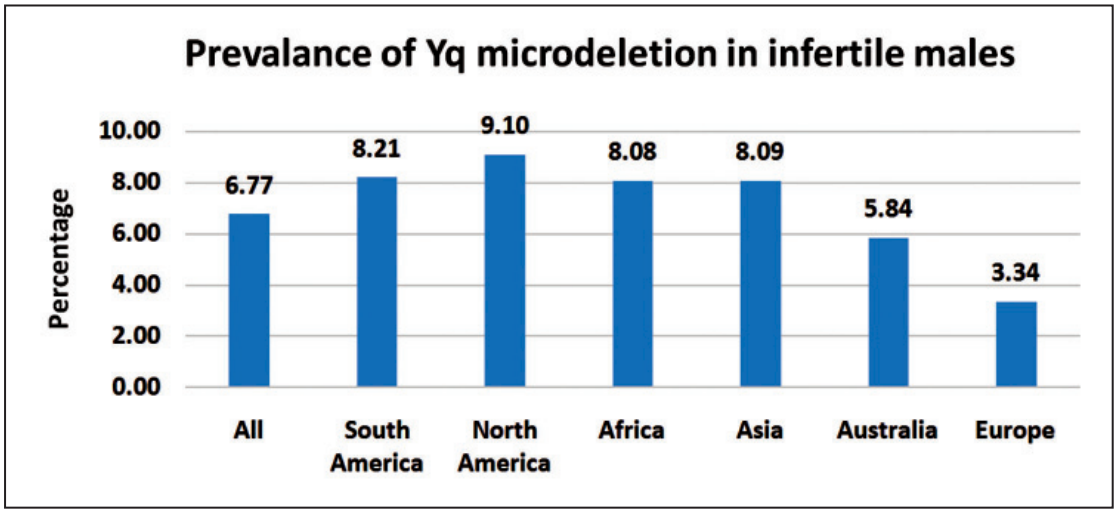


Figure 3.5.2 : Prevalence of Yq microdeletions in infertile men.

Distribution of Yq microdeletions in the Asian region is shown in Figure 3.6.4. Amongst the Asians we observed that the highest prevalence of Yq microdeletions is amongst the East and South East Asians and lowest amongst South Asians (Fig.3.5.3) (Colaco S et. al., 2018).[38]

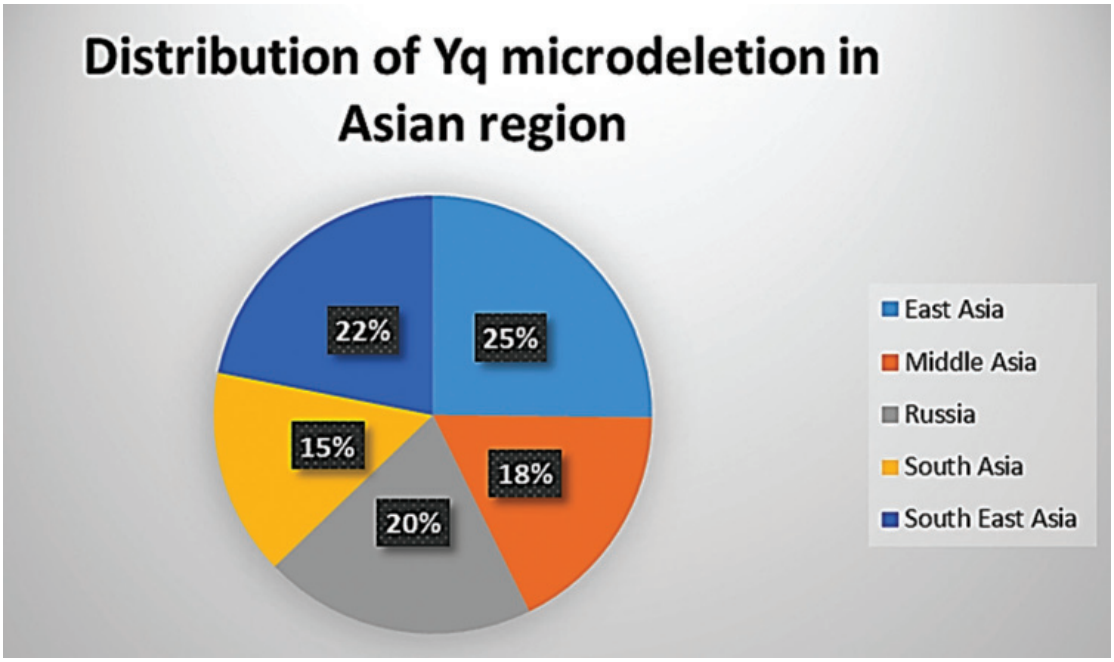


Figure 3.5.3 : Prevalence of Yq microdeletions in infertile men in Asian Region

Irrespective of the population understudy, the AZFc is most frequently deleted locus in infertile men [60–70%] followed by AZFa [0.5–4%], AZFb [1–5%] and AZFb+c [1–3%] deletion (Hinch AG et. al., 2014).[92] While these are global estimates, it is intriguing that the frequencies of the various AZF loci that are deleted in infertile men differ amongst various populations. While the frequency of AZFc deletion is lower in Indian population as compared to western counter parts (45% versus 60%), the frequency of AZF a deletion is almost double (11 versus 5%). Further, the frequency of double deletions (AZF a + b, b + c) is also higher in the Indian population as opposed to world literature (Sen S et. al., 2013).[88] Also there are unusual deletions in AZFa along with AZFc are only observed in some populations (O' Donnell L. et. al., 2001, Sen S et. al., 2013, Massart A et. al., 2012, Simoni M et. al., 2008).[77, 88, 93, 94]

It is suggested that since the unusual combination of deletions are detected only

by isolated markers and not confirmed by additional analyses these are perhaps methodological artefacts (Krauz et. al., 2014).[53] Additional data will be needed to understand the molecular basis of such deletions.

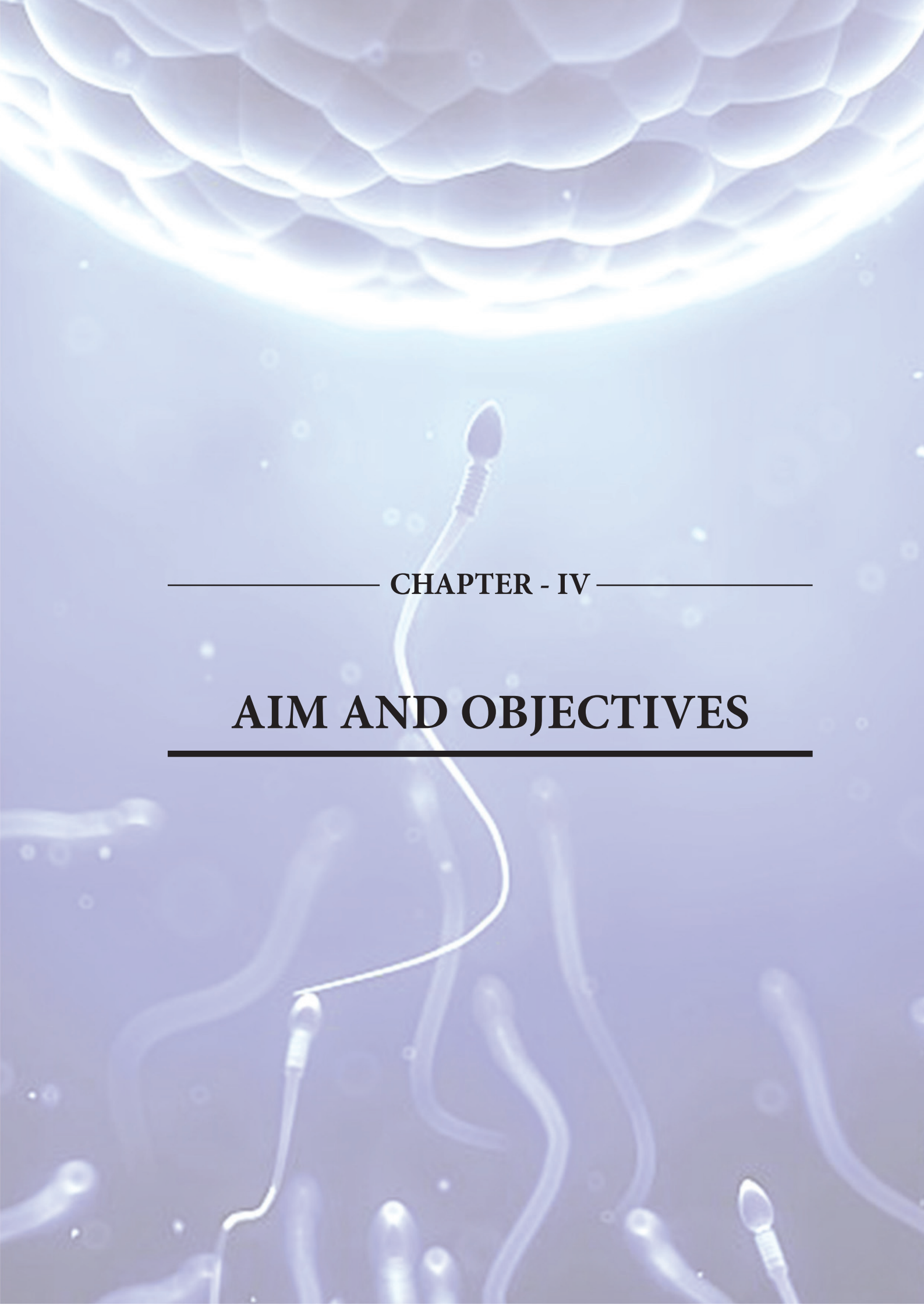
3.6 Statement of the Problem

Statistical genetics holds incredible promise in study of male infertility. By studying infertile men, genetic markers can be identified as markers for infertility.

For genetic studies performed in different ethnic/geographic origins are needed to determine if there are population differences between the frequency, position or extent of the deletions. No study to identify genetic markers is reported in Kolhapur district, Maharashtra.

Semen analysis, hormone assay and histology of testis will give a clue for further genetic studies of Y chromosome microdeletion, especially in azoospermic and severe oligozoospermic males. Such studies are less in Indian population and no such study is reported from Kolhapur district.

Testing for Yq micro deletion is very important clinically in terms of establishing the diagnosis and required for appropriate genetic counselling and clinical management of the infertile cases.



CHAPTER - IV

AIM AND OBJECTIVES

AIM

To detect Azoospermic Factor (AZF) microdeletions in azoospermic and severe oligospermic males in Kolhapur district.

OBJECTIVES

1. To find immature spermatogenic cells in semen by Leishman's stain.
2. To correlate between hormones assay and semen analysis and microdeletion.
3. To correlate between micro deletions of AZF and histopathology of testis.
4. To compare European Andrology Association markers to Non-European Andrological markers to detect of microdeletion of azoospermic factor (AZF) loci of Y chromosome in infertile males.

CHAPTER - V

RESEARCH METHODOLOGY

5 RESEARCH METHODOLOGY

5.1 Ethical Considerations

5.2 Study Sample Size and Detailed history

5.3 Selection Criteria of Study Population

5.4 Study Plan

5.5 Collection of samples

5.5.1 Collection of semen samples

5.5.2 Collection of whole blood

5.5.3 Testicular Biopsy

5.6 Parameters for evaluation

5.7 Semen Analysis

5.7.1 Determination of Sperm Count and Motility

5.7.2 Counting of Immature Germ cells in Semen

5.8 Hormone Analysis

5.9 Extraction of genomic DNA and quantification

5.10 Primer Design

5.11 Y Chromosome Microdeletion analysis using PCR

5.11.1 Primer stock preparation

5.11.2 PCR reaction mixture for 6 primer sets

5.11.3 Procedure

5.11.4 Visualization of PCR products

5.12 Statistical Analysis

5.1 Ethical Considerations

The present study was approved by the Ethics Committee of D. Y. Patil Education Society, Institution deemed to be university, Kolhapur. Approval letter no DMCK/74/2017 dated 14th March 2017 (Copy attached). All participants were informed about the study aim, procedure, risks and benefits before voluntarily signing the consent form.

5.2 Study Sample Size and Detailed history

The sample size was determined based on convenient sampling, as per number of patients visiting the facility Dr. Rahul Gune, Consultant Andrologists, Gune Chambers, Kolhapur, Maharashtra, India between March 2017 to March 2019. Cases were collected from the Kolhapur district and due to the sensitive nature of the study, questionnaires and sample collection were carried out by the lab technicians in complete privacy to avoid discomfort and to maintain anonymity. Consenting participants were provided with a questionnaire (attached) and 10 ml venous blood was collected for chromosome analysis.

The detailed history was collected (Case report attached), including questions on coital frequency and timing, duration of infertility and prior fertility, childhood illnesses and development, past medical or surgical or sexual history, and gonadal toxin or environmental exposure. Also, included in the medical history was a thorough review of medications and allergies, lifestyle exposures (smoking and consumption of alcohol), family reproductive history, sexually transmitted diseases and previous infections.

5.3 Selection Criteria of Study Population

The study population included 100 infertile men.

Inclusion criteria : Azoospermic infertile males, infertile males with severe Oligospermia that is sperm count less than 5 million per ml from Kolhapur District, visiting hospital from March 2017 to March 2019.

One fertile male and one female as control to perform microdeletion study.

Exclusion criteria : Men with a previous history of severe testicular trauma or infection, chronic testicular diseases, surgery, congenital anomalies or chemotherapy were excluded. Also males not willing for genetic studies were excluded.

Written informed consent (attached) was taken from every participant. All participants were divided into two groups based on the semen count.

5.4 Study Plan

A detailed history was taken to determine their eligibility for the study. One fertile male DNA was used as a positive control. One fertile male was considered as those with sperm concentrations more than 20 million/ml, normal hormone profiles and two or more children conceived naturally. Female DNA was used as a negative control.

5.5 Collection of samples

5.5.1 Collection of semen samples

The semen samples were collected in a clean, wide-mouthed, non-toxic, plastic container, stored between 20-37°C to avoid large changes in temperature which may affect spermatozoa after ejaculation. The prior labeled collection tubes were

incubated at 37°C for liquefaction to take place. All semen samples were collected during the abstinence period of 2–5 days.[8]

5.5.2 Collection of whole blood

Venous blood (10 ml) of study participants were collected under aseptic conditions into standard blood tubes by the technician. All blood samples were stored in aseptic measures. Samples were allowed to clot for 5 minutes and serum was extracted by centrifugation at 1000 rpm for 10 minutes at room temperature. Serum was stored at -20^{0}C until analysis. Blood sample for genomic DNA extraction were immediately transferred to an EDTA vial and thoroughly mixed by gentle rotation in an 8 cm diameter circle.[95]

5.5.3 Testicular Biopsy

Testicular biopsy was performed on male patients with history of infertility who gave consent to the study (consent for operation attached) All biopsies were performed under general anesthesia. Each testis was delivered through scrotal incision. Biopsy material was obtained through small incision in tunica albugenia of testis and teasing out the seminiferous tubules. All biopsies were collected in 10% Bouin's fluid and routinely processed and stained with Haematoxylin and Eosin (H and E). Epididymis and vas deferens were examined for any abnormalities.

All slides were examined microscopically and various histological patterns were evaluated and categorized according to the histopathological patterns and Modified Johnson scoring was performed. The testicular biopsies were categorized into different histopathological patterns [96] as follows:-

1. Normal Spermatogenesis
2. Hypospermatogenesis
3. Germ cell maturation arrest
4. Sertoli cell only syndrome
5. Seminiferous tubule hyalinization
6. Mixed pattern
7. Discordant pattern

5.6 Parameters for evaluation

For the study of semen, blood and testicular biopsy samples following parameters were studied as shown in Table 5.6.1

Table 5.6.1 : Different parameters used for the present study

Type of Study	Sample	Parameters
Morphological	Semen	Sperm Count
		Sperm Motility
		Round cells Count
Biochemical	Blood	Hormones Assay - FSH,LH, testosterone & Prolactin
Biopsy	Testis	Histology - H & E
Molecular	Blood (DNA)	Microdeletion

5.7 Semen Analysis

Semen analysis involved morphological evaluation to determine sperm volume motility and percentage of immature Germ cells.

5.7.1 Determination of Sperm Count and Motility

Assessment of sperm motility was performed on the liquefied of semen sample, (30-60 minutes post liquefaction following ejaculation) according to World Health Organization (WHO) methodology. (WHO laboratory manual, 2010)[8]

The semen was evaluated for the following parameters : semen volume (ml), sperm count (millions/ml), motility (%) according to WHO guidelines.[8] The sperm concentration was determined by using Markler counting Chamber (Semen Analysis Chamber, ISO 9001:2000, by Sefi Medical Instruments, Heifa, Israel).[97] Morphological examination of the collected sperm sample was performed using Olympus® Binocular microscope (10X). Confirmation of azoospermic samples was performed by examining the pellet of centrifuged semen sample. The test was repeated on atleast two occasions at eight weeks interval. The normal values (reference) for semen volume (mL), motility on wet mount slide (%) and sperm count (millions/mL) is 1.4-3.7, 38-42, and 12-16, respectively.[98]

5.7.2 Counting of Immature Germ cells in Semen

Smears of the collected semen sample were prepared by the feathering method described in the 5th edition of the WHO manual for semen analysis.[8] Smears were stained by using Leishman stain.[98]

The round cells were counted and differentiated into immature germ cells and leucocytes. Smears were observed under oil immersion lens (100x) of microscope for differential counts of round cells into immature germ cells and leucocytes. Cells were identified according to their size, shape, and morphology.[99, 100] Round cells were

counted as counted as percentage of the total count.

For data analysis, the patients were grouped based on their seminal parameter as severe oligozoospermic (SOAS, $\leq 5 \times 10^6$ million spermatozoa/mL of ejaculate) and azoospermia (no spermatozoa / ejaculate).[101] Sugar analysis was conducted as per standard procedure.

5.8 Hormone Analysis

From blood samples, levels of serum follicle-stimulating hormone (FSH), luteinizing hormone (LH), testosterone (T), prolactin (PRL) were measured using chemiluminescence immunoassay. Normal valve of FSH mIU/ml:1.5-12, LH mIU/ml :1.1-7, Testosterone ng/ml : 3-10.6, Prolactin ng/ml :1.5 -19.[102]

5.9 Extraction of genomic DNA and quantification

DNA extraction from whole blood was performed using the Genei Pure™ Blood Genomic DNA Purification kit (Merck-Millipor) Genomic DNA was used to determine Y chromosome microdeletion. Chemicals and procedure for DNA extraction as suggested were performed. [95]

Quantitative analysis included the assessment of purity and concentration of DNA by measuring the optical density with a spectrophotometer (Thermo Scientific Nanodrop LITE).

DNA concentration (µg/ml) = A260 × 50 / 2 (volume factor) ×2 (dilution factor)

Extracted DNA was visualized on 1% agarose gel. Purity of the genomic DNA was

spectrophotometrically quantified with a Biospectrometer (Eppendorf, USA).

5.10 Primer Design

Allele-specific polymerase chain reaction (PCR) was used to screen the Y chromosome for STS that are recommended by the European Academy of Andrology (EAA) as shown in Table 5.10.1. Other STS were referred as Non-European Academy Academy of Andrology markers.[88]

A total of 18 forward and reverse primers, shown in Table 5.10.1 and 5.10.2, were included in the study.

Table 5.10.1 : Details of Sequences of STS of EAA markers

	STS	Sequences	Bp
AZFa EAA markers	sY84	5-AGA AGG GTC TGA AAG CAG GT-3	326
		5-GCC TAC CTG GAG GAG GCT TC-3	
	sY86	5-GTG ACA CAC AGA CTA TGC TTC-3	320
		5-ACA CAC AGA GGG ACA ACC CT-3	
AZFb EAA markers	sY127	5-GGC TCA CAA ACG AAA AGA AA-3	274
		5-CTG CAG GCA GTA ATA AGG GA-3	
	sY134	5-GTC TGC CTC ACC ATA AAA CG-3	301
		5-CCG TGT GCT GGA GAC TAA TC-3	
AZFc EAA markers	sY254	5-GGG TGT TAC CAG AAG GCA AA-3	400
		5-GAC CGT ATC TAC CAA AGC TGC-3	
	sY255	5-GTT ACA GGA TTC GGC GTG AT-3	126
		5-CTC GTC ATG TGC AGC CAC-3	

Table 5.10.2 : Details of Sequences of STS of Non EAA markers

	STS	Sequences	Bp
AZFa non EAA markers	sY746	F: TTG ACT GCT TAT TCT ACA CAA GGC	216
		R: CAG GGG AAA TTG GGT TTT	
	sY82	F: TACAGTCGGACGCGT CCCTC ATCCTGCCCTTCTGAATCTC	304
		R: CTGGTCCGTACTACCGTGCG CAGTGTCCACTGATGGATGA	
AZFb Non EAA markers	sY121	F: AGTTCACAGAATGGAGCCTG	190
		R: CCTGTGACTCCAGTTTGGTC	
	sY128	F: TACAGTCGGACGCGTCCCTC GGATGAGACATTTTTGTGGG-3	268
		R: CTGGTCCGTACTACCGTGCG AGCCCAATGTAAACTGGACA	
	sY130	F: AGAGAGTTTTCTAACAGGGCG	173
		R:TGGGAATCACTTTTGCAACT	
	sY143	F: GCAGGATGAGAAGCAGGTAG	311
		R: CCGTGTGCTGGAGACTAATC	
AZFc non EAA markers	sY145	F: TACAGTCGGACGCGTCCCTCC AACACAAAAACACTCATATACTCG	183
		R: CTGGTCCGTACTACCGTGCGTT GAGAATAATTGTATGTTACGGG	
	sY160	F: TAC GGG TCT CGA ATG GAA TA	236
		R: TCA TTG CAT TCC TTT CCA TT ‘	

5.11 Y Chromosome Microdeletion analysis using PCR

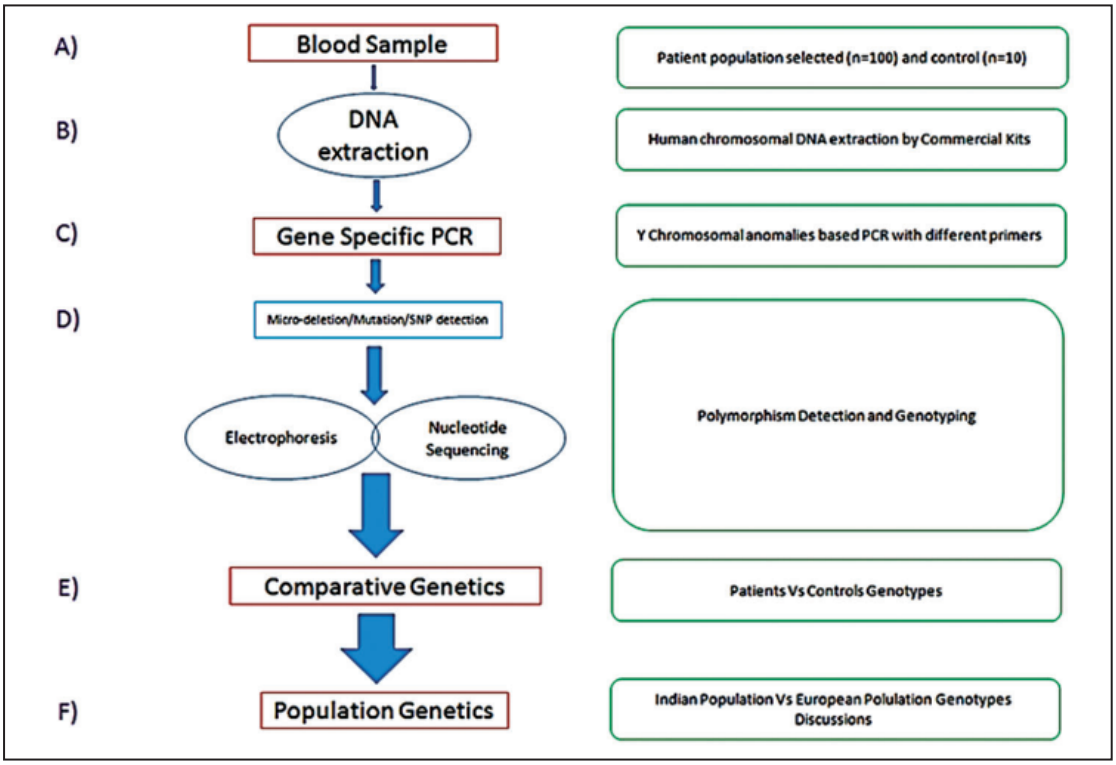


Figure 5.11.1 : Methodology of Y chromosome deletion determination

5.11.1 Primer stock preparation

The main primer stock vials were diluted to 10 pMol, by adding PCR water according to the molar concentration of the primers.

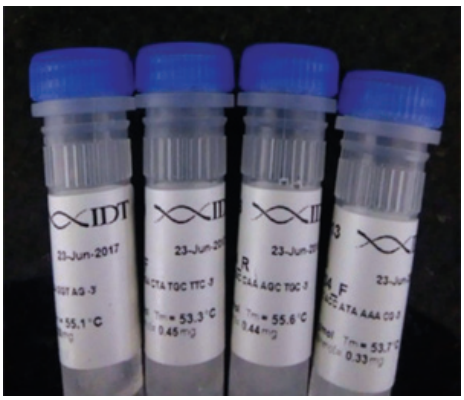


Figure 5.11.2 : Primers : sy143, sy86, sy134, sy254

5.11.2 PCR reaction mixture for 6 primer sets

1. The reagents for 6 primer sets and their respective concentration mentioned below : All calculations were for 25µl reaction mixture as shown in Table 5.11.1.

Table 5.11.1 : Concentration and reagents of Primer sets

How many samples	**1**	**6**
Components		
AmpliTaq	12.5 µl	75 µl
F primer	1µl	1 µl of each unique F primer in separate PCR tube*
R primer	1µl	1 µl of each unique R primer in separate PCR tube*
Template	2 µl	2 µl of each 6 different template DNA to be amplified in separate PCR tube
D/w	8.5	51 µl

2. Initially the master mix was prepared for 6 samples:

1. 75 µl of Ampli Taq polymerase was added to PEG tube (500 µl tube).
2. 51 µl of D/W was added to it.
3. The above mixture of 126 µl, was distributed equally into 6 PCR tubes, 21 µl each
4. Tubes were labelled according to the primer sets.
5. 1 µl of A_sY84_F and A_Sy84_R was added to the tube labelled as sy84*
6. As mentioned above 1 µl of each primer was added to the respective PCR tube.
7. Finally 2 µl of template DNA was added to the tube, that made reaction of 25 µl 21 µl (Ampli Taq+D/w) + 2 µl (1µl F+1µl R, primers) + 2 µl (Template DNA) = 25 µl.
8. Finally PCR reaction was set up on PCR machine (Figure 5.11.2.1) at various temperature as perform the following protocol.

5.11.3 Procedure

All the DNA samples were processed for Yq microdeletions analysis using PCR (TC-512 gradient thermocycler, ABI Bio systems™) Figure 5.11.3 .Each sample was analyzed using 14 sets of primers. The PCR conditions were as shown in Table 5.11.3.

Figure 5.11.3 : PCR Machine

Table 5.11.2 : Conditions of PCR

Initial denaturation	Annealing temperature	Extension	Final extension
5 min at 94 °C for 35 cycles	50 °C to 64 °C (Please refer table no.1) for 1 min	72 °C for 1 min.	7 min at 72°C

The PCR products were analyzed on a 1.5% agarose gel containing ethidium bromide (0.5 µg/mL) and gel images were captured on Synegen G Box; and STS or gene was considered absent after the amplification failures.

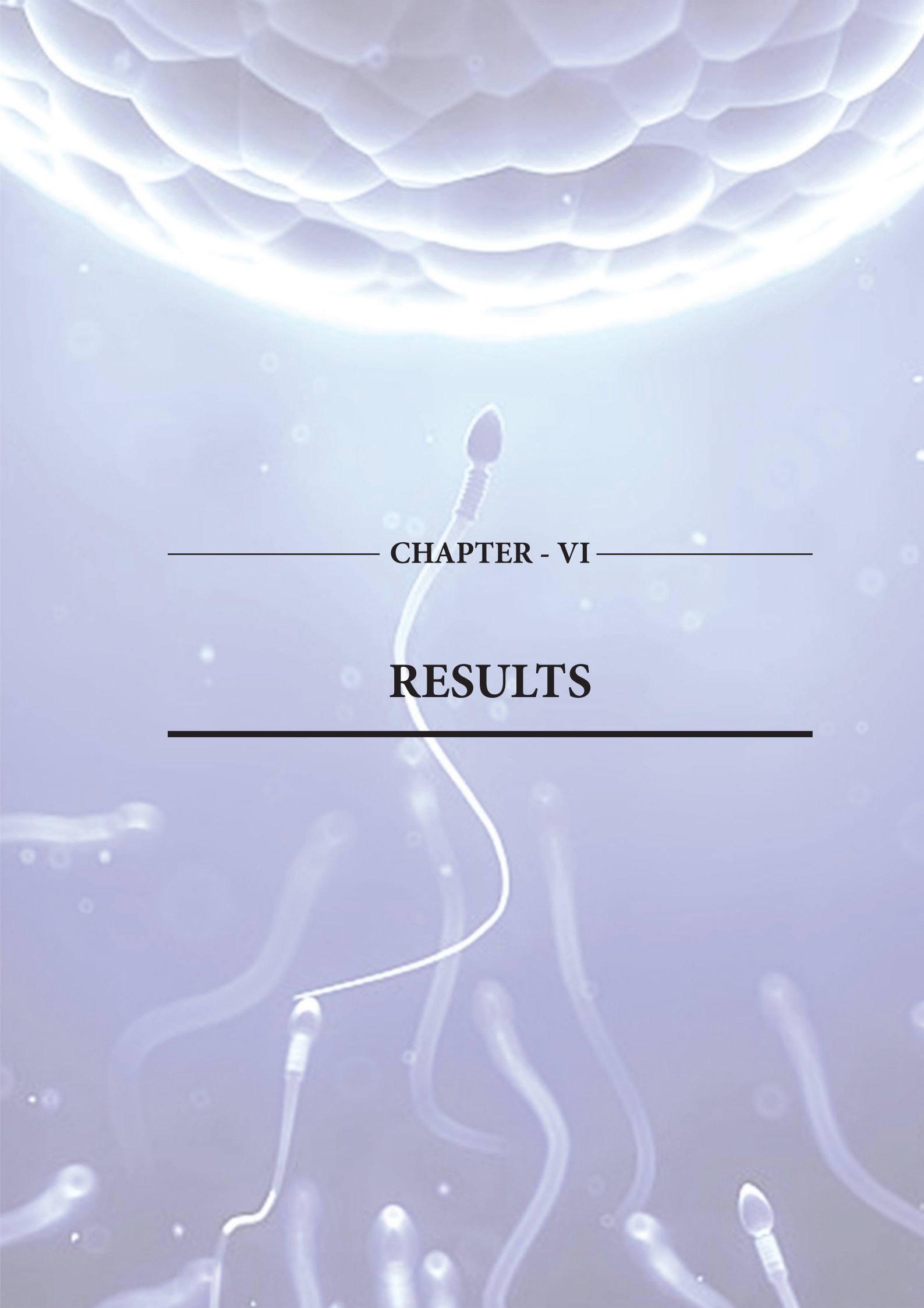
5.11.4 Visualization of PCR products

To remove unused primers and dNTPs, PCR products were purified by gel band

excision. The bands were excised from the agarose gel and purified using a GFX-PCR DNA gel band purification kit (Merck-Millipore) according to the manufacturer's instruction. The sample bands were checked against positive (normozoospermic man) and negative (female DNA sample) genomic DNA samples to rule out DNA contamination a long side the PCR products a 100 bp DNA ladder is loaded to estimate the band size. PCR was performed in triplicates to confirm primer specific deletion. The samples were loaded serially and the absence of the band in the completed gel was considered as deletion.

5.12 Statistical Analysis

Master chart was prepared by using MS-Excel 2010. Data was grouped as Azoospermic and Oligospermic groups. The mean, standard deviation (SD), the standard error of mean (SEM) are calculated for quantitative variables using MS-Excel 2010. Means of quantitative variables for different groups were compared by using unpaired t-test and % of quantitative variables for different groups were compared by using Z-test for proportion. $p \leq 0.05$ was considered statistically significant at 5% level of significance. All the data obtained during the period of investigation were statistically analysed to determine the effect of Y-chromosome microdeletion on the reproductive factors. Effect of microdeletion on histopathology of testis of azoospermic group was also analysed.



CHAPTER - VI

RESULTS

6 RESULTS

6.1 History

From the history, the mean age of the men in both groups was similar (33 years). In addition, the family reproductive history treated for infertility in the Oligospermic group was approximately four times higher than the azoospermic group as shown in Table 6.1.

Table 6.1 : Baseline patient characteristics

	Total (N=100)	Age in years			Family reproductive history
		Mean	Min	Max	
Azoospermic	32	33.16	29	38	2 Father treated for infertility
Oligospermic	68	33.23	30	39	8 Father treated for infertility

6.2 Sperm count and motility

6.2.1 Determination of Sperm Count and Motility

In this study out of total 100 infertile males, 68 were severe Oligospermic while 32 were Azoospermic. Semen volume, semen count and motility were recorded. Out of 32 Azoospermic, 31 semen were Fructose positive one was Fructose negative (table 6.2.1).[101]

Table 6.2.1 : Sperm count and motility

	Total	Volume (ml)	Count (millions/ml)	Motility (%)	Fructose
Azoospermic	32	1.437 ± 0.281	0	0	Positive in 31
Oligospermic	68	2.464 ± 0.371	3.144 ± 0.558	34.558 ± 8.141	Positive in 68

6.2.2 Counting of Immature Germ cells in Semen

The round cells were counted and differentiated into immature germ cells and leucocytes.

- Immature germ cells were noticeable as primary spermatocyte, identified by their large size, large spherical nucleus with woolly appearance and evenly distributed chromatin granules [Figure 6.2.1].
- Spermatids were smaller, round to oval cells with a dark nucleus [Figure 6.2.2].
- Leucocytes were differentiated by their smaller size and multilobed nuclei [Figure 6.2.1].

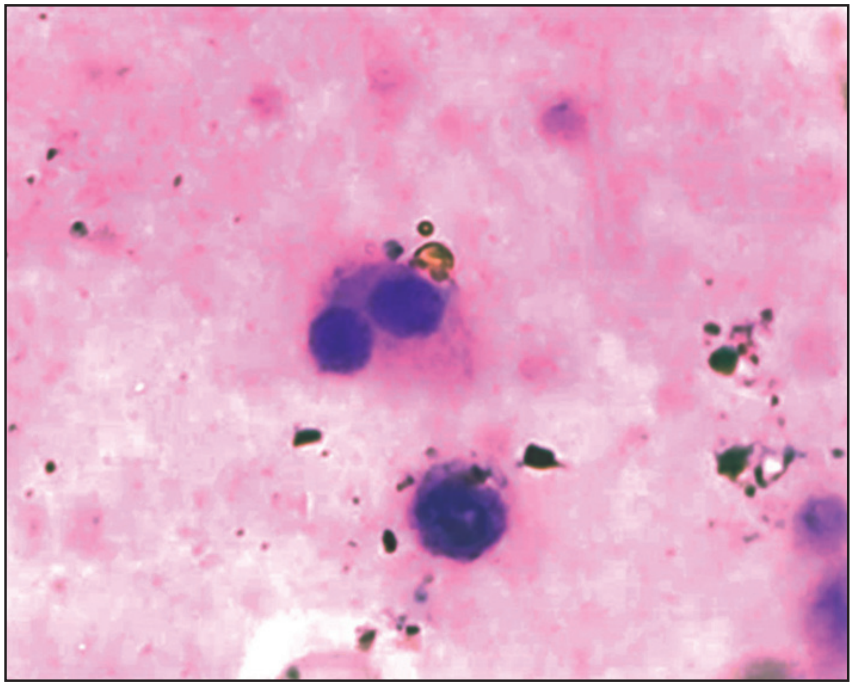


Figure 6.2.1 : Leucocyte and Primary Spermatocyte (Leishman Stain, x100)

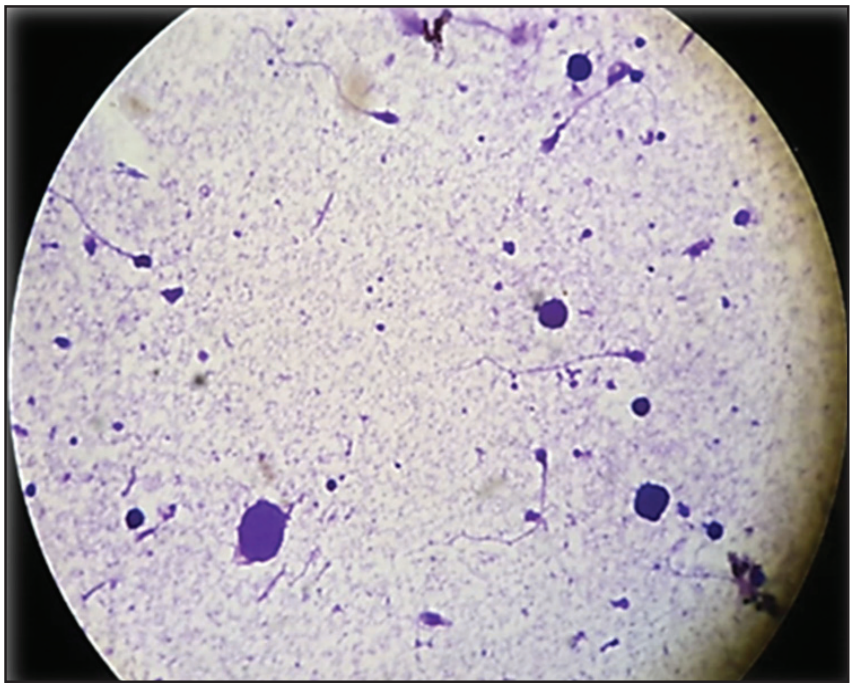


Figure 6.2.2 : Sperm, Spermatid and Primary Spermatocyte (Leishman Stain, x100)

The percentage of immature germ cells was higher in Oligospermic as compared to azoospermic males (Table 6.2.2). A variation between leucocyte percentages between the two groups was observed as well.

Table 6.2.2 : Round cell count and percentage of cells

Group	Semen Leishman Stain		
	Total cells million/ml	Immature germ cells %	Leucocytes %
Azoospermic (32)	1.928 ± 0.267	70.40 ± 16.77	30.87 ± 6.74
Oligospermic (68)	2.533 ± 0.429	82.632 ± 6.958	17.36 ± 6.95

6.3 Histopathological Patterns

A total 32 cases of testicular biopsies from azoospermic males and 3 from severe Oligospermic males were evaluated. Bilateral testicular biopsy was performed after receiving a written informed consent (operative copy attached). On microscopic observation (100X), the following histopathological patterns were observed:

- **Normal Spermatogenesis :** The seminiferous tubules were lined by thin basement membrane and the germinal epithelium showed normal progression from spermatogonia to spermatocytes along with spermatids and spermatozoa (fig 6.3.1).

- **Hypospermatogenesis :** The germinal epithelium showed all the stages of germ cells but the number was reduced (fig 6.3.2).

- **Germ cell maturation arrest :** At a specific cell stage, being the level of primary or secondary spermatocytes, the process of spermatogenesis was arrested. It was further observed as complete (fig 6.3.3) and incomplete (fig 6.3.4).

- **Sertoli cell only syndrome :** The tubules exclusively contained only Sertoli cells and no other cells of spermatogenesis (fig 6.3.5).

- **Seminiferous tubule hyalinization :** The tubules had thickened basement membrane with a smaller diameter along with tubular collagenisation. There was no germinal epithelium observed (fig 6.3.6).

- **Mixed Pattern :** Various histopathological patterns were observed in the same testicular biopsy. These were germ cell maturation arrest along with Sertoli cell syndrome (fig 6.3.7) and seminiferous tubule along with hyalinization germ cell maturation arrest (fig 6.3.8).

- **Discordant pattern :** A variation in the histopathological pattern of right and left testis was observed

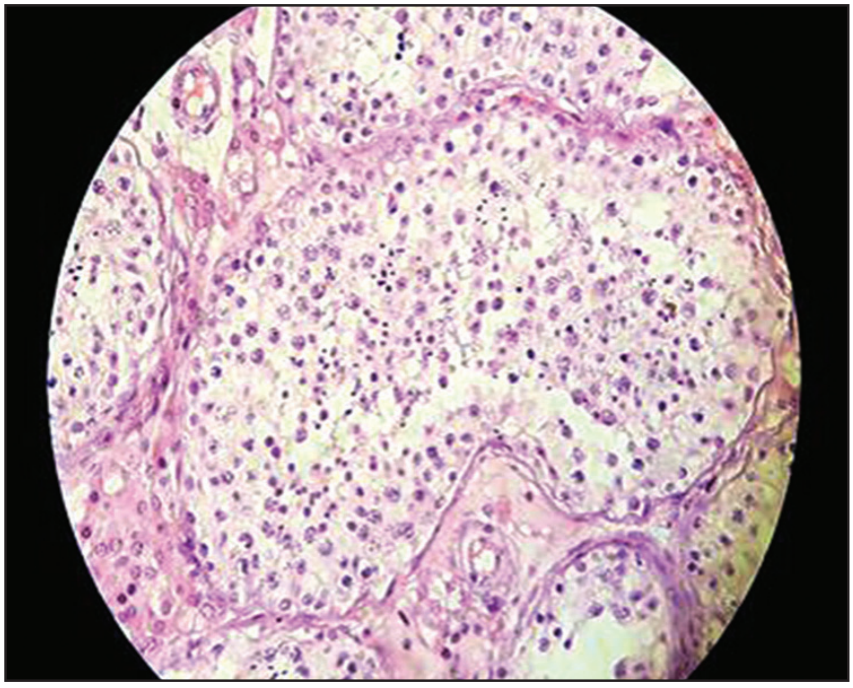


Figure 6.3.1 : Normal Spermatogenesis

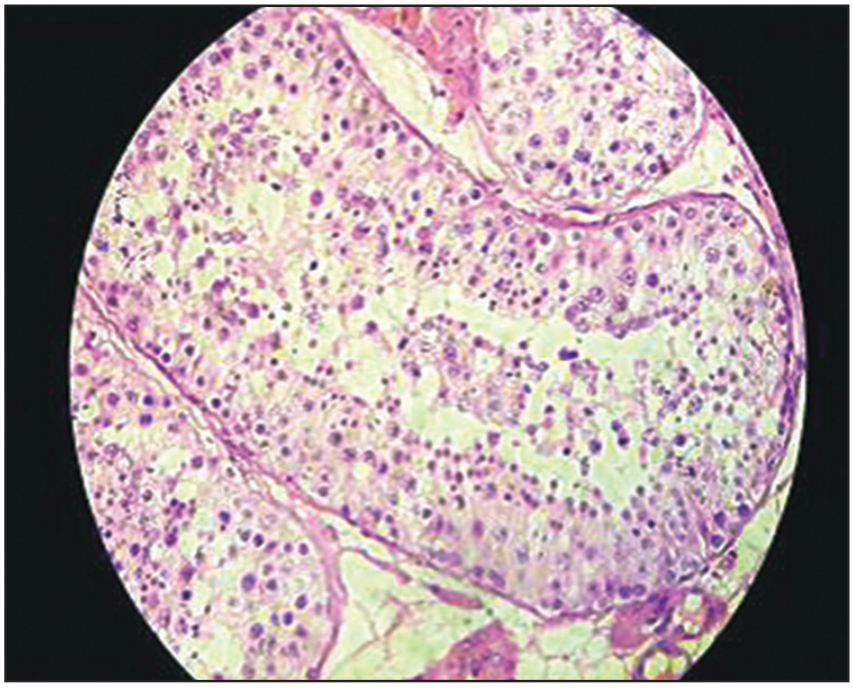


Figure 6.3.2 : Hypospermatogenesis

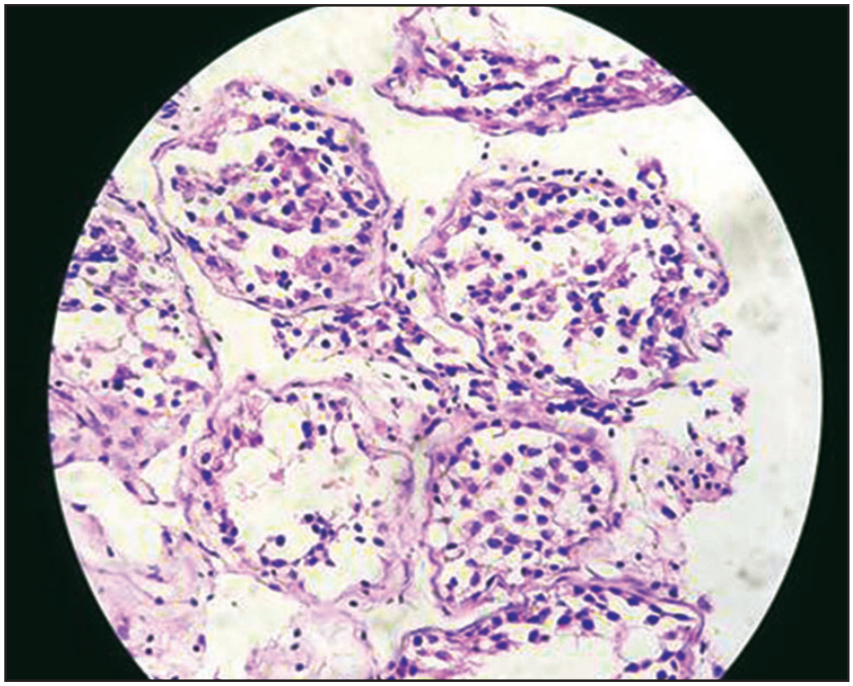


Figure 6.3.3 : Complete Maturation Arrest

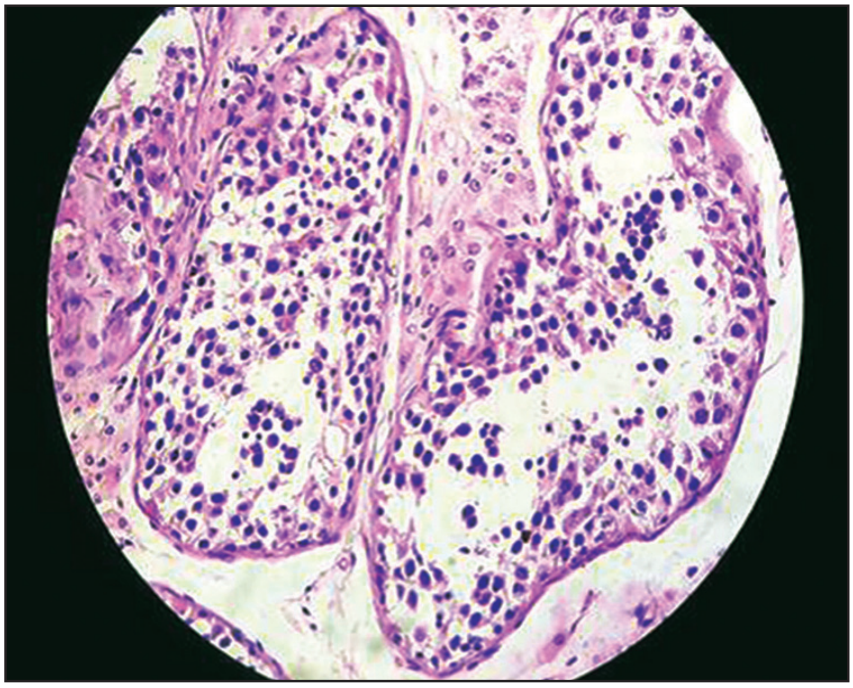


Figure 6.3.4 : Incomplete Maturation Arrest

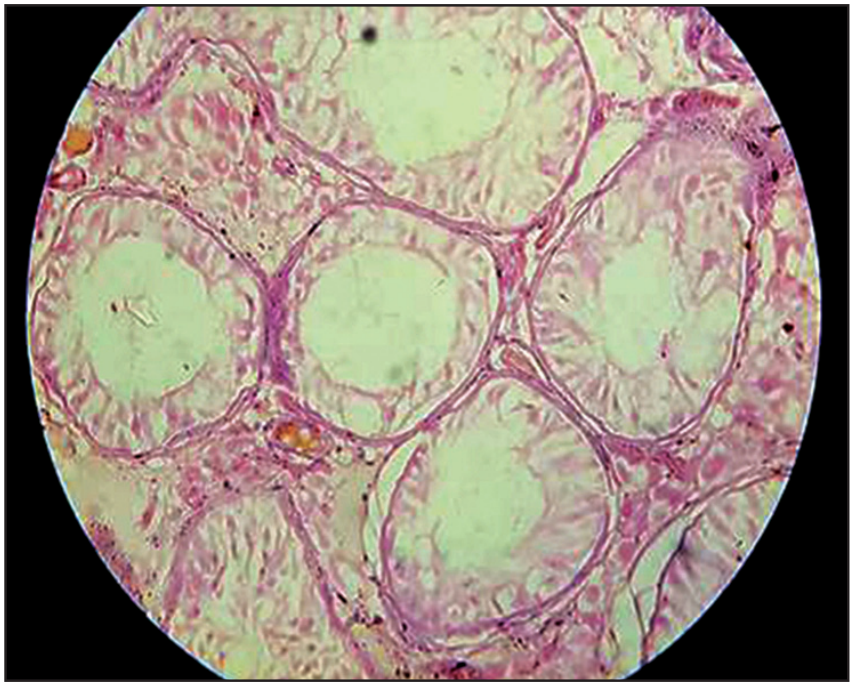


Figure 6.3.5 : Sertoli cell only syndrome

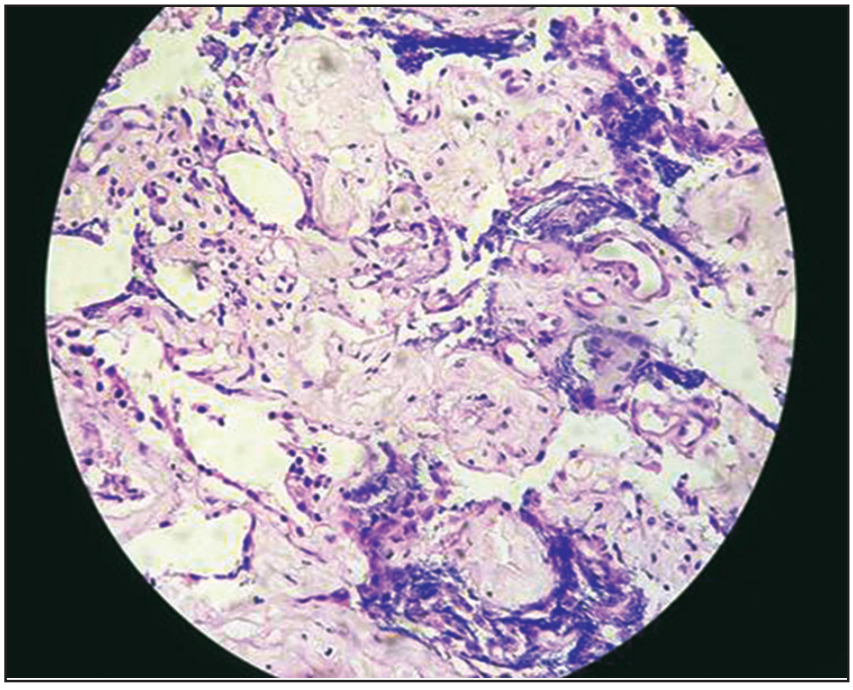


Figure 6.3.6 : Seminiferous tubule hyalinization

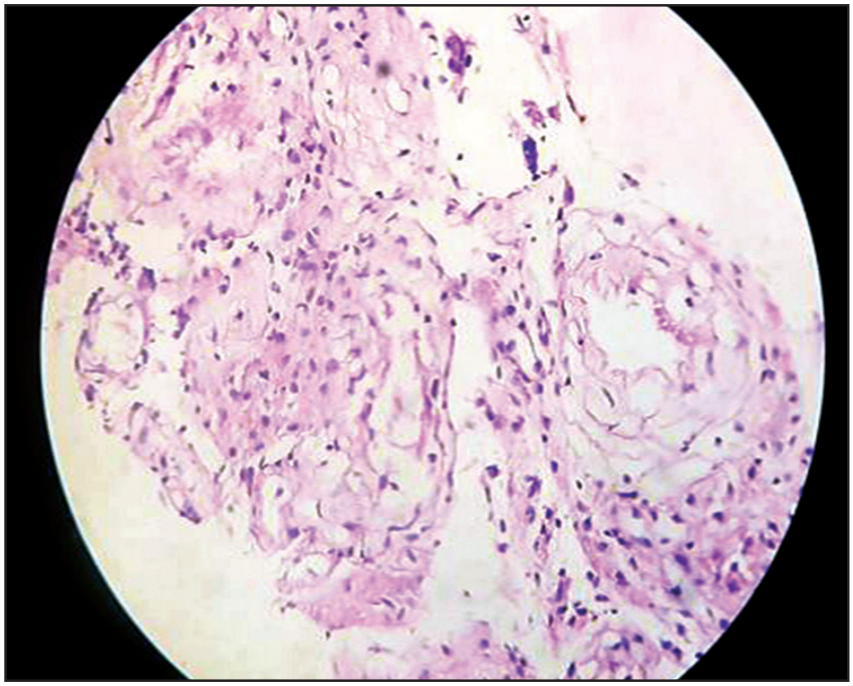


Figure 6.3.7 : Mixed pattern, Left side Sertoli cell and Right tubule shows maturation arrest at spermatogonia

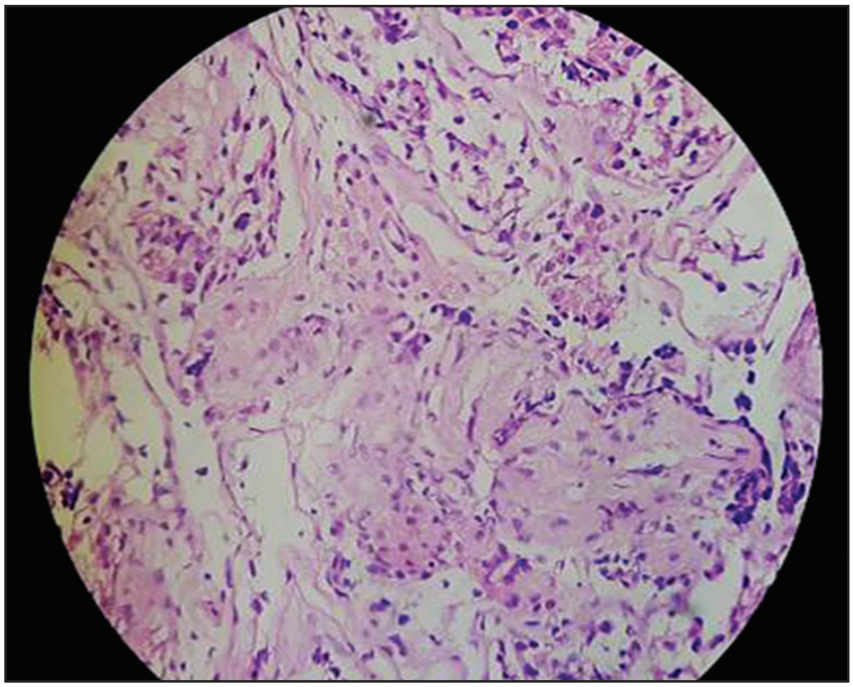


Figure 6.3.8 : Mixed Pattern, seminiferous tubule alongwith hyalinization germ cell maturation arrest

The number of cases and their percentages for various histopathological patterns were calculated (table 6.3.1). In histopathology of testicular bilateral biopsies in azoospermic infertile men, the most frequent pattern was seminiferous tubule hyalinization (34.375%), germ cell maturation arrest (25%), Sertoli cell only syndrome (18.75%), mixed pattern (9.35%), and discordant pattern (6.25%).[103]

Table 6.3.1 : Histopathological classification of testicular biopsies in men

	Azoospermic* n (%)	**Oligospermic+n (%)**	**Total (N=35)**
Normal spermatogenesis	1 (3.125)	1 (33.33)	2
Hypospermatogenesis	1 (3.125)	1 (33.33)	2
Germ cell maturation arrest	8 (25)	1 (33.33)	9
Sertoli cell only syndrome	6 (18.75)	-	6
Seminiferous tubule hyalinization	11 (34.375)	-	11
Mixed Pattern	3 (9.35)	-	3
Discordant pattern	2 (6.25)	-	2

* n number of azoospermic men : 32, +n number of Oligospermic men : 3

Out of the 3 cases that showed mixed pattern (table 6.3.1), 2 were germ cell maturation arrest along with Sertoli cell syndrome (Figure 6.3.7) and one was seminiferous tubule hyalinization along with germ cell maturation arrest (Figure 6.3.8).

Among the two cases which showed discordant pattern :

- A case of incomplete maturation arrest up to spermatids on right testis and left testis showed hypospermatogenesis
- A case of Sertoli cell only syndrome on the right testis and germ cell maturation arrest on left testis; were observed.

On categorization of testicular biopsies according Johnson scoring system, the most frequent pattern was of seminiferous tubule hyalinization which was correlated to Johnson score 1; followed by germ cell maturation arrest which was correlated to Johnson score from 8-3; and lastly, Sertoli cell only syndrome which was correlated to Johnson score 2. Hypospermatogenesis was correlated to Johnson score 9 and Normal spermatogenesis was correlated to Johnson score 10.[103] As per scoring, categorization of testicular biopsies of Azoospermic males was done, as shown in table 6.3.2

Table 6.3.2 : Categorization of testicular biopsies of Azoospermic males according to Johnson scoring system

Johnson scoring	No of cases (32)*n	Scoring %
10	1	3.125
9	5	15.625
8	4	12.5
7	0	0
6	0	0
5	1	3.125
4	0	0
3	5	15.625
2	6	18.75
1	10	31.25
Total	32	100

* n number of biopsy : 64

6.4 Hormone Analysis

FSH and LH levels were increased in both azoospermic as well as severe Oligospermic group as compared to the normal values; while testosterone levels were on lower range of normal value in both groups. Serum prolactin levels, however, were normal in both the groups (table 6.4.1).

According to the hormone assay, azoospermic and Oligospermic cases were further classified as hypergonadotropic hypogonadism or primary testicular failure cases.

Comparison between azoospermic and severe Oligospermic group revealed that among FSH, LH, testosterone and prolactin difference was noted in LH Levels (1.85 mIU/ml) as shown in Table 6.4.1.

Table 6.4.1 : Hormone levels in azoospermic and Oligospermic males

	Normal Value[102]	**Azoospermic (n=32)**	**Oligospermic (n=68)**
FSHmIU/ml	1.5-12	16.01 ± 3.69	16.15 ± 1.95
LHmIU/ml	1.1-7	15.65 ± 4.12	17.50 ± 1.18
Testosteroneng/ml	3-10.6	4.78 ± 1.22	5.38 ± 1.46
Prolactinng/ml	1.5 -19	14.91 ± 1.73	13.73 ± 1.55

6.5 Extraction of Genomic DNA and Quantification

The presence of DNA when checked on agarose gel electrophoresis (1.0 %), in our study DNA was confirmed in all 100 samples as shown in Figure 6.5.1

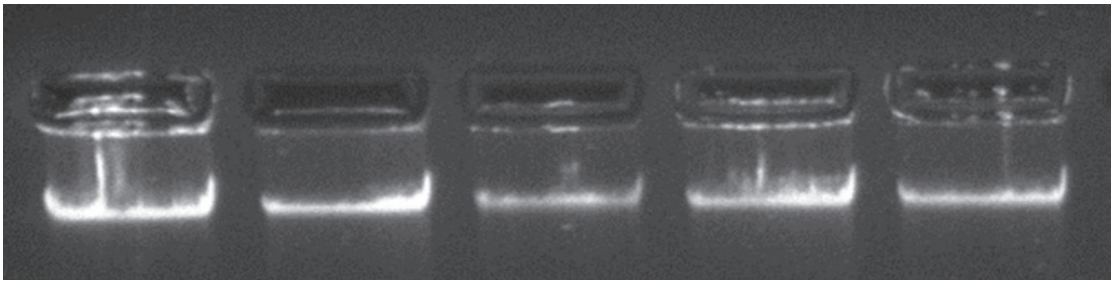


Fig 6.5.1 : DNA bands visualized on Agarose gel (1%)

The purity and concentration of all isolated DNA samples were found to be within normal ranges (purity: 1.7-1.85 and concentration: >30 ng/µl) and stored at -20˚C until required for PCR (table 6.5.1) (fig 6.5.2).

Table 6.5.1 : Quantification of extracted DNA

Sample	Result (ng/ μ L)	A_{260}/1 mm	A_{260}/A_{280}
1	375.4	0.751	1.85
2	267.5	0.535	1.78

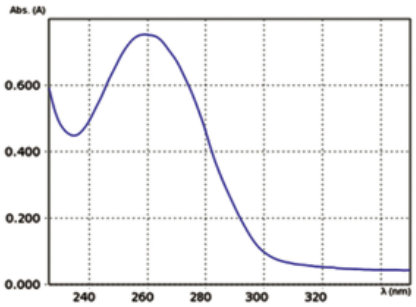


Fig 6.5.2 : A_{260} of extracted DNA

6.6 Chromosome Microdeletion Analysis Results

Microdeletions were studied considering 14 STS, 6 STS recommended by European Association of Andrologists (EAA) and 8 selected STS were referred as Non-European Academy of Andrology markers. Microdeletions in the Y chromosome were confirmed by gel electrophoresis of the PCR amplified products in azoospermic and Oligospermic men. Deletions were observed as missing bands from the respective primers on the agarose gel electrophoresis (fig 6.6.1 - 6.6.7).

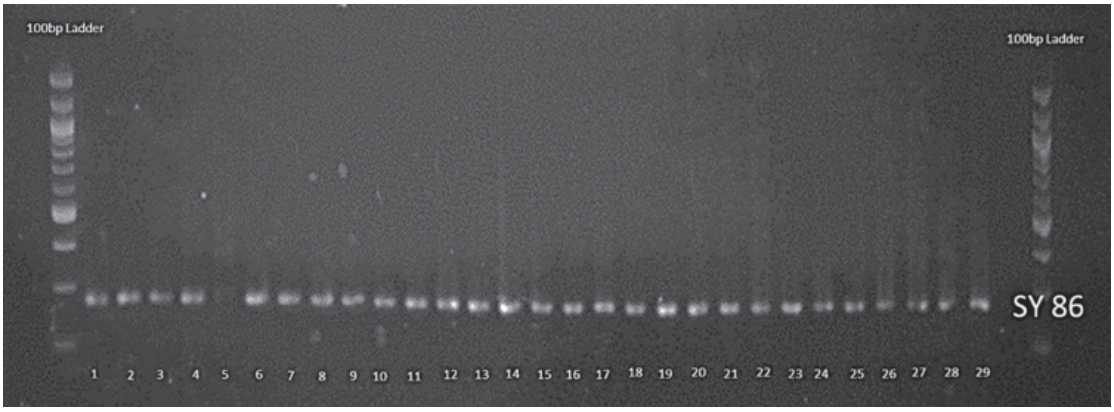


Figure 6.6.1 : Sy86 deletion in sample number 5 in Azoospermic

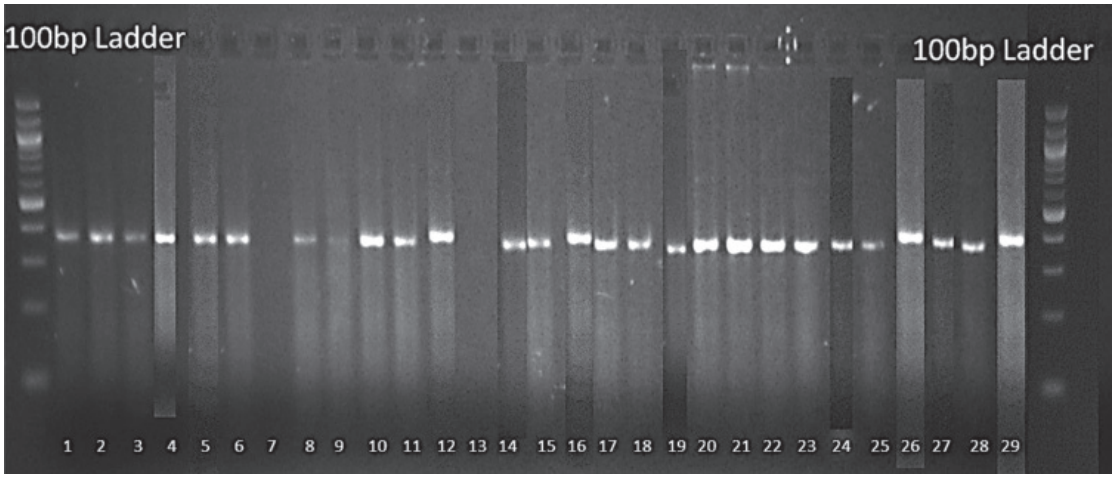


Figure 6.6.2 : Sy254 microdeletion in sample numbers 7, 13 in Azoospermic

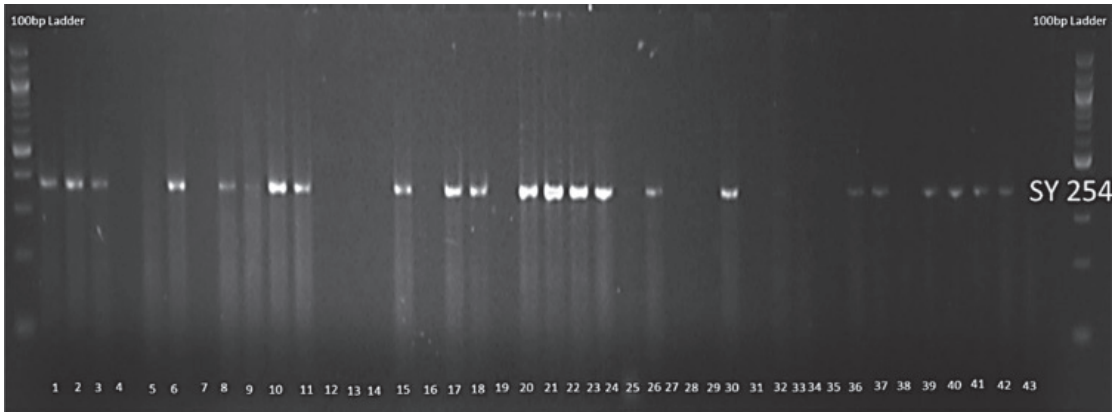


Figure 6.6.3 : Sy254 deletions in sample numbers 4, 5, 7, 12, 13, 14, 16, 19, 25, 27, 28, 29, 31, 32, 33, 34, 35, 38, 43, 63 in Oligospermic

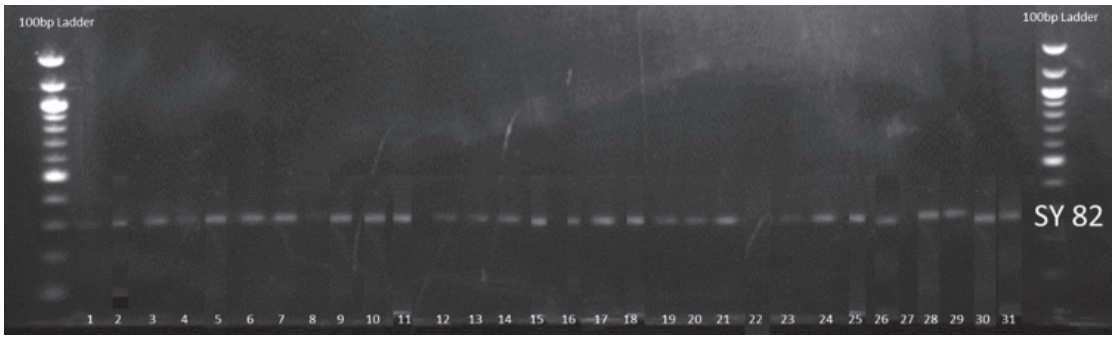


Figure 6.6.4 : Sy82 deletions in sample numbers 8, 22, 34, 56 in Oligospermic

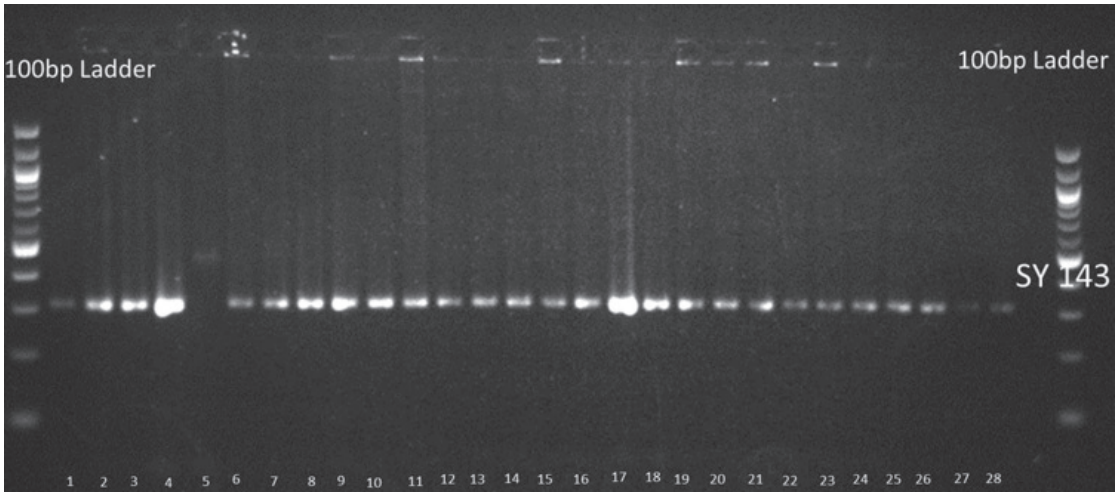


Figure 6.6.5 : Sy143 deletion in sample numbers 5 and 29 in Oligospermic

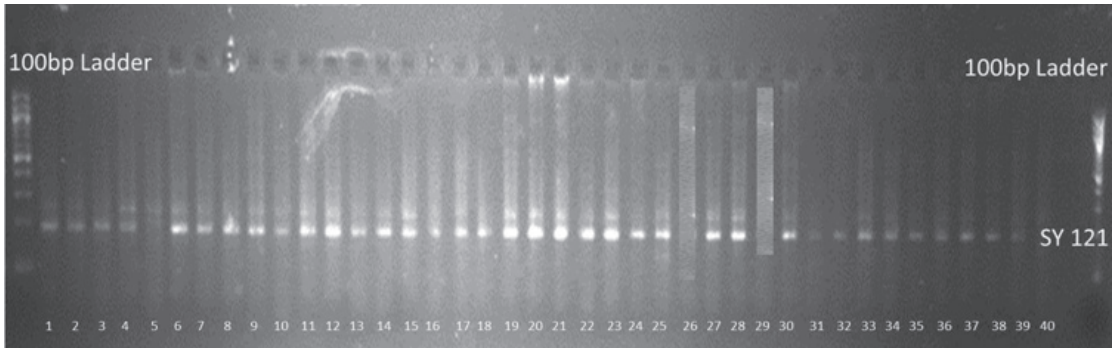


Figure 6.6.6 : Sy121 microdeletions in sample numbers 26 and 29 in Oligospermic

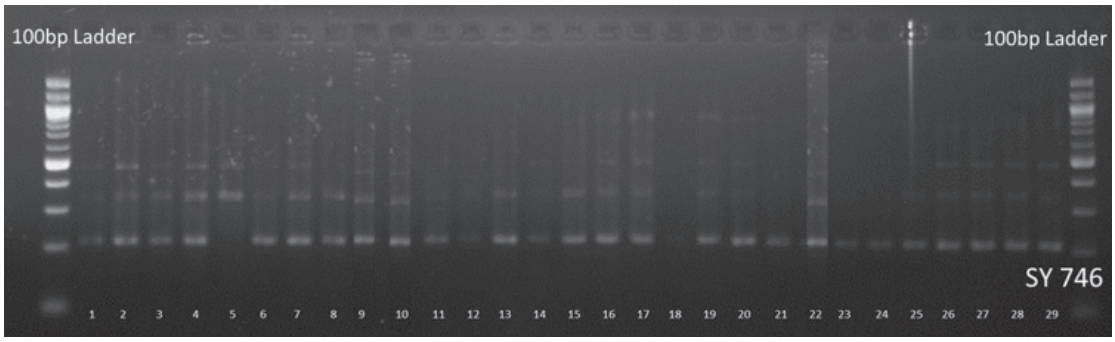


Figure 6.6.7 : Sy746 deletions in sample number 18 in Oligospermic

Microdeletions documented in azoospermic and Oligospermic groups were 7 (n=32) and 25 (n=68), respectively. Primer based observable deletions are tabulated below (table 6.6.1)

Table 6.6.1 : Primer based observable deletions

Markers	STS	Azoospermic (n=32)		Oligospermic (n=68)		Total (N=100)
		Sample numbers	No of cases with deletion	Sample numbers	No of cases with deletion	Total No of cases with deletion
AZFa EAA markers	sY84	-	-	-	-	-
	sY86	5	1	32, 33	2	3
AZFb EAA markers	sY127	32	1	32	1	2
	sY134			5	1	1
AZFc EAA markers	sY254	7,13	2	4, 5, 7, 12, 13, 14, 16, 19, 25, 27, 28, 29, 31, 32, 33, 34, 35, 38, 43, 63	20	22
	sY255	-	-	5	1	1
AZFa non EAA markers	sY746	-	-	18	1	1
	sY82	2,4	2	8, 22, 34, 56	4	6
AZFb Non EAA markers	sY121	26	1	26,29	2	3
	sY128	-	-		-	-
	sY130	-	-		-	-
	sY143	-	-	5,29	2	2
AZFc non EAA markers	sY145	-	-	32	1	1
	sY160	-	-	-	-	-

Total frequency of Y chromosome microdeletions in Azoospermic and Oligospermic males was 32%. Region wise deletion of AZFc region was the most frequent n (%): AZFc 17 (53.13%); followed by AZFa, 7 (21.88%); followed by AZFb and AZFac,

3 (9.375%); and least frequent was AZFbc 2 (6.25%) (Table 6.6.2). Region wise microdeletion is graphically represented in azoospermic (fig 6. 6.8) and Oligospermic men (fig 6.6.9).

Table 6.6.2 : Frequency of microdeletions in azoospermic and Oligospermic men

Markers	Azoospermic (n=7) (%)	Oligospermic (n=25) (%)	Total (n=32) (%)
AZFa	3 (42.85%)	4 (16%)	7 (21.88%)
AZFb	2 (28.57%)	1 (4%)	3 (9.375%)
AZFc	2 (28.57%)	15 (60%)	17 (53.13%)
AZF b,c	0	2 (8%)	2 (6.25%)
AZFa, c	0	3)12%)	3 (9.375%)

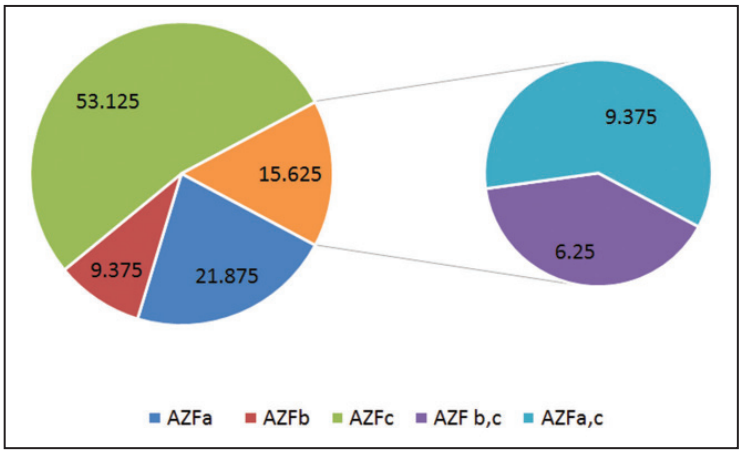


Figure 6.6.8 : Region wise percentage of Microdeletion

In both azoospermic and Oligospermic groups, no significant difference in semen volume and count, between deleted and non-deleted samples, were observed. However, there was a significant increase in motility in deleted as compared to non-deleted cases in the Oligospermic group (p=0.049). Total cell count and percentage of immature germ cells in azoospermic group decreased as compared to Oligospermic group. A significant increase in percentage of leucocyte in samples with deletion compared to non-deleted in azoospermic group was observed (p=0.0001). There

was significant increase in levels of FSH, LH in deleted samples of both the groups. But, the level of testosterone significantly decreased in samples with deletion of Oligospermic group ($p<0.0001$). Johnson scores of samples with deleted cases also decreased significantly in azoospermic males (table 6.6.3).

Table 6.6.3 : Effect of Y chromosome microdeletion in azoospermic and oligospermic men

	Parameters	Overall	Deletion +	Deletion -	P-value
Semen analysis					
Azoospermia	Volume	1.437 ± 0.28	1.37 ± 0.29	1.46 ± 0.29	0.45
	Count/ml	0	0	0	-
	Motility	0	0	0	-
Oligospermia	Volume	2.464±0.37	2.35±0.36	2.53 ± 0.36	0.5
	Count/ml	3.144 ± 0.55	3.27 ± 0.50	3.6 ± 0.58	0.12
	Motility	34.558 ± 8.14	37.2±8.56	33.02 ± 7.57	0.049*
Leishman staining					
Azoospermia	Total cells	1.926±0.26	1.72±0.14	1.98 ± 0.27	0.0028*
	Immature	70.40 ± 16.7	56.86 ± 2.4	74.2 ± 17.12	0.0001**
	Leucocyte	30.87 ± 6.74	43.14 ± 2.14	27.44 ± 17.45	0.0001**
Oligospermia	Total cells	2.533±0.429	2.46 ± 9.45	2.57 ± 0.42	0.3
	Immature	82.632 ± 6.95	83.32 ± 6.95	82.23 ± 7.01	0.5
	Leucocyte	20.308 ± 25.65	16.68 ± 6.95	22.41 ± 31.78	0.2
Hormone analysis					
Azoospermia	Testosterone	4.78 ± 1.22	4.65 ± 1.24	4.82 ± 1.24	0.75
	FSH	16.01 ± 3.69	18.63 ± 2.75	15.28 ± 3.63	0.02**
	LH	15.65 ± 4.12	18.05 ± 2.95	14.99 ± 4.19	0.047*
	Prolactin	14.91 ± 1.73	15.4 ± 1.73	14.78 ± 1.74	0.42
Oligospermia	Testosterone	5.38 ± 0.66	3.71 ± 0.66	6.36 ± 0.74	<0.0001*
	FSH	16.15 ± 1.95	17.13 ± 1.71	15.57 ± 1.87	<0.0001*
	LH	17.50 ± 1.18	18.33 ± 0.89	16.02 ± 1.06	<0.0001*
	Prolactin	13.73 ± 1.55	13.45 ± 1.5	13.89 ± 1.58	0.26
Testicular Biopsy					
Azoospermia	Johnson scoring	-	1.42 ± 0.78	4.76 ± 3.42	0.0001*

* Significant **highly significant

In azoospermic males with deletion, histology of testis mostly showed Seminiferous tubule hyalinization in maximum case in 5 cases, Germ cell maturation arrest in one and Discordant pattern Sertoli cell only syndrome Germ cell maturation arrest in one. While in males with non-deletion, complete and incomplete maturation arrest at primary spermatogonia were seen in maximum cases.

6.7 Semen Analysis and Microdeletion

On analysis of semen volume, it was found that there was negligible difference in deleted and non-deleted cases in Azoospermic as well as Oligospermic group as represented in Table 6.7.1 and 6.7.2. Sperm count in severe Oligospermic group did not show significant difference (table 6.7.2). In Oligospermic group motility showed significant difference, motility observed was more in cases with deletion as shown in Table 6.7.2

Table 6.7.1 : Effect of microdeletion in semen parameters in azoospermic group

Azoospermic (n=32)	Semen	Deletion + ve (n=7)	Deletion –ve (n=25)	T value	P value
Semen Analysis	Volume	1.37 ± 0.24	1.46 ± 0.29	0.77	0.45
	Count/ml	0	0		
	Motility	0	0		
Semen Leishman Stain	Total cells	1.72 ± 0.14	1.98 ± 0.27	3.4	0.0028**
	Immature	56.86 ± 2.41	74.2 ± 17.12	4.89	P<0.0001**

Table 6.7.2 : Effect of microdeletion in semen parameters in oligospermic group

Oligospermic	Semen	Deletion + ve (25)	Deletion –ve (43)	T value	P value
Semen Analysis	Volume	2.35±0.36	2.53±0.36	2	0.5
	Count/ml	3.27±0.50	3.6±0.58	1.56	0.12
	Motility	37.2±8.56	33.02±7.57	2.02	0.049*
Semen Leishman Stain	Total cells	2.46±0.45	2.57±0.42	1.006	0.31
	Immature	83.32±6.95	82.23±7.01	0.62	0.53

Observation of Leishman stain on semen smear showed significant difference of total cell count and percentage of immature germ cells in deleted and non-deleted samples of azoospermic group (table 6.7.1). Total cells as well as percentage of immature germ cells were more in non-deleted group. Contrarily, no significant difference was observed in the total cell count and percentage of immature germinal cells in deleted and non- deleted samples of oligospermic groups (table 6.7.2).

6.8 Hormones and Microdeletion

In azoospermic group, the FSH level in patients with deletions was found to be significantly elevated as compared to subjects with no deletions (p=0.02). Serum levels of LH in patients with deletions was found to be significantly elevated as compared to subjects with no deletions (p=0.047). Serum testosterone level was not influenced by microdeletions (table 6.8.1).

Table 6.8.1 : Effect of microdeletion on hormone levels in azoospermic group

Azoospermic (32)	Deletion (+) (n=7)	Deletion (-) (n=25)	T VALUE	P VALUE
Testosterone	4.65±1.24	4.82±1.24	0.32	0.75
FSH	18.63±2.75	15.28±3.63	2.64	0.02**
LH	18.05±2.95	14.99±4.19	2.19	0.047*
Prolactin	15.4±1.73	14.78±1.74	0.83	0.42

* Significant **highly significant

In severe Oligospermic group, serum FSH and LH levels were significantly elevated between the deleted and non-deleted groups (p<0.0001). Contrarily, serum testosterone levels were significantly reduced in deleted group (p<0.0001). Serum level of prolactin was not influenced by microdeletions (table 6.8.2)

Table 6.8.2 : Effect of microdeletion on hormone levels in oligospermic group

Oligospermic (68)	Deletion (+) (25)	Deletion (-) (43)	T value	P value
Testosterone	3.71±0.66	5.06±0.70	15.2	P<0.0001**
FSH	17.13±1.71	15.57±1.87	16.16	P<0.0001**
LH	18.33±0.89	16.02±1.06	14.67	P<0.0001**
Prolactin	13.45±1.5	13.89±1.58	1.13	0.26

* Significant **highly significant

6.9 Johnson's score and microdeletion

Johnson's score was significantly varied owing to microdeletions (p= 0.0001). An elevation in the score was observed in the non-deleted group (table 6.9.1).

Table 6.9.1 : Effect of microdeletion on Johnson's score in azoospermic men

Testicular biopsy in azoospermic (n=32)	Deletion (+) (n=7)	Deletion (-) (n=25)	T value	P value
Johnson's Score	1.42±0.78	4.76±3.41	4.46	0.0001**

**Highly significant

6.10 EAA and non-EAA primer specific microdeletion

A total of 32 cases recorded deletions for 6 EAA primers and 8 Non EAA primers. In azoospermic group (n=7), EAA primer deletions were 4 and 3 in non-EAA primers. In Oligospermic group (n=25), the EAA, Non EAA and both (EAA and Non EAA) primers, deletions were 18, 4 and 3, respectively (table 6.10.1)

Table 6.10.1 : Number of EAA and non-EAA primer specific Microdeletion

	Deletion (+)	Only EAA	Only Non EAA	Both EAA and Non EAA
Azoospermic (32)	7	4	3	0
Oligospermic (68)	25	18	4	3
Total	32	22	7	3

In terms of specific STS primers used (n=14), the microdeletions, in the descending order, were recorded for 14 sY254 (22), sY82 (6), sY121 (4), sY86 and sY127 (3 each), and sY134, sy255, sy746, sY145 (1 each) (table 6.10.2)

Table 6.10.2 : STS Specific microdeletions

Markers	STS Total 14	Azoospermic (n=32) Deletions (+ 7)	0ligospermic (n=68) Deletions (+35)
AZFa EAA	sY84	0	0
AZFa EAA	sY86	1	2
AZFb EAA	sY127	1	1
AZFb EAA	sY134	0	1
AZFc EAA	sY254	2	20
AZFc EAA	sY255	0	1
AZFa non EAA	sY746	0	1
AZFa non EAA	sY82	2	4
AZFb non EAA	sY121	1	2
AZFb non EAA	sY128	0	0
AZFb non EAA	sY130	0	0
AZFb non EAA	sY143	0	2
AZFc non EAA	sY145	0	1
AZFc non EAA	sY160	0	0

The percentage of STS microdeletions specific for EAA and non-EAA primers was 25% and 11%, respectively. A significant difference in the nature of primer microdeletions between EAA and Non-EAA STS was recorded (p=0.0098) The nature of the primer (EAA or non-EAA) did not significantly affect the microdeletions in the azoospermic group. Contrarily, a significant difference for the same was observed in the Oligospermic group (table 6.10.3).

Table 6.10.3 : Percentage of STS microdeletions specific for EAA and non-EAA primers

	Deletion (+) %	EAA %	NON-EAA %	P VALUE
Azoospermic (32)	21.87%	12.50%	9.33%	0.68
Oligospermic (68)	36.76%	31%	12%	0.00652**
Total (100)	36%	25.00%	11.00%	0.0098**

**Highly significant

CHAPTER - VII

DISCUSSION

7 DISCUSSION

7.1 Semen Analysis

Semen analysis is routinely performed to evaluate the possibility of infertility in the male partner of infertile couples. According to WHO (2010)[8], the lower reference limit of sperm concentration and sperm motility for normal men is 15 million/ml and 40%, respectively. In the azoospermic cases, owing to the lack of sperm cells in the semen, both the sperm concentration and sperm motility values were found to be zero. Contrarily, as compared to the lower limit, in the Oligospermic group, lower sperm motility was observed (34.55).

The differential counts of round cells showed that of the total round cells, 70% to 80% were immature germ cells and 10% to 20% were leucocytes (Gune et. al., 2019).[101] The results presented are in agreement with previous studies (Gandini et. al.,1999, Ariagno et. al., 2002).[104-105]

In the previous study conducted by Patil P S et. al.,[98] of the total round cells, majority of cells were immature germ cells (80-90%) which supports the present study, where

the immature germ cells were 65- 85% and 75-85% in azoospermic and Oligospermic groups, respectively.

For understanding the etiology of infertility, if in semen report percentage of immature germ cell are mentioned will definitely be a useful tool. Also this could be a good indicator of a dysfunction at the testicular level, by providing an insight on germ cell maturation arrest.

The absence of immature germ cells on staining is suggestive of an obstruction. In such cases, invasive procedures, such as testicular biopsy can be avoided (Gandini et. al., 1999).[104] In agreement with this statement in our study confirmation of obstruction of azoospermic sample which was negative for fructose and no immature germ cells were found. Thus, the conclusion that presence of germ cells can easily and definitely differentiate non-obstructive from obstructive azoospermia holds true (WHO).[8] These immature germ cells in semen can be used for diagnostic and Cytogenetic studies. Cytogenetic study on isolated germ cells can be performed for numerical and structural chromosomal aberrations**.** For Intra-cytoplasmic sperm injection procedure, these spermatids (immature germ cells) can be retrieved by performing TESA (testicular sperm extraction). So this information is really important especially in azoospermic (Kahraman et. al., 1998).[106]

7.2 Testicular Biopsy

The occurrence of male infertility and the consequent histological findings in testicular biopsies are dissimilar due to the few fundamental etiological elements including social propensities, hereditary causes and ecological conditions, for

example, hidden contaminations, synthetic concoctions, radiation and exposure to warm environment (Lunenfeld et. al., 2004, Saradha et al., 2006).[107-08]

Despite the fact that a fundamental male fertility assessment requires an extensive history and physical examination alongside semen investigation, testicular biopsy remains the key examination for every single testicular reason for infertility (Levi et. al., 1979).[109] The present outcomes demonstrated concurrence with some International investigations and disparities with others and these outcomes are analyzed as pursue:

Hypospermatogenesis represented 3.125% out of the 32 azoospermic cases. This is similar to the outcome reported by Meinhard et. al[110], 5%. Different investigators detailed higher frequency Nagpal et. al., [111] detailing 16%, and Haddad et. al., [112] at Jordon which revealed 11.2% for obstructive azoospermia, Brannen and Roth[113] detailed (35%).

Spermatogenic arrest was subdivided into two gatherings: Complete and Incomplete. All tissue tests indicating spermatogenic arrest spoke to 25% which is equivalent with concentrate done by Rashed et. al., (2008)[114] who detailed spermatogenic capture at a recurrence of 28% in Egyptian men, Glina et. al., (2005)[115] revealed a higher occurrence contrasted with referenced investigations (37.5%) in Brazil, however there are studies with outcomes lower as compared to our studies, Brannen and Roth (1979)[113] indicated (12.5%) in United States, Al-Rayess and Al-Rikabi (2000)[116] concluded (11%) for Soudian network and Thomas (1990)[117] revealed (5%) in Nigeria, while Haddad et. al., (2004)[112] announced an exceptionally low frequency of spermatocyte development capture (1.7%) in Jordon.

Sertoli cell in our study amounted to 18.75%, which was lower than the observed 34% in Egyptian study (Rashed et. al.,2008)[114] and higher than 12.5% in united States study (Brannen and Roth.,1979).[113] The lowest reported occurrence of SCOS is 2.9% (Haddad et. al., 2004).[112] SCO may result from various causes, for example, cryptorchidism, cytotoxic medications, or light. The non attendance of germ cells might be because of variables present amid fetal life (Skakkebaek et. al., 1994).[118]

Seminiferous tubule hyalinization group represented percentage (34.375%) which is the highest in our study. On comparison incidence of 28.4% was reported in Haddad et. al.[112] study, and 23% in Thomas study (1990).[117] May the higher percentage in our study is because of inclusion of severe Oligospermic and Azoospermic males. These blended outcomes might be because of some hereditary variables and also inclusion criteria of only Azoospermic infertile males. More investigations and bigger number of testicular samples are expected to understand the numerous patterns brought from this heterogeneity.

7.3 Hormone Analysis

The purpose of the hormonal evaluation of an infertile male patient is to identify the endocrinological disorders that adversely affect male reproduction and to gain prognostic information. An evaluation of pituitary-gonadal axis provides valuable information about the sperm production in infertile men. It reveals the problem associated with the pituitary axis that causes infertility. In sub fertile men whose sperm count is less than $<10\times10^6$ sperm /ml, it is very much essential to evaluate the status of FSH and Testosterone levels (Sigman et. al., 1997).[119] LH and prolactin levels are evaluated in cases which showed decline in testosterone and FSH

production in order to differentiate hypogonadotropic hypogonadism from testicular insufficiency (Bourcigaux et. al., 2008).[120]

Even though FSH is required for the initiation of spermatogenesis, the hormone by itself cannot be used as a marker for spermatogenesis. FSH twice the normal is indicative of germinal epithelial destruction, but normal or elevated FSH is associated with both normal as well as aberrant spermatogenesis, where testicular histology could show SCO syndrome, Hypo is seen where the quantity of spermatogenesis is decreased and is known to be associated with either normal or elevated FSH (Bablok et. al., 1978).[121]

The serum Testosterone level reflects the overall endocrine balance whereas the FSH levels in men shows the status of sperm production. Studies has shown males with low TT, have abnormal sperm morphology so have lower live birthrates (Trussell et. al., 2019).[122]

The present study shows that the sperm count is inversely associated with serum levels of FSH and LH. Serum LH and T levels were statistically not significant, but serum FSH levels were significantly higher in the Oligospermic group compared to the control group (Subhan et. al, 1995).[123] Our findings for FSH and sperm concentrations are consistent with those in several previous studies.[124,125,126,127,128] Jensen et. al., (1997)[125] have reported significant correlations of FSH, LH with sperm concentration among 1558 young Danish men who reported for military service. In another study, Uhler et. al., (2003)[129] also have reported significant bivariate correlations of FSH with sperm concentration among 145 men in couples with no known fertility impairment, although they found no associations between LH or

testosterone and semen quality parameters and again the associations were not tested further in multivariate analyses.

7.4 Frequency of Microdeletions of Y chromosome.

In our study, the overall percentage of microdeletion was 32%, while the percentage of microdeletion in azoospermic and severe Oligospermic groups were 21.87% and 36.7%, respectively. A similar study by Zhu et. al., 2008[130] conducted on Chinese men reported an overall AZF microdeletions on the Y chromosome to be 11.5%, while the percentage of microdeletions were 13.99% and 20.30% in azoospermic and severely oligozoospermic patients, respectively. However, literature reviews have uncovered a high frequency (51.6%) of microdeletions among azoospermic patients and a low frequency (less than 5%) in men with severe oligozoospermia (Ferlin et. al., 2007).[131] According to earlier report Yq microdeletion frequency prevalence differs in a wide range from 1% (Vander VK et. al., 1997),[132] to 55% (Foresta et. al., 1998).[133] It has been reported in the literature that the frequency of Y chromosomal microdeletions in infertile men varies between 0.98% and 55.5%, depending on the inclusion criteria and the STS regions used (Krausz et. al., 2014).[53] This discrepancy and wide variation in deletion frequency estimates might be due to ethnic differences, sample sizes, patient selection criteria, methodological aspects, and even the type and number of markers used in the studies.

Prevalence of Yq Microdeletions in India

Studies from India have also reported a wide variation in the prevalence of Yq microdeletions in infertile males. The frequency of Y chromosome microdeletions

in the Indian population reported the range from 3.3 (Pandey et. al., 2010)[134] to 36% (Suganthi et. al., 2013)[21] by some workers (Sen et. al., 2013).[88] A study by Suganthi et. al., 2013[21] reported a higher microdeletion frequency of 36% when they examined South Indian infertile males.

Table 7.4.1 : Geographic variations in frequency of Yq microdeletions in India.

Area	Percentage of Deletions	Number of cases deleted/ total number of cases studied
Delhi	5.2%	81/1547
Uttar Pradesh, Western Bihar, Lucknow, Varanasi	6.0%	39/643
Assam	5%	5/100
Kolkata	8.5%	29/340
Mumbai	3.3%	20/587
Hyderabad	6.5%	23/369
Tamil Nadu	13.4%	26/193
South India	36%	18/50

A more recent study noted that 8.3% of azoospermic males and 2.3% oligozoospermic males showed the presence of Y chromosome microdeletions (Mascarenhans et. al., 2016).[135] An extensive meta-analysis of the data available on the prevalence of Yq microdeletions in the Indian population by Sen et. al., 2013[88] revealed a prevalence rate of 5.8%. On segregating these data based on the type of AZF deletion and sperm count, it was observed that the AZFc microdeletion frequency is about 35% in azoospermic males and 70% in oligozoospermic males.

It has been noted that the prevalence of Y chromosome microdeletions varies with geographical locations, the highest occurring among South Indian males and the

lowest among Northern and Western parts of India. This suggests that ethnicity might also influence the prevalence of Y chromosome microdeletions. Due to these findings, it is necessary to test Indian infertile male for microdeletion study all classes of males. No phenotype parameters are predicated surely as guidance for considering microdeletion test to be conducted. So, screening for Y chromosome microdeletions should be considered as diagnostic test to know etiology of male infertility in India.

7.4.1 Regional distribution of microdeletions

This study shows that AZFc region deletion was the most frequent (53.12%) followed by AZFa region (21.87%), AZFb and AZFac (9.37%) and AZFbc (6.25%). Our findings were almost similar to a Korean study that showed a high frequency of microdeletions in the AZFc (56.55%) as compared to deletions in AZFa, 7.74%, AZFb, 5.95%, AZFbc, 22.02%, and AZFabc, 7.74% (Silber et. al., 1997).[136] The AZFc deletion was the most common pattern of AZF microdeletions in patients with azoospermia and Oligospermia; this finding was consistent with previous reports (Machev et. al., 2004).[137] AZFc region has many fertility genes, presence of many repetitive sequences present in this region could be cause of frequent deletion of AZFc region. It has been suggested that men with AZFc deletion can produce sperm, but some patients do not have any sperm inside their seminiferous tubules (Kim SY et. al., 2017).[138] Previous study have suggested spermatogenic failure is due to deletion in the AZFc region. However, other researchers have disagreed, proposing that this is simply a polymorphic deletion with no clinical ramifications (Repping et. al., 2003).[139]

7.4.2 EAA and Non EAA STS

In the determination of Y chromosome microdeletion frequency selection of an appropriate combination of sequence tagged site (STS) loci is a critical factor.

The European Academy of Andrology suggest the use of six STS loci (sY84, sY86, sY127, sY134, sY 254, and sY255) for detection of up to 95% of all reported Y chromosome microdeletions (Simoni et. al., 2004).[89]

In our study, when both EAA and non-EAA markers were used, 32% microdeletions were observed, while EAA showed 25% and non-EAA markers showed 11% microdeletions. The study conducted by Sen et. al., 2013[88] in Indian population, however, reported only 8.5% deletion when both EAA and non-EAA markers were used, 5.4% and 3.1% deletions by EAA and non-EAA, respectively.

Abobakr et. al., 2009[140]; Sheikh et. al., 2009[141] and Pandey et. al., 2010[134] used EAA recommended markers and reported a lower frequency of microdeletions of Y chromosome (0%) 40 cases, (1.7%) 4/100 cases (4%) and 2/64 (3%) respectively.

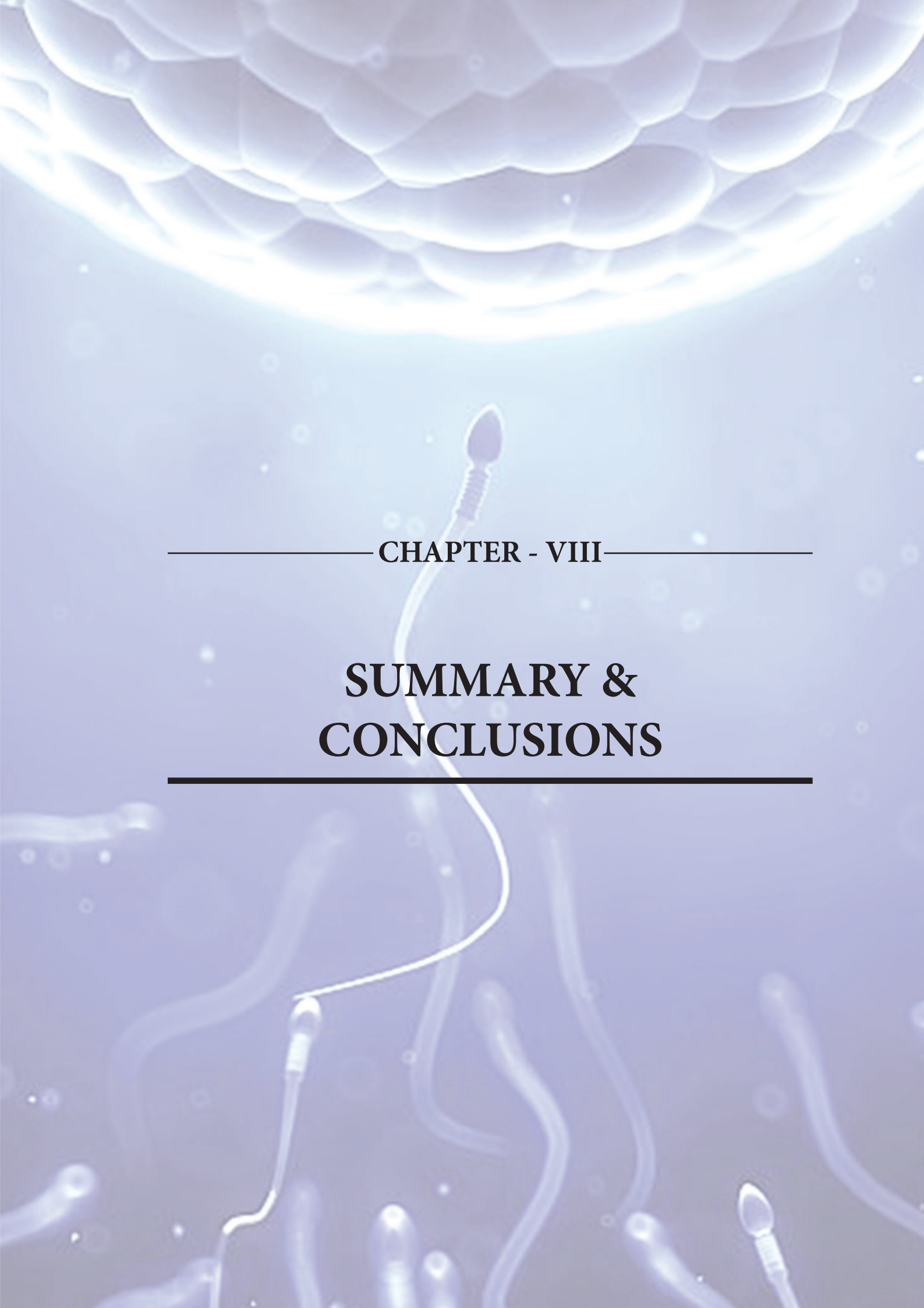
7.5 Microdeletion and Semen Analysis and Hormone assay and Testicular Biopsy

In our study, on comparing semen volume (ml) there was no significant difference seen in cases with microdeletion or cases without microdeletion in both azoospermic group and Oligospermic groups.

Studies have shown changes in male hormone physiology according to levels of FSH, LH, and T, as well as modification of gonadal morphology in individuals affected by

microdeletions of the Y chromosome (Vog PH et. al., 1996, Abid et. al., 2008).[50,142] In our study too, there were significant high levels of FSH and LH in infertile males with deletion of Y chromosome when compared to non-deletions in both azoospermic and Oligospermic groups. The levels of testosterone in males with deletions were significantly lower than that in males with non-deletions in Oligospermic group while in case of azoospermic males, it was non-significant. In contrast to our findings, the levels of FSH and T in patients with Y microdeletions were significantly lower than that in patients without Y microdeletions, whereas the levels of LH were significantly higher in another study (Park SH et. al., 2013).[143]

When we correlated microdeletion study to histopathology of testis of the azoospermic males, males with deletion revealed seminiferous tubule hyalinization this finding was supported by the study conducted by Singh K et. al., 2005[144], he also reported the same result with deletion of AZFc region.



CHAPTER - VIII

SUMMARY & CONCLUSIONS

8 SUMMARY AND CONCLUSIONS

8.1 Summary

8.2 Conclusions

8.3 Justification and use of results

8.4 Challenges for the study

8.5 Work that can be done further

8.1 SUMMARY

In the present study, an attempt has been made to study microdeletion of Y chromosome as causative factor in infertile males. This topic was selected to fill the existing gaps in data for these disorders in Kolhapur district. Also, an attempt has been made to correlate hormone levels, histology of testis to microdeletion.

In our study out of 100 infertile males, Azoospermic were 32 while severe Oligospermic were 68.

Semen analysis involved evaluation of semen volume, sperm count, and motility. Leishman staining of semen smear was done to count percentage of immature Germ cells.

Testicular biopsy was performed on 32 azoospermic men and 3 Oligospermic men with their consent for the procedure.

Hormone levels of serum follicle-stimulating hormone (FSH), luteinizing hormone (LH), testosterone (T), prolactin (PRL) were measured using chemiluminescence immunoassay on blood samples.

The present study explored the markers recommended by the European Academy of Andrology (EAA) along with 8 other markers to detect the microdeletions of Y chromosome in the Indian infertile males. Total 14 STS markers that are EAA (sY84, sY86, sY127, sY134, sY254, sY255) and Non EAA (sY746, sY82, sY121, sY128, sY130, sY143, sY145, sY160) were used. Lastly comparison of EAA to Non EAA markers was done.

The following results were noted

1. The mean age of the men in both groups was similar (33 years). The family reproductive history treated for infertility in the Oligospermic group was approximately four times higher than the azoospermic group.

2. Out of 32 Azoospermic, 31 semen were Fructose positive, one was Fructose negative

3. Leishman Staining of semen revealed presence of 70.40% immature germ cells in Azoospermic and 82.63% in Oligospermic.

4. In histopathology of bilateral testicular biopsies in Azoospermic males, most frequent pattern was of seminiferous tubule hyalinization, followed by Germ cell maturation arrest, followed by Sertoli cell only syndrome as shown in figure 8.1. Out of 32 cases, 3 showed mixed pattern: 2 cases showed Germ cell maturation arrest along with Sertoli Cell Syndrome and one case showed Seminiferous tubule hyalinization along with Germ cell maturation arrest. In the same group 2 cases showed discordant pattern: in one subject right testis showed Incomplete Maturation arrest up to spermatids and left testis showed

Hypospermatogenesis. Another case showed Sertoli cell only syndrome on right and Germ cell maturation arrest on left side.

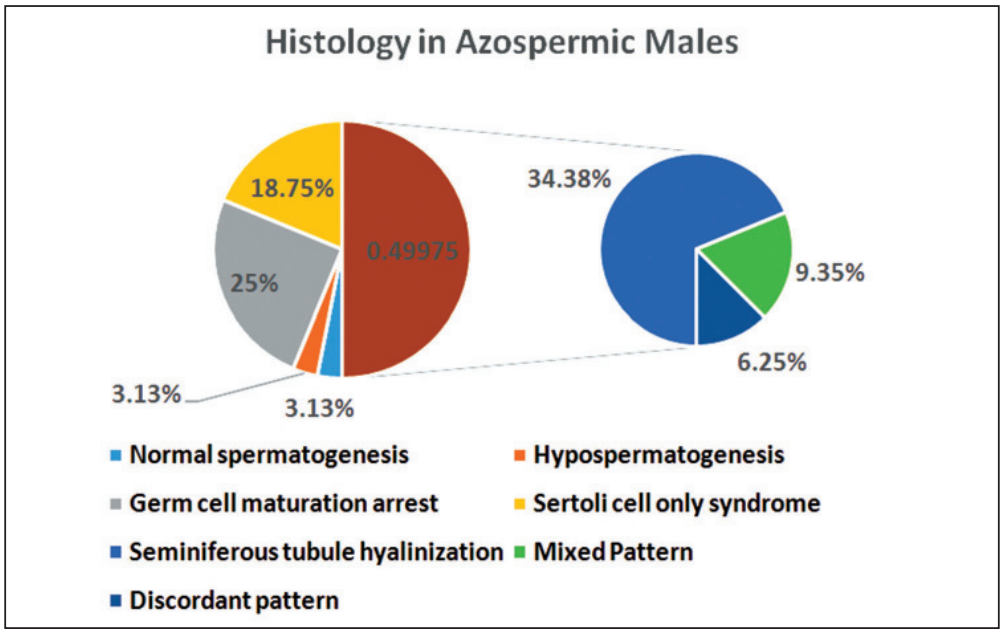


Figure 8.1 : Histology in Azoospermic Males

5. On categorization of testicular biopsies according Johnson scoring system, Johnson score 1 (Seminiferous tubule hyalinization) was observed in 31.35%. Johnson score 2 (Sertoli cell Syndrome) was observed in 18.75%. Johnson score 3 (Germ cell maturation arrest) was observed in 15.625%. Johnson score 9 (Hypospermatogenesis) was observed in 15.625%. Johnson score 10 (Normal Spermatogenesis) was observed in 3.125%.

6. In severe Oligospermic group out of 68 males, 3 consented for biopsy. Out of three biopsies Normal spermatogenesis, Hypospermatogenesis and Germ cell maturation arrest were observed

7. FSH and LH levels were definitely increased in both azoospermic as well

as severe Oligospermic group as compared to the normal values; while testosterone levels were on lower range of normal value in both groups. Serum prolactin levels, however, were normal in both the groups

8. The overall frequency of Y chromosome microdeletions was 32% (32/100).

9. In 2 Azoospermic and 8 Oligospermic males, there was a family history of father treated for infertility and all these subjects had microdeletion in Y chromosome.

10. On segregating these data based on the type of AZF deletion, it was observed that the AZF microdeletion percentage was 21.88% in azoospermic males and 36.76% in oligozoospermic males. Of the males with Y-chromosome microdeletions, deletion of the AZFc region was the most frequent, followed by the AZFa, AZFb and AZFac and AZFbc as shown in Figure 8.2.

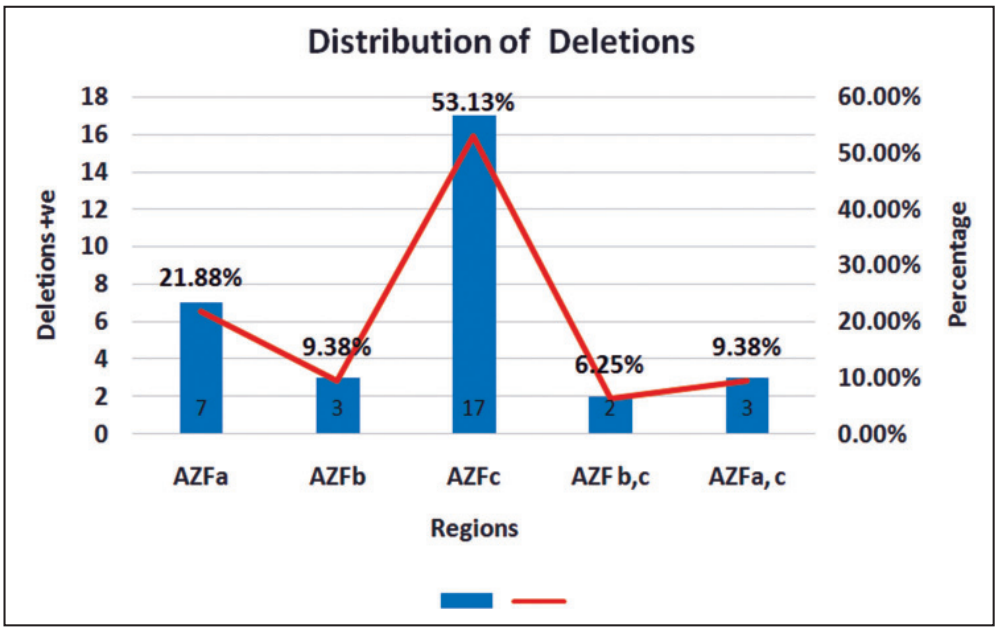


Figure 8.2 : Region wise Distribution of Deletions

11. We observed a significant difference between EAA and non-EAA markers. In azoospermic group out of 7 deletions, EAA primer deletions were 4 and 3 in non- EAA primers. In Oligospermic group out of 25 deletions, the EAA, non EAA primer both deletions (EAA & Non EAA) were 18, 4 and 3, respectively as shown in Figure 8.3

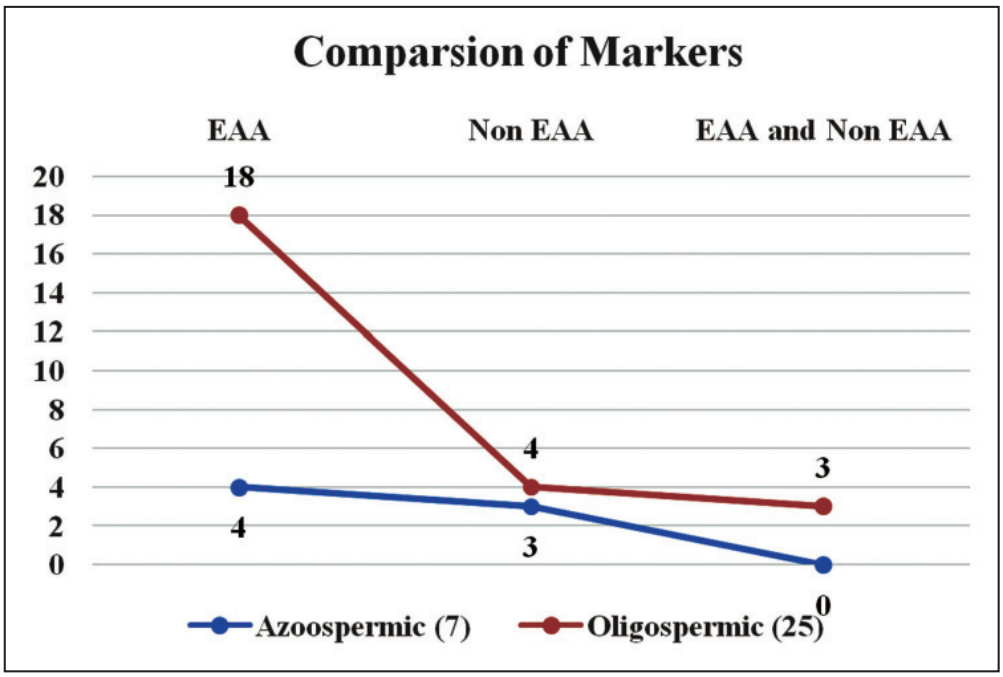


Figure 8.3 : Comparison of Markers

12. Comparing semen volume and sperm count in Oligospermic groups having Y chromosome microdeletion with non-deletion cases no significant difference was observed but increased sperm motility was seen in samples with Y chromosome microdeletion

13. In azoospermic males, percentage of immature cells was less in Y chromosome microdeletion present cases compared with non-deleted cases as shown in Figure 8.4

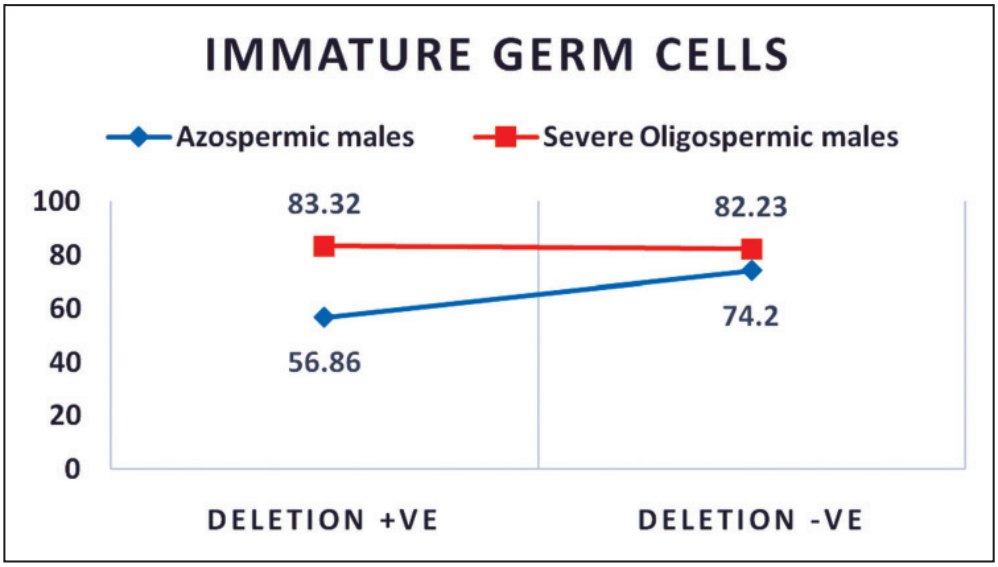


Figure 8.4 : Comparison of Immature germ cells

14. There was significant increase in levels of FSH & LH in Y chromosome microdeletion samples of both the groups. And levels of testosterone were significantly decreased in Y chromosome microdeletion Oligospermic group.

15. In azoospermic males with Y chromosome microdeletion, histology of testis mostly showed Seminiferous tubule hyalinization in maximum cases, Germ cell maturation arrest in one and Discordant pattern Sertoli cell only syndrome along with Germ cell maturation arrest in one. While in males with Y chromosome non-deletion, complete and incomplete maturation arrest at primary spermatogonia were seen in maximum cases as shown in Table 8.1.

Table 8.1 : Comparison of Histopathological pattern of testicular biopsies in deleted and non-deleted Azoospermic infertile men

Histology in Azoospermic	Total	Microdeletion -ve	Microdeletion +ve
Normal spermatogenesis	1	1	0
Hypospermatogenesis	1	1	0
Germ cell maturation arrest	8	7	1
Sertoli cell only syndrome	6	6	0
Seminiferous tubule hyalinization	11	6	5
Mixed Pattern	3	3	0
Discordant pattern	2	1	1

16. Decreased Johnson score was seen in Y chromosome microdeletion cases in azoospermic males.

8.2 CONCLUSIONS

1. In semen analysis round cells should also be given due importance, as they can be differentiated into leucocytes and immature germ cells. The count of immature germ cells could be obtained by simple staining procedures and will add clinically relevant information to the semen report.

2. Further, the immature germ cells in semen can be separated and isolated for diagnostic, cytogenetic studies and can be used for in-vitro culture.

3. Absence of both fructose and immature germ cells in semen and testicular biopsy showing normal spermatogenesis confirms distal Obstructive azoospermia or CAVD.

4. Biopsy of Testis is vital diagnostic test in determining the gonadal causes of azoospermia, reproductive prognosis and therapeutic considerations for Azoospermic men.

5. Testicular biopsy is useful for the recovery of mature spermatozoon from men who are opting for ICSI treatment to conceive. For ICSI the surgeon needs to understand that there can be discordant and mixed pattern, so retrieval of germ cells from multiple sites of both the testis will increase chances of success.

6. Hormone level as one of the investigations may be one option as they are simple. However, correlation between Y chromosome deletion and hormone levels is not clear.

7. Since EAA markers were found to be more effective in detection of Y chromosome microdeletion compared to non-EAA markers, these can be used in future for detection of microdeletions. Finally, the study recommended that combinations of EAA and Non EAA should be used in India to maximize detection of Y chromosome microdeletion.

8. In conclusion patients with azoospermia or severe Oligospermia (sperm count < 5 million/ml), family history of treated infertility, significantly elevated levels of FSH & LH, lower levels of testosterone, histology pattern of Seminiferous tubule hyalinization with low Johnson's score should be selected for AZF screening program.

8.3 JUSTIFICATION AND USE OF RESULTS

1. This study has given baseline statistics of microdeletion of Y chromosome and also helped us to identify genetic markers in Kolhapur district population. Report of Y chromosome microdeletion will provide cause for infertility.

2. This will help couples with long standing infertility to resolve stress, blame or feelings of guilt.

3. Knowledge of the type of Yq deletion may assist the clinician in determining the chances of finding sperm on testicular biopsy or microTESE for best type of ART treatment.

4. Testicular biopsy is useful for the recovery of mature spermatozoon from men who are opting for ICSI treatment to conceive. For ICSI the surgeon needs to understand that there can be Discordant and mixed pattern, so retrieval of germ cells from multiple sites of both the testis will increase chances of success.

5. Male with Y chromosome microdeletion will transmit this genetic microdeletion to his male offspring. So information should be offered to such couples that their male offspring will almost certainly be subfertile and require reproductive monitoring from the time of sexual maturation.

6. To conclude result of this study will help detection of microdeletions which will provides great advantage in guiding clinical diagnosis, selection of treatment schemes and Genetic counselling of families.

8.4 CHALLENGES FOR THE STUDY

There were many challenges in conducting this study, namely:

1. For detection of Y chromosome microdeletion, more than 300 STS are available. To select STS to be included in this study was challenging, as no

previous study is reported in Kolhapur.

2. Standardization of PCR conditions was difficult as few STS were very close and bit overlapping

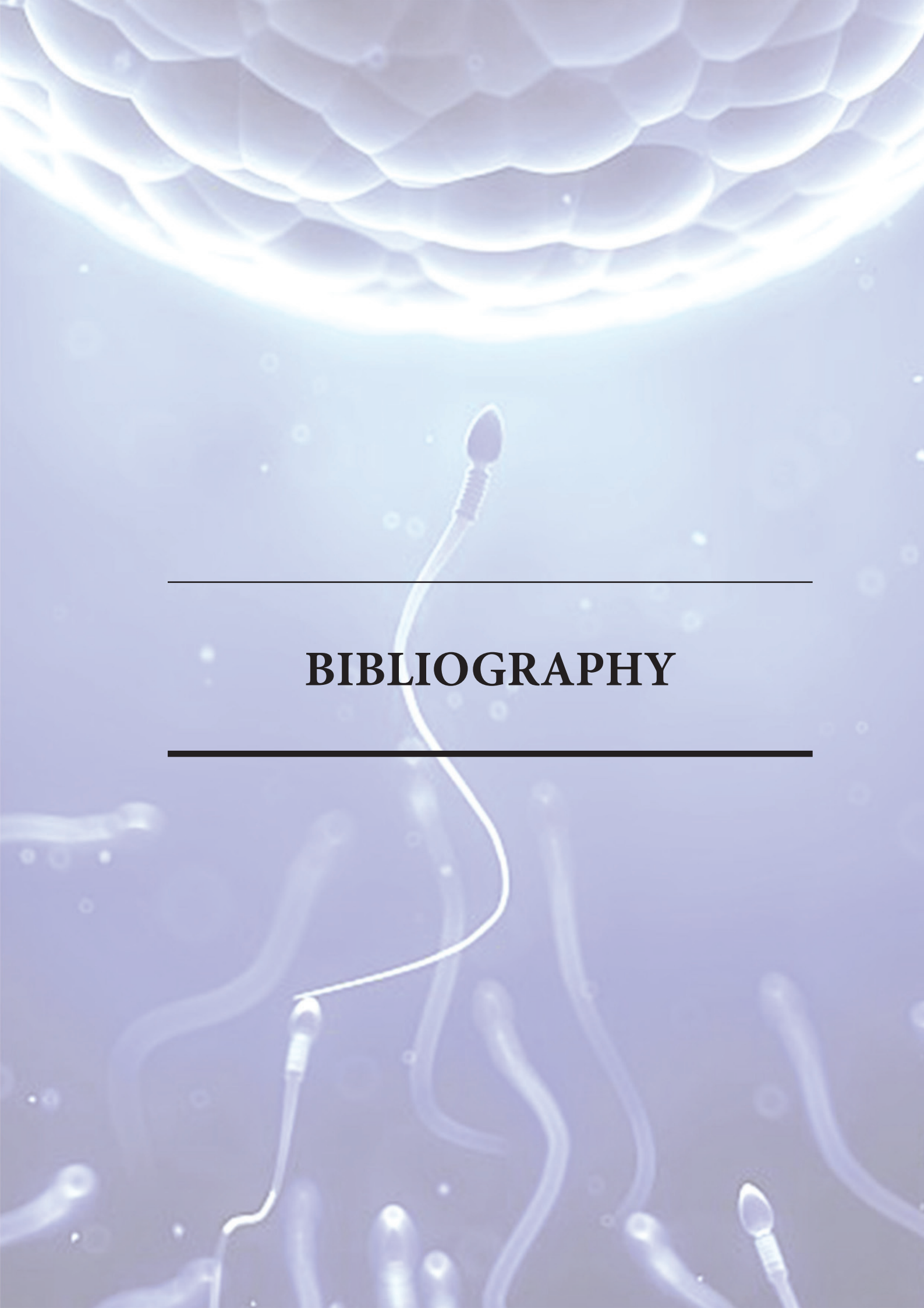
3. Convincing participants was difficult as they were interested only in results and achieving off springs

8.5 WORK THAT CAN BE DONE FURTHER

We have to enhance the same type of work especially in India, as in developed country they have already worked on sub deletion of AZF regions.

We can design a microchip device with incorporated STS for Detection of microdeletion in infertile male.

If we can design medication specifically related to deleted region after studying RNA, Proteins, Enzymes missing in these subjects, will be very useful clinically for treatment of male infertility with Y chromosome microdeletions.



BIBLIOGRAPHY

1. Infertility : An Overview. A Guide for Patients. American Society for Reproductive Medicine. https://www.asrm.org. Revised 2017.

2. Inhorn MC, Patrizio P. Infertility around the globe : new thinking on gender, reproductive technologies and global movements in the 21st century. Human Reproductive Update. 2015; 21 (4),411–426.

3. More R, Borate S, Gangane SD. Cytogenetic study in couples with primary Infertility. Journal of Medical Science Clinical Research. 2016;4(1),8941–8944.

4. Case AM. Infertility evaluation and management. Strategies for family physicians. Canadian Family Physician. 2003; 49,1465–1472.

5. Foresta C, Ferlin A, Gianaroli L, Dallapiccola B. Guidelines for the appropriate use of genetic tests in infertile couples. European Journal of Human Genetics.2002; 10 (5), 303–312.

6. Kamali M, Baghestani AR, Kashfi F, Kashani H, Tavajohi S, Amirchagmaghi E. A survey on infertility in Royan Institute. Iranian Journal of Fertility and Sterility. 2007; 1(1):23–26.

7. Poongothai J, Gopenath TS, Manonayaki S. Genetics of human male infertility. Singapore Medical Journal. 2009; 50: 336-347.

8. Laboratory manual for the examination and processing of human semen. 5thed. Geneva : World Health Organization; 2010.

9. Agarwal A, Mulgund A, Hamada A, Chyatte MR. A unique view on male infertility around the globe. Reproductive Biology Endocrinology. 2015;13:37–46.

10. Hotaling J, Carrell DT. Clinical genetic testing for male factor infertility : current applications and future directions. Andrology. 2014;2:339–50.

11. Colaco S, Lakdawala A, Modi D. Role of Y chromosome microdeletions in the clinical evaluation of infertile males. MGM Journal of Medical Science. 2017; 4:79–88.

12. Mierla D, Jardan D, Stoian V. Chromosomal abnormality in men with impaired spermatogenesis. International Journal of Fertility Sterility. 2014;8(1):35–42.

13. Jaganathan S, Smita BK, Kamala S, Muthiah SS, Geetha H, Chandra RS. Chromosomal Abnormalities in Infertile Men from Southern India. Journal of Clinical Diagnosis and Research. 2015;9(7):GC05-GC10.

14. Jarow J, Kolettis PN, Lipshultz LR, Mcclure RD, Nangia AK. The optimal evaluation of the infertile male: American Urology Association best practice statement. American Urology Association Education and Research, Inc. 2010.

15. Değirmencia B, Solakb M, Yildizb SH, Erdoganc MO, Elmasb M, Fistikb T. Evaluation of cytogenetic and Y chromosome microdeletion analyzes in Infertile cases. Meta Gene. 2019;19:78–81

16. Costa M, Cavani S, Pisaturo V. Routine karyotyping in infertile couples: is it really mandatory? Proposal from experience on 7,196 infertile Italian couples. Italian Journal of Gynecology Obstetrics.2017; 29 (1):13-18

17. LuC, Jiang J, Zhang R, Wang Y, XuM, Qin Y, Lin Y, Guo X, Ni B, Zhao Y, Diao N. Gene copy number alterations in the azoospermia-associated AZFc region

and their effect on spermatogenic impairment. Molecular Human Reproduction. 2014; 20:836–43.

18. Zhang F, Lu C, Li Z, Xie P, Xia Y, Zhu X, Wu B, Cai X, Wang X, Qian J,Wang X. Partial deletions are associated with an increased risk of complete deletion in AZFc: a new insight into the role of partial AZFc deletions in male infertility. Journal of Medical Genetics. 2007; 44:437–44.

19. Krausz C, Escamilla AR, Chianese C. Genetics of male infertility: from research to clinic. Reproduction. 2015; 150:R159–74.

20. Lopes AM, Aston KI, Thompson E, Carvalho F, Gonçalves J, Huang N, Matthiesen R, Noordam MJ, Quintela I, Ramu A, Seabra C. Human spermatogenic failure purges deleterious mutation load from the autosomes and both sex chromosomes, including the gene DMRT1. PLOS Genetics. 2013; 9(3):e1003349

21. Suganthi R, Vijesh V, Jayachandran S, Fathima Benazir JA. Multiplex PCR based screening for microdeletions in azoospermia factor region of Y chromosome in azoospermic and severe oligozoospermic south Indian men. Iran Journal of Reproductive Medicine. 2013;11(3):219-226.

22. Karkanaki A, Praras N, Katsikis I, Kita M, Panidis D. Is the Y chromosome all that is required for sex determination? Hippokratia. 2007;11(3):120–123.

23. Potter SJ, DeFalco T. Role of the testis interstitial compartment in spermatogonial stem cell function. HHS Public Access Author manuscript Reproduction. 2017 April; 153(4):R151–R162.

24. Witstuba J, Stukenborg J.B., Luetjens C.M. Mammalian spermatogenesis. Functional Development Embryology. 2007;1(2):99−117.

25. Bryan Principles of anatomy & physiology. Gerard J. Tortora, Bryan Derrickson. Hoboken, NJ Wiley, 13th ed.2012.

26. Schlatt S, Ehmcke J. Regulation of spermatogenesis : An evolutionary biologist's perspective. Sem Cell Development Biology. 2014;29:2−16.

27. Hexx RA, de Franca LR. Spermatogenesis and cycle of the seminiferous epithelium. Molecular mechanism in spermatogenesis. New York : Springer. 2008; p.1−15.

28. Sharpe RM, McKinnell C, Kivlin C, Fisher JS. Proliferation and functional maturation of Sertoli cells, and their relevance to disorders of testis function in adulthood. Reproduction. 2003; 125:769−784.

29. Principles of anatomy and physiology. Tortora, Gerard J, Grabowski, Sandra Reynolds, Sanner, Martha DePecol, Lancraft, Thomas M, Tortora, Gerard J . Wiley, New York; Chichester, 9th ed. 2000.

30. Holstein A, Schulze W, Davidoff M. Understanding spermatogenesis is a prerequisite for treatment. Reproductive Biology and Endocrinology. 2003;1(107).

31. Molecular Cell Biology. Section 19.4, Cilia and Flagella : Structure and Movement. New York : W. H. Freeman; 4th ed. 2000.

32. Molecular Biology of the Cell. Alberts B, Johnson A, Lewis J, et al. New York :

Garland Science; 4th ed. 2002.

33. Gudeloglu A, Parekattil SJ. Update in the evaluation of the azoospermic male. Clinics (Sao Paulo). 2013; 68 Suppl1:27-34.

34. Sussman EM, Chudnovsky A, Niederberger CS. Hormonal evaluation of the infertile male: has it evolved? Urological Clinical North America. 2008; 35(2):147, vii-55.

35. Hughes JF, Page DC. The biology and evolution of mammalian Y chromosomes. Annual Revision of Genetics. 2015;49:507–27.

36. Navarro-Costa P, Gonçalves J, Plancha CE. The AZFc region of the Y chromosome: at the crossroads between genetic diversity and male infertility. Human Reproductive Update. 2010;18:525–42.

37. Bellott DW, Hughes JF, Skaletsky H, Brown LG, Pyntikova T, Cho TJ, Koutseva N, Zaghlul S, Graves T, Rock S, Kremitzki C. Mammalian Y chromosomes retain widely expressed dosage-sensitive regulators. Nature. 2014;508:494–9.

38. Colaco S, Modi D. Genetics of the human Y chromosome and its association with male infertility. Reproductive Biology and Endocrinology. 2018;16(1):14.

39. Lorente-Galdos B,Lao O, Serra-Vidal G, et. al. Whole-genome sequence analysis of a Pan African set of samples reveals archaic gene flow from an extinct basal population of modern humans into sub-Saharan populations. Genome Biology. 2019; 20(1):77. 1684-5.

40. Vogt PH. AZF deletions and Y chromosomal haplogroups: history and update based on sequence. Human Reproduction Update. 2005; 11(4):319–336

41. Georgiou I, Syrrou M, Pardalidis N, Karakitsios K. Genetic and epigenetic risks of intracytoplasmic sperm injection method. Asian Journal of Andrology. 2006; 8: 643- 673.

42. Willard HF. Tales of the Y chromosome. Nature. 2003;423(6942):810-13.

43. Cocuzza M, Alvarenga C, Pagani R. The epidemiology and etiology of azoospermia. Clinics (Sao Paulo). 2013; 68(1):15–26.

44. Foresta C, Moro E, Ferlin A. Y chromosome microdeletions and alterations of spermatogenesis. Endocrinology Revision. 2001;22:226–39.

45. Skaletsky H, Kuroda-Kawaguchi T, Minx PJ, Cordum HS, Hillier L, Brown LG, Repping S, Pyntikova T, Ali J, Bieri T. The male-specific region of the human Y chromosome is a mosaic of discrete sequence classes. Nature. 2003;423:825–37.

46. Li G, Davis BW, Raudsepp T. Comparative analysis of mammalian Y chromosomes illuminates ancestral structure and lineage-specific evolution. Genome Res. 2013; 23(9):1486–1495.

47. Kuroda-Kawaguchi T, Skaletsky H, Brown LG, Minx PJ, Cordum HS, Waterston RH, Wilson RK, Silber S, Oates R, Rozen S, Page DC. The AZFc region of the Y chromosome features massive palindromes and uniform recurrent deletions in infertile men. National Genetics. 2001;29:279–86.

48. Cerrone M, Cantile M, Collina F. Molecular strategies for detecting chromosomal translocations in soft tissue tumors (review). International Journal of Molecular Medicine. 2014;33(6):1379–1391.

49. Bachtrog D, Charlesworth B. Towards a complete sequence of the human Y chromosome. Genome Biology. 2001;2(5):1016.

50. Vog PH, Edelmann A, Kirsch S, Henegariu O, Hirschmann P, Kiesewetter F, Köhn FM, Schill WB, Farah S, Ramos C, Hartmann M. Human Y chromosome azoospermia factors (AZF) mapped to different sub regions in Yq11. Human Molecular Genetics. 1996;5:933–43.

51. Reijo R, Lee TY, Salo P, Alagappan R, Brown LG, Rosenberg M, Rozen S, Jaffe T, Straus D, Hovatta O, Dela Chapelle A. Diverse spermatogenic defects in humans caused by Y chromosome deletions encompass in a novel RNA–binding protein gene. National Genetics. 1995;10:3383–93.

52. Yu XW, Wei ZT, Jiang YT, Zhang SL. Y chromosome azoospermia factor region microdeletions and transmission characteristics in azoospermic and severe oligozoospermic patients. International Journal of Clinical Expert Medicine. 2015; 8(9):14634–14646.

53. Krausz C, Hoefsloot L, Simoni M, Tuttelmann F. EAA/EMQN best practice guidelines for molecular diagnosis of Y-chromosomal microdeletions : state-of-the-art. Andrology. 2014;2:5–19.

54. Elfateh F, Rulin D, Xin Y, Linlin L, Haibo Z, Liu RZ. Prevalence and patterns of Y chromosome microdeletion in infertile men with azoospermia and

oligozoospermia in Northeast China. Iran Journal of Reproductive Medicine. 2014; 12(6):383–388.

55. Boivin J, Bunting L, Collins JA, Nygren K. International estimates of infertility prevalence and treatment-seeking : potential need and demand for infertility medical care. Human Reproduction. 2007; 22(6): 1506–1512.

56. Samli H, Samli MM, Solak M, Imirzalioglu N. Genetic anomalies detected in patients with non-obstructive azoospermia and oligozoospermia. Archives of Andrology. 2006; 52:4,263-267.

57. Statistics by country for male infertility. US census bureau population estimates and US census bureau, international database in 2004.

58. National, Regional, and Global Trends in Infertility Prevalence since 1990 : A Systematic Analysis of 277 Health Surveys. Mascarenhas M, Flaxman S, Boerma T, Vanderpol S, Stevens G. Public Library of Science Medicine. 2012.

59. Esteves S, Agarwal A. The azoospermic male : current knowledge and future perspectives 2013. Clinics (Sao Paolo). 2013; 68(1):1-4.

60. Agarwal A, Mulgund A, Hamada A, Chyatte M. A unique view on male infertility around the globe. Reproductive Biology and Endocrinology. 2015;13(37).

61. Gianotten J, Westerveld GH, Leschot NJ, Tanck MWT, Lilford RJ, Lombardi MP, Vander Veen F. Familial clustering of impaired spermatogenesis : no evidence for a common genetic inheritance pattern. Human Reproduction.2004; 9(1):71-76.

62. Dada R, Kumar R, Shamsi MB, Tanwar M, Pathak D, Venkatesh Kumar M, Singh H, Singh K, Aron M, Kumar R, Singh G, Sharma RK and Gupta NP . Genetic screening in couples experiencing recurrent assisted procreation failure. Indian Journal of Biochemical Biophysics. 2008;45:116-120.

63. Agarwal A, Varghese AC and Sharma RK. Markers of oxidative stress and sperm chromatin integrity. Methods Molecular Biology. 2009;590:377-402.

64. Adiga SK, Jayaraman V, Kalthur G, Upadhya D, Kumar P. Declining semen quality among south Indian infertile men : A retrospective study. Journal of human reproductive sciences. 2008; 1(1):15.

65. Feki NC, Abid N, Rebai A, Sellami A, Ayed BB, Guermazi M, Bahloul A, Rebai T, Ammar LK. Semen quality decline among men in infertile relationships : experience over 12 years in the South of Tunisia. Journal of Andrology. 2009; 30:541-547.

66. Mishra P, Negi MPS, Srivastava M, Singh K, Rajender S. Decline in seminal quality in Indian men over the last 37 years. Reproductive Biology and Endocrinology. 2018; 16(1):103.

67. John AR, Mark BA. Oxidative stress and male reproductive biology. Reproduction, Fertility and Development. 2004;16:581-588.

68. Sha YW, Ding L, Li P. Management of primary ciliary dyskinesia/Kartagener's syndrome in infertile male patients and current progress in defining the underlying genetic mechanism. Asian Journal of Andrology. 2013;16(1):101–106.

69. Flynn K, Varghese A, Agarwal A. The Genetic Causes of Male Factor Infertility : A review. Fertility and Sterility. 2010; 93:1-12.

70. Storm DW, Hogan MJ, Jayanthi VR. Initial experience with percutaneous selective embolization : a truly minimally invasive treatment of the adolescent varicocele with no risk of hydrocele formation. Journal of Pediatric Urology. 2010; 6: 567-571.

71. Ferlin A, Zuccarello D, Zuccarello B, Chirico MR, Zanon GF. Foresta C. Genetic alterations associated with cryptorchidism. JAMA.2008; 19:2271-2276.

72. Daniel Justa DG, Granberg CF, Villanueva C, Baker LA. Contemporary Review of Testicular Torsion: New Concepts, Emerging Technologies and Potential Therapeutics. Journal of Pediatric Urology. 2013 December; 9(6):12.

73. Sigman M, Jarow JP. Male Infertility. In : Wein AJ, ed. Campbell-Walsh Urology. 9th ed. Philadelphia, Pa: Sanders Elsevier. 2007; 609–653.

74. Little C. Testicular torsion. Nursing. 2010; 30-33.

75. Nordlander E. Diethylstilbestrol in Mumps Orchitis. Acta Obstetricia et Gynecologica Scandinavia. 2011; 38:586-589

76. Brugh V, Lipshutz. Male factor infertility : evaluation and management. Medicine Clinics of North America. 2004; 88:367-85.

77. Endocrinology of the Male Reproductive System and Spermatogenesis. O'Donnell L, Stanton P, de Kretser DM. Feingold KR, Anawalt B, Boyce A,

et. al., editors. https://www.ncbi.nlm.nih.gov/books/NBK279031 Endotext : Updated 2017. South Dartmouth (MA): MDText.com,Inc.;2000

78. Zeqiraj A, Gashi Z, Elezaj S, Berisha S, Shabani A. Determination of Infertility in Infertile Men in the Dukagjin Region in Republic of Kosovo. Internal Medicine. 2017; 7: 244.

79. Sussman EM, Chudnovsky A, Niederberger CS. Hormonal evaluation of the infertile male : has it evolved? Urology Clinics of North America. 2008;35(2):147, vii-55.

80. European Association of Urology Guidelines on Male Infertility. Jungwirth A, Diemer T, Dohle GR, Giwercman A, Kopa Z, Krausz C. European Association of Urology; 2018.

81. Hwang K, Yatsenko AN, Jorgez CJ, et al. Mendelian genetics of male infertility. Ann N Y Academy Science. 2010;1214:E1–E17.

82. Speiser PW, Azziz R, Baskin LS, et. al. Congenital adrenal hyperplasia due to steroid 21-hydroxylase deficiency : an Endocrine Society clinical practice guideline. Journal of Clinical Endocrinology Metabolism. 2010; 95(9):4133–4160.

83. Whirledge S, Cidlowski JA. Glucocorticoids, stress, and fertility. Minerva Endocrinol. 2010; 35(2):109–125.

84. Fitzgerald P, Dinan T G. Prolactin and dopamine: What is the connection? A Review Article. Journal of Psychopharmacology. 2008; 22(2):12–19.

85. Harton GL, Tempest HG. Chromosomal disorders and male infertility. Asian Journal of Andrology. 2011;14(1):32–39.

86. Lin XZ, Tang YG, Zhing LX. Chromosomal abnormality and Y chromosome microdeletions in patients with azoospermia. Zhong Yi Xwe Y Chuan Xuei. 2010; 27: 460- 462.

87. Repping S, Skaletsky H, Lange J, Silber S, van der Veen F, Oates RD, Page DC, Rozen S. Recombination between palindromes P5 and P1 on the human Y chromosome causes massive deletions and spermatogenic failure. Am J Hum Genetics. 2002;71:906–22.

88. Sen S, Pasi AR, Dada R, Shamsi MB, Modi D. Y chromosome microdeletions in infertile men: prevalence, phenotypes and screening markers for the Indian population. Journal of Assisted Reproductive Genetics. 2013;30:413–22.

89. Simoni M, Bakker E, Krausz C. EAA/EMQN best practice guidelines for molecular diagnosis of Y-chromosomal microdeletions. International Journal of Andrology. 2004;27:240–9.

90. Chang PL, Sauer MV, Brown S. Y chromosome microdeletion in a father and his four infertile sons. Human Reproduction. 1999;14:2689–94.

91. Gatta V, Stuppia L, Calabrese G, Morizio E, Guanciali-Franchi P, Palka G. A new case of Yq microdeletion transmitted from a normal father to two infertile sons. Journal of Medical Genetics. 2002; 39:e27

92. Hinch AG, Altemose N, Noor N, Donnelly P, Myers SR. Recombination in the human pseudoautosomal region PAR1. PLOS Genet. 2014;10:e1004503.

93. Massart A, Lissens W, Tournaye H, and Stouffs K. Genetic causes of spermatogenic failure. Asian Journal of Andrology. 2012;14:40–8.

94. Simoni M, Tuttelman F, Gromoll J, Nieschalg E. Clinical consequences of microdeletions of the Y chromosome : the extended Munster experience. Reproductive Biomedical Online. 2008;16:289–303.

95. Preparation and Analysis of Eukaryotic Genomic DNA. Sambrook J. and Russell DW. In Molecular Cloning a Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York. 3rd ed.2001.

96. Nistal M, Paniagua R. Testicular biopsy. Contemporary interpretation. Urology Clinics of North America. 1999;26:555-93

97. Vasan SS. Semen analysis and sperm function tests: How much to test? Indian Journal of Urology. 2011;27(1):41–48.

98. Patil PS, Humbarwadi RS, Patil AD, Gune AR. Immature germ cells in semen - correlation with total sperm count and sperm motility. Journal of Cytology. 2013; 30:185-9.

99. Jassim A, Festenstein H. Immunological and morphological characterization of nucleated cells other than sperm in semen of Oligospermic donors. Journal of Reproductive Immunology. 1987;11:77-89.

100. Clinical atlas of sperm morphology. Phadke AM. New Delhi : Jaypee Brothers; 1st ed. 2007; section 2: pg.43.

101. Gune AR, Patil AD, Patil RS. Correlation of Immature Germ Cells in Semen in Severe Oligospermic and Azoospermic Males. International Journal of Scientific Research and Reviews. 2019; 8(2):2329-2333.

102. Babu S, Sadhnani M, Swarna M, Padmavathi P, Reddy P. Evaluation of FSH, LH and testosterone levels in different subgroups of infertile males. Indian journal of clinical biochemistry. 2014; 19:45-9.

103. Gune AR, Patil AD, Gune RP, Patil RS. Histopathological Patterns of Testicular Biopsies in Azoospermic Infertile Males. International Journal of Medical Health Science Research. 2019;9(5):22-27

104. Gandini L, Lenzi A, Lombardo F, Pacifici R, Dondero F. Immature germ cell separation using a modified discontinuous Percoll gradient technique in human semen. Human Reproduction. 1999;14:1022-7.

105. Ariagno J, Curi S, Mendeluk G, Grinspon D, Repetto H, Chenio P. Shedding of immature germ cells. Archives of Andrology. 2002;48:127-31.

106. Kahraman S, Polat G, Samli M. Multiple pregnancies obtained by testicular spermatid injection in combination with intracytoplasmic sperm injection. Human Reproduction. 1998;13:104-110.

107. Lunenfeld B, Steirteghem VA. Infertility in the third millennium : Implications for the individual, family and society: Condensed meeting report from the Bertarelli foundation's second global conference. Human Reproduction Update. 2004; 10:317-26.

108. Saradha B, Mathur PP. Effect of environmental contaminants on male reproduction. Env Tox Pharma 2006;21:34-41.

109. Levin HS. Testicular biopsy in the study of male infertility : Its current use fullness, histologic techniques, and prospects for the future. Human Pathology. 1979; 10:569-84.

110. Meinhard E, Raecu MC, and Chisholm GD : Testicular Biopsy in Evaluation of Male Infertility British Medical journal, 1973, 3,577-581.

111. Nagpal BL, Manjari M, Kapoor K, Dhaliwal US. Testicular biopsy in cases of male infertility : A retrospective study. Journal of Indian Medical Association. 1993; 91:171-4.

112. Haddad FH, Omari AA, Malkawi OM, et. al; Patterns of testicular cytology in men with primary infertility : any change since the Gulf War Acta Cytology. 2004 Nov-Dec;48(6):807-12.

113. Brannen GE and Roth RR. Testicular abnormalities of the subfertile male. Journal of Urology. 1979; 122:757.

114. Rashed MM, Ragab NM, Shalaby AR, Ragab WK. Patterns of testicular histopathology in men with primary infertility. The Internet Journal of Urology. 2008; 2(5):1-4.

115. Glina S, Soares J, Nelson J. Testicular histopathological diagnosis as a predictive factor for retrieving spermatozoa for ICSI in non-obstructive azoospermic patients. International Brazil Journal of Urology. 2005;31(4):338-41

116. Al-Rayess MM, Al-Rikabi AC. Morphologic patterns of male infertility in Saudi patients A University Hospital experience. Saudi Medical Journal. 2000; Vol. 21 (7): 625-628.

117. Thomas JO. Histological pattern of testicular biopsies in infertile males in Ibadan, Nigeria. East African Medical Journal. 1990; 67:578-584.

118. Skakkebaek NE; Giwercman A; de Kretser D: Pathogenesis and management of male infertility. Lancet. 1994; 3433:1473.

119. Sigman M, Jarow JP. Endocrine evaluation of infertile men. Urology. 1997; 50:659–664

120. Bourcigaux N, Christin-Maître S. Hormonal evaluation in infertile men. Gynecology Obstetric Fertility. 2008; 36: 551– 556.

121. Bablok L, Janczewski Z, Kwiatkowska Z, Fracki S. The relation-ship between plasma FSH, testosterone levels and testicular histology in males with azoospermia. Andrologia 1978;10:502–505.

122. Trussell JC, Coward RW, Santoro N, Stetter C, Kunselman A, Diamond MP. Reproductive Medicine Network. Association between testosterone, semen parameters and live birth in men with unexplained infertility in an intrauterine insemination population. Fertility Sterility 2019; 111:1129–34.

123. Subhan F, Tahir F, Ahmad R, Khan, Z. Oligospermia and its relation with hormonal profile. Journal of Pakistan Medical Association. 1995; 45:246-247.

124. Sina D, Schuhmann R, Abraham R, Taubert HD, Dericks-Tan JSE. Increased serum FSH levels correlated with low and high sperm counts in male infertile patients. Andrologia. 1975; 7(1):31-37.

125. Jensen TK, Andersson AM, Hjollund NHI, Scheike T, Kolstad H, Giwercman A, McNeilly A. Inhibin B as a serum marker of spermatogenesis: correlation to differences in sperm concentration and follicle-stimulating hormone levels. A study of 349 Danish men. The Journal of Clinical Endocrinology & Metabolism. 1997; 82(12): 4059-4063.

126. Mahmoud AM, Comhaire FH, Depuydt CE. The clinical and biologic significance of serum inhibins in subfertile men. Reproductive Toxicology. 1998; 12(6): 591- 599.

127. Pierik F, Stijnen T, de Jong F, Weber R, Vreeburg J. Serum inhibin B as a marker of spermatogenesis. Journal of clinical endocrinology and metabolism. 1998; 83 (9):3110-3114.

128. Mabeck LM, Jensen MS, Toft G, et al. Fecundability according to male serum inhibin B a prospective study among first pregnancy planners. Human Reproduction. 2005; 20:2909-2915.

129. Uhler ML, Zinaman MJ, Brown CC, Clegg ED. Relationship between sperm characteristics and hormonal parameters in normal couples. Fertility Sterility. 2003; 79:1535-1542.

130. Zhu YJ, Liu SY, Wang H, Wei P, Ding XP. The prevalence of azoospermia factor microdeletion on the Y chromosome of Chinese infertile men detected by

multi-analyte suspension array technology. Asian Journal of Andrology. 2008; 10(6):873-81.

131. Ferlin A, Arredi B, Speltra E, Cazzadore C, Selice R, Garolla A, Lenzi A, Foresta C. Molecular and clinical characterization of Y chromosome microdeletions in infertile men : a 10-year experience in Italy. Journal of Clinical Endocrinology and Metabolism. 2007;92(3):762-70.

132. Vander VK, Montag M, Peschka B, Leygraaf J, Schwanitz G, Haidl G, Krebs D, van der Van H. Combined cytogenetic and Y-chromosome microdeletion screening in males undergoing intracytoplasmic sperm injection. Molecular Human Reproduction. 1997;3:699–704

133. Foresta, C., Ferlin, A., Garolla, A., Moro, E., Pistorello, M., Barbaux, S., & Rossato, M. High frequency of well-defined Y-chromosome deletions in idiopathic Sertoli cell-only syndrome. Human reproduction. 1998; 13(2):302-307.

134. Pandey LK, Pandey S, Gupta J, Saxena AK. Loss of the AZFc region due to a human Y-chromosome microdeletion in infertile male patients. Genetics Molecular Research. 2010; 9(2):1267-1273.

135. Mascarenhas M, Thomas S, Kamath MS, Ramalingam R, Kongari AM, Yuvarani S, Srivastava VM, George K. Prevalence of chromosomal abnormalities 12 and Y chromosome microdeletion among men with severe semen abnormalities and its correlation with successful sperm retrieval. Journal of Human Reproductive Science. 2016;9(3):187-193.

136. Silber SJ, Nagy Z, Devroey P, Tournaye H, Van Steirteghem AC. Distribution of spermatogenesis in the testicles of azoospermic men : the presence or absence of spermatids in the testes of men with germinal failure. Human Reproduction. 1997;12(11):2422–28

137. Machev N, Saut N, Longepied G, Terriou P, Navarro A, Lévy N, Guichaoua M, Metzler-Guillemain C, Collignon P, Frances AM, Belougne J. Sequence family variant loss from the AZFc interval of the human Y chromosome, but not gene copy loss, is strongly associated with male infertility. Journal of Medical Genetics. 2004 1;41(11):814-25

138. Kim SY, Kim HJ, Lee BY, Park SY, Lee HS, Seo JT. Y chromosome microdeletions in infertile men with non-obstructive azoospermia and severe oligozoospermia. Journal of Reproductive Infertility.2017:18(3):307.

139. Repping S, Skaletsky H, Brown L, van Daalen SK, Korver CM, Pyntikova T, Kuroda-Kawaguchi T, de Vries JW, Oates RD, Silber S, van der Veen F. Polymorphism for a 1.6-Mb deletion of the human Y chromosome persists through balance between recurrent mutation and haploid selection. National Genetics. 2003;35(3):247.

140. Abobakr RAM, Mostafa RM, Mahmoud SH, Abdallah HY, Ibrahim GH. Detection of azoospermia factor (AZF) microdeletion on Y chromosome in infertile men with azoospermia or severe oligozoospermia. Egypt Journal of Derm & Andrology. 2009; 29:65-72.

141. Sheikh M, Nower S, Rasol H. Prevalence of Y chromosome microdeletions in

males with azoospermia and severe oligospermia in Egypt. Kasr Aini Med J. 2009; 12:47–50.

142. Abid S, Maitra A, Meherji P, Patel Z, Kadam S, Shah J, Shah R, Kulkarni V, Baburao V, Gokral J. Clinical and laboratory evaluation of idiopathic male Infertility in a secondary referral center in India. Journal of Clinical Laboratory Analysis. 2008; 22(1):29-38.

143. Park SH, Lee HS, Choe JH, Lee JS, Seo JT. Success rate of microsurgical multiple testicular sperm extraction and sperm presence in the ejaculate in Korean men with Y chromosome microdeletions. Korean Journal of Urology. 2013;54(8):536–40.

144. Singh K, Raman R. Male infertility : Y-chromosome deletion and testicular aetiology in cases of azoo-/Oligospermia. Indian Journal of Experimental Biology. 2005:43(11),1088-92

ANNEXURE - I

SHIVAJI UNIVERSITY, KOLHAPUR
PLAGIARISM CHECK REPORT NO. 1/2/3/4

1.	Name of the Research Scholar	DR. ANITA RAHUL GUNE			
2.	Faculty and Subject	PH.D ANATOMY			
3.	Title of the Thesis/ Dissertation	Detection of Azoospermic Factors(AZF) Microdeletions in Azoospermic and Severe Oligospermic Males.'			
4.	Name of the Research Guide	DR. ASHALATA DEEPAK PATIL			
5.	Name of the Co- Guide	—			
6.	Department / Institution/ College	DEPARTMENT OF ANATOMY, D.Y. PATIL MEDICAL COLLEGE, KOLHAPUR.			
7.	Similarity Content (%) (up to 30% acceptable)	1st	2nd	3rd	4th
		2%	%	%	%
8.	Plagiarism detection tool applied	Thenticate			
9.	Date of plagiarism check	10-11-2020			

2 Yr. As Per Revised UGC Notification

Checked by

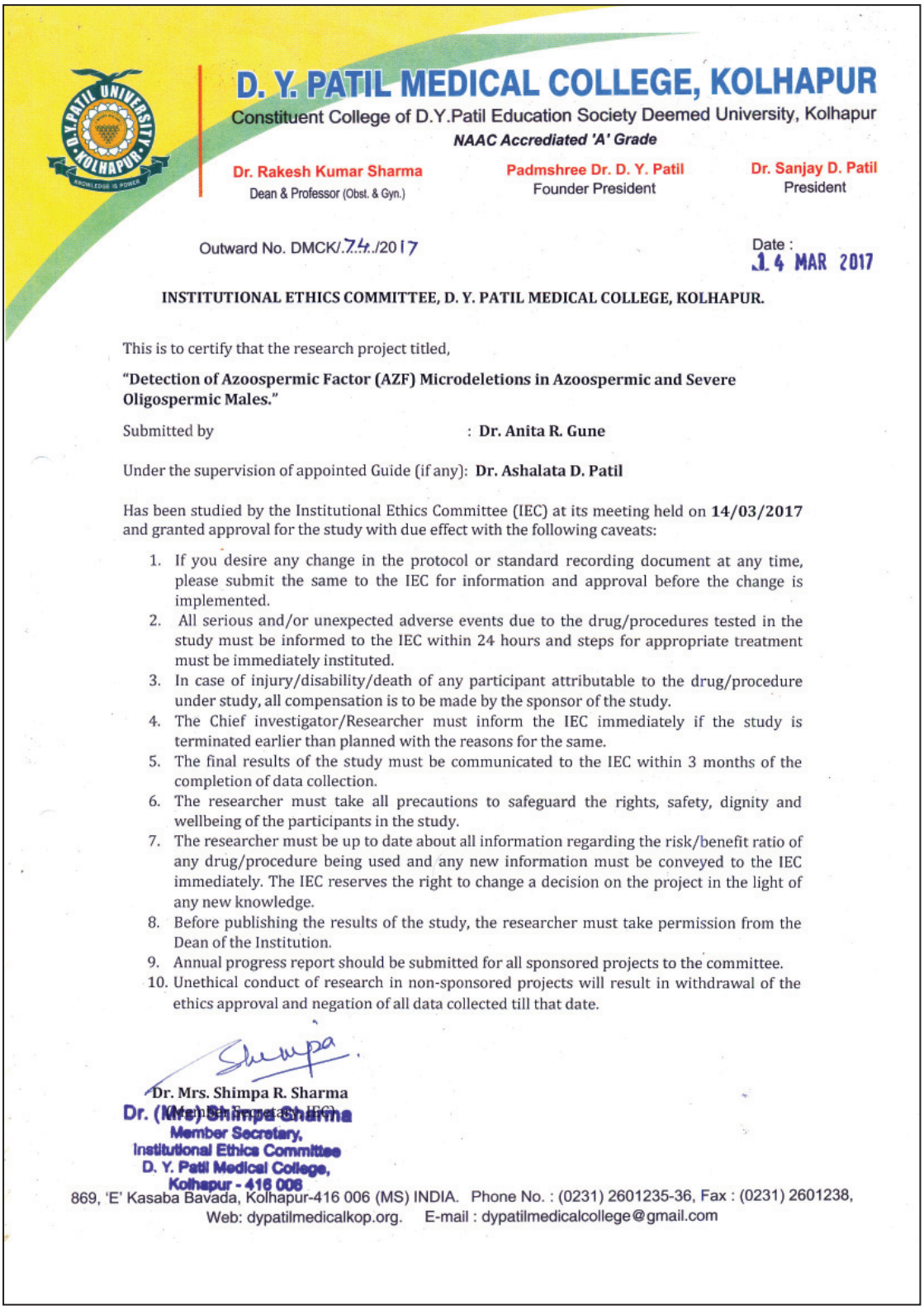
Name: Dr. J. B. Yadav

Designation: Scientific Officer

Signature: USIC Shivaji University, Kolhapur

Date:- 10-11-2020

Ethical Committee Clearance Certificate



D. Y. PATIL MEDICAL COLLEGE, KOLHAPUR

Constituent College of D.Y.Patil Education Society Deemed University, Kolhapur

NAAC Accrediated 'A' Grade

Dr. Rakesh Kumar Sharma
Dean & Professor (Obst. & Gyn.)

Padmshree Dr. D. Y. Patil
Founder President

Dr. Sanjay D. Patil
President

Outward No. DMCK/74/2017

Date : 14 MAR 2017

INSTITUTIONAL ETHICS COMMITTEE, D. Y. PATIL MEDICAL COLLEGE, KOLHAPUR.

This is to certify that the research project titled,

"Detection of Azoospermic Factor (AZF) Microdeletions in Azoospermic and Severe Oligospermic Males."

Submitted by : **Dr. Anita R. Gune**

Under the supervision of appointed Guide (if any): **Dr. Ashalata D. Patil**

Has been studied by the Institutional Ethics Committee (IEC) at its meeting held on **14/03/2017** and granted approval for the study with due effect with the following caveats:

1. If you desire any change in the protocol or standard recording document at any time, please submit the same to the IEC for information and approval before the change is implemented.
2. All serious and/or unexpected adverse events due to the drug/procedures tested in the study must be informed to the IEC within 24 hours and steps for appropriate treatment must be immediately instituted.
3. In case of injury/disability/death of any participant attributable to the drug/procedure under study, all compensation is to be made by the sponsor of the study.
4. The Chief investigator/Researcher must inform the IEC immediately if the study is terminated earlier than planned with the reasons for the same.
5. The final results of the study must be communicated to the IEC within 3 months of the completion of data collection.
6. The researcher must take all precautions to safeguard the rights, safety, dignity and wellbeing of the participants in the study.
7. The researcher must be up to date about all information regarding the risk/benefit ratio of any drug/procedure being used and any new information must be conveyed to the IEC immediately. The IEC reserves the right to change a decision on the project in the light of any new knowledge.
8. Before publishing the results of the study, the researcher must take permission from the Dean of the Institution.
9. Annual progress report should be submitted for all sponsored projects to the committee.
10. Unethical conduct of research in non-sponsored projects will result in withdrawal of the ethics approval and negation of all data collected till that date.

Dr. Mrs. Shimpa R. Sharma
(Member Secretary, IEC)

Dr. (Mrs) Shimpa Sharma
Member Secretary,
Institutional Ethics Committee
D. Y. Patil Medical College,
Kolhapur - 416 006

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Result of Pre-Ph.D. exam

D.Y. PATIL UNIVERSITY 6966

D.Y. PATIL EDUCATION SOCIETY, KOLHAPUR

(Deemed to be UNIVERSITY Declared u/s 3 of the UGC Act 1956 vide Notification No. F.9-26/2004-U.3 dt. 01-09-2005 of the GOI)

Accredited by NAAC with 'A' Grade

MAHARASHTRA STATE (INDIA)

Statement of Marks

Name Shri./Smt. : Gune Anita Rahul **Seat No. :** 3806

Mother's Name : Leela

PRN : 2015000247

EXAM. : Pre. Ph.D. July – 2017

College/Department : Centre for Interdisciplinary Research, Kolhapur.

Subject		Marks Max.	Marks Min.	Marks Obtained	Grade $
Paper - I	Research Methodology and Computer Basics	100	40	63	A
Paper - II	Recent Topics in Medical Science	100	40	61	A
Paper - III	Advances in Anatomy	100	40	82	O
	Total Marks/ Average Grade	**300**	**150**	**206**	**A**

Grand Total (In Words) :- Two hundred Six

Result :- Pass **Grade :-** 'A' **Percentage :-** 68.67 %

$ 40 – 50% = **C Grade,** 51 – 60% = **B Grade,**
61 – 75% = **A Grade,** and above 76% = **O Grade**
Minimum grade required for passing in each head of passing - **'C'**

AB:- Absent **FFF:-** Fail **P:-** Previous Pass

Controller of Examinations

Result declared on:- 28.08.2017 **Note:-** Correction Not Allowed

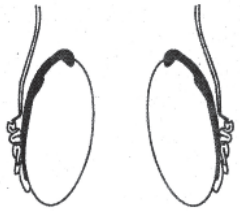
Case Record

ANDROLOGY

CASE RECORD

DR. RAHUL GUNE
F.C.P.S; M.N.A.M.S; D.N.B.(URO)

Ref. By Dr. Add.	Patient's Name : Patient's Address :		Reporting Date : Age : Sex : Phone No.
History	Wife/Developmental/Family History	Examination	INVESTIGATION
MARITAL DETAILS Duration Try Time	WIFE : Name : Age:	GENERAL EXAMINATION Built	URINE
Pregnancy	Medical History	Growth	
Sexual History		Hair	BLOOD
Libldo	Obs. History		C. B. C. :
Frequency	No. of Preg. Abortion	Pulse BP	Bl. Sugar (F) (PP)
Lubrication	Menstr. History		BUL S. Creat.
Penetration	Periodicity Leuco.	SYSTEMIC EXAMINATION	PREVIOUS SEMEN ANALYSIS
Orgasm	DEVELOPMENTAL HISTORY :		Date / Vol. / Count. / Moti. / Fru. / Treatment
Ejaculation		LOCAL EXAMINATION	
Deposition	FAMILY HISTORY :	R L	
PERSONAL HISTORY :		Hernia	
Occupation		TESTICLES	
Alcohol/Smoking/Tobacco		Volume	
Hot Bath/Sauna/Steam		Shape	SP. TEST
Underclothing		Consistency	Hormonal Assay
Emotional Stress		Epidi	FSH LH
MEDICAL HISTORY :			Test. Prol.
Mumps/T.B./Small Pox			TESTICULAR BIOPSY :
UTI/STD/Leprosy			
Epididymitis(Non/-Sp.)		Vas	RADIOLOGY :
Rec. Cold & Cough			
Thyroid/Renal Disease			DOPPLER STUDY :
Jaundice/HT/DM/IHD		Varicocele	
Major illness/Radiation			INVASIVE TEST :
Testicular : Trauma /		Penis :	
Torsion / Surg. / Swelling		Prostated Seminal Vesicles	
OTHER :			HIV :



PRELIMINARY IMPRESSION :

PLAN OF ACTION :

SEMEN ANALYSIS & FOLLOW-UP CHART :

DATE	VOL (ml)	COUNT Mill / ml.	MOTILITY iv	iii	ii	i	O	FRUC.	WBC	ONGOING TREATMENT	

Proforma

Name of Patient : _____

Age : _____ Address : _____

_____Phone No : mobile _____

History

Occupation:
Married : years
Infertility : years
Past relevant History :
Mumps / Hydrocele / Operative
Family History :

Investigations

Semen Analysis

- Semen volume (ml) – 1.4-1.7 (normal valve)
- Wet mount slide - motility : 38 – 42 % (normal valve)
- Sperm count : millions/ml – 12 -16 (normal valve)
  Semen staining with Leishman's stain
- round cells – 3-4 % (normal valve)
- Immature Germ Cells -

Hormone Assay

FSH
LH
Testosterone
Prolactin

Testicular Biopsy if Done

Blood for Micro deletion study:

3 ml in EDTA tube : Label and Date:

Consent form (Marathi)

रुग्ण संमती पत्र

डी. वाय. पाटील मेडीकल कॉलेज आणि
पद्मश्री डी. वाय. पाटील हॉस्पिटल व रिसर्च सेंटर, कोल्हापूर.

मी श्री./सौ./कु. _____

लिंग : _____ वय : _____ राहणार : _____

_____

या पत्राद्वारे खात्री देतो की,

१) मला डी. वाय. पाटील मेडीकल कॉलेजच्या वैद्यकीय डॉक्टर संशोधक यांच्याकडून विचारले गेले आहे की, मेडीकल कॉलेजच्या सहकार्याखाली संशोधन अभ्यासात माझी भाग घ्यायची इच्छा आहे का?

२) वैद्यकीय डॉक्टर संशोधक यांच्याकडून केल्या जाणाऱ्या संशोधन अभ्यासाचे स्वरूप व त्यामध्ये माझ्या सहभागाचा कालावधी याविषयी व्यवस्थितपणे मला समजणाऱ्या भाषेत सांगितले आहे.

३) संशोधन अभ्यासादरम्यान उद्‌भवणारे धोके आणि परिणाम व्यवस्थितपणे मला समजणाऱ्या भाषेत समजावून सांगितले आहेत.

४) मला हे सुद्धा माहिती आहे की, माझा अभ्यासातील सहभाग फक्त वैद्यकीय संशोधन क्षेत्राच्या प्रगतीकरिता फायदा होण्यासाठी आहे, ना की मेडीकल कॉलेज किंवा संशोधन कर्त्यांकडून पैशाच्या फायद्याकरिता.

५) मला याची पण कल्पना दिली आहे की, मी कोणत्याही स्थितीत सहभागासाठी बांधील नाही आणि एकदा मी अभ्यासात सहभागासाठी सहमती दिली तरी मी माझा अभ्यासातील सहभाग कोणत्याही वेळी विहीत नमुन्यात मेडीकल कॉलेजला लेखी अर्ज करून कोणतेही कारण नमूद न देता रद्द करू शकतो.

६) माझ्यामध्ये आणि संशोधनकर्ते किंवा डी. वाय. पाटील मेडीकल कॉलेज यांच्यात अभ्यासात सहभागासाठी कोणताही आर्थिक व्यवहार असणार नाही.

७) मला याची पण कल्पना दिली आहे, की माझ्या अभ्यासातील सहभागातून जी काही माहिती गोळा गेली जाईल त्याचा वापर फक्त शैक्षणिक हेतू करिता आणि किंवा पुढील वैद्यकीय संशोधनाकरिताच होईल.

८) मला याची पण खात्री दिली आहे की, अभ्यासाच्या काळात गोळा केलेल्या माहितीचे सार्वजनिक प्रसारण किंवा त्यांचा परिणामांचे सार्वजनिक प्रसारण नाव न जाहीर करता केले जाईल आणि कोणत्याही परिस्थितीत माझी स्वत:ची ओळख दाखवली जाणार नाही. कोणतीही वैयक्तिक माहिती माझी वैयक्तिक ओळख दाखविण्याची शक्यता असेल तर नेहमीच गुप्त राखली जाईल.

९) या संमती पत्रातील मजकूर आणि त्याचा परिणाम मला समजणाऱ्या भाषेत व्यवस्थित समजावून सांगितला आहे.

रुग्ण संमती पत्र

डी. वाय. पाटील मेडीकल कॉलेज इथे माझी सही किंवा अंगठा करून मी स्वतःच्या इच्छेने व मोकळेपणाने मेडीकल कॉलेजच्या सहकार्याखाली पदवीत्तर शिक्षणासाठीच्या वैद्यकीय डॉक्टर संशोधकांच्या अभ्यासात सहभागासाठी संमती देत आहे. मी हे ही अधोरेखित करतो की कोणत्याही प्रकारे माझा वैयक्तिक गोष्टी गुप्त राखण्याचा अधिकार मोडला जाणार नाही.

सहभाग्याची सही / अंगठा

दिनांक :

साक्षीदार

१. नाव :

हुद्दा / पात्राता :

सही :

२. नाव :

हुद्दा / पात्राता :

सही :

Consent form (English)

PATIENT CONSENT FORM

I, Mr. / Mrs. / MS …….....

Gender ..... Age: ..... residing at .....

.....

do hereby confirm that:

(i) I have been asked by the student/researcher of D. Y. Patil Medical College, Kolhapur whether I wish to participate in a study under the aegis of the Medical College;

(ii) The nature of the study being undertaken by the student / researcher, as well as the extent of my participation in it, have been duly explained to me in a language that I understand;

(iii) The potential risks and consequences associated with this study have also been duly explained to me in a language that I understand;

(iv) I also understand that my participation in this study is only for the benefit of advancement in the field of medical research and that at no point in time is my participation being solicited for any pecuniary gain by the researcher or the Medical College;

(v) I have also been explained that I am in no way obliged to participate in the study and that, once I have agreed to participate in the study, I am still free to withdraw from participation in the study at any point in time upon notifying the Medical College in writing in the prescribed form without assigning any reason;

(vi) There will be no financial transaction between myself, the researcher and/or the Dr. D. Y. Patil Medical College for my participation in that study;

(vii) I have been explained that any data collected out of my participation in the study will only be used for academic purposes and/or for further medical research;

(viii) I have also been reassured that any publication of the data collected during the

course of the study or any publication of its conclusions, shall be done on a 'no names' basis and shall under no circumstances reveal my personal identity. Any personal details likely to reveal my personal identity shall at all times remain confidential;

(ix) The contents and effect of this consent form have also been duly explained to me in a language that I understand;

By affixing my signature/thumb print hereto, I am therefore freely and voluntarily signifying my consent, intent and willingness to participate in the study of the student / researcher for the purposes of the Ph. D. thesis under the egis of the D. Y. Patil Education Society, Deemed to be institute, Kolhapur. I also certify that my right to privacy has not been infringed in any manner.

[Signature / Thumb print of participant]

Date:

Witnessed by :

(1) Name :

Title/capacity :

Signature :

(2) Name :

Title/capacity :

Signature :

Operative Consent form

Consent for Surgery

Patient Information	Relative's Information
Name : _____	Name : _____
Gender :_____ Age : _____	Gender :_____ Age : _____
OPD No. :_____ IPD No. :_____	Add : _____
Diagnosis : _____	_____
Operation's Title :_____	Relationship with Pt. _____

I undersigned, _____ give consent for _____ My Own _____ Aforementioned patient's above mentioned operation and / or medication / investigation / anaesthesia / therapy procedure etc.

The necessity of this medication / investigation / anesthesia / operation / therapy / procedure, the ill effects if this is not performed, hazards and complication in the therapeutic modalities other than operation, have been expplained to me by Dr.

I have been explained clearly that any medication / investingation / operation / therapy is not totally safe and that such procedure or anaethesia can be a risk to life of an otherwise healthy person also.

Doctors have explained to me that excessive bleeding, infection, cardiac arrest, pulmonary embolism and complications like this can arise suddenly and unexpectedly while undergoing medication / investigation / operation / therapy / procedure or anaesthesia.

I give consent for any change in the ananesthesia or operative procedure as well as for removal of any organ as deemed necessary by the Doctors at the time of medication / Investigation / Operation / Therapy / Procedure and anaesthesia, instead of desired enefit, some complication may arise e.g.

_____

And I belive that to avoid such complications, if any appropriate care shall be taken by Surgeon Dr. _____ , Anaesthetist Dr. _____or any other doctors suggested by them.

I have read the above writing / The above writing has been read out to me. I have understood the aforesaid and I am giving my consent willingly.

Surgeon Signature Name :_____ _____	Witness Sign Name _____ Age Relation with Pt _____ Address _____ _____ Dt.	Patient / Relative Sign or Thumb impression Dt. @ AM/PM

ANNEXURE - II

LIST OF PUBLICATIONS

1. Gune Anita R., Patil Ashalata D., Patil Rajendra S. Correlation of Immature Germ Cells in Semen in Severe Oligospermic and Azoospermic Males. International Journal of Scientific Research and Reviews. 2019; 8(2):2329-2333.

2. Gune A. R., Patil A. D., Gune RP et.al. Histopathological patterns of testicular biopsies in azoospermic infertile males. Int J Health Sci Res. 2019; 9(5):22-27.

3. Priya S. Patil, Rajendra S. Humbarwadi, Ashalata D. Patil, Anita R. Gune. Immature germ cells in semen – correlation with total sperm count and sperm motility. Journal of Cytology. 2013; 30 (3) 50-54.

Gune Anita R et al., IJSRR 2019, 8(2), 2329-2333

Research article Available online www.ijsrr.org ISSN: 2279–0543

International Journal of Scientific Research and Reviews

Correlation of Immature Germ Cells in Semen in Severe Oligospermic and Azoospermic Males

Gune Anita R[1*], Patil Ashalata D[2], Gune Rahul P[3] and Patil Rajendra S[4]

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[2]Professor, Department of Anatomy, D.Y. Patil Medical College, Kolhapur.
[3]Consultant Urologist, Nirmal Nursing Home, Mirjakar Tikkti, Kolhapur.
[4]Associate Professor, Department of Pathology, D.Y. Patil Medical College, Kolhapur.

ABSTRACT

Recent studies suggest suggests that male factor contributing to infertility is up to 30% of the total cases. Semen analysis report "round cells" as without further differentiating them into leucocytes or immature germ cells. The aim of this work was to study a method for differentiating the round cells in semen into immature germ cells and leucocytes and to correlate shedding of immature germ cells in severe Oligospermic and Azoospermic males. Semen samples from 100 males, who had come for investigation for infertility, were collected, semen parameters recorded, and stained with Leishman smears studied for different round cells. In this study, out of 100 cases 32 were Azoospermic whereas 68 were severe Oligospermic males. The round cells were counted as percentage of the total count and it was seen that their percentage of immature germ cells were more in Oligospermic as compared to Azoospermic males. The differential counts of round cells showed 70% were immature germ cells in Azoospermic males and 80% in Severe Oligospermic males.In semen analysis round cells should also be given due importance, as they can be differentiated and counted into immature germ cells. Presence of immature germ cells in Azoospermic males rules out Obstructive type. The differential counts mentioned in a semen report give valuable and clinically relevant information.

KEYWORDS: Immature germ cells; round cells; semen; total sperm count.

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International Journal of Health Sciences and Research
www.ijhsr.org ISSN: 2249-9571

Original Research Article

Histopathological Patterns of Testicular Biopsies in Azoospermic Infertile Males

Dr. Anita R Gune[1], Dr. Ashalata D Patil[2], Dr. Rahul P Gune[3], Dr. Rajendra S Patil[4]

[1]Associate Professor, Department of Anatomy, D.Y. Patil Medical College, Kolhapur.
[2]Professor, Department of Anatomy, D.Y. Patil Medical College, Kolhapur.
[3]Consultant Urologist, Nirmal Nursing Home, Mirjakar Tikkti, Kolhapur.
[4]Associate Professor, Department of Pathology, D.Y. Patil Medical College, Kolhapur.

Corresponding Author: Dr. Anita R Gune

ABSTRACT

Introduction: Testicular biopsy is important in categorizing patients with Azoospermia and provides useful information and guidelines for further treatment. Histopathological findings of testicular biopsies are of significant importance in making decision for selection of cases for Intracytoplasmic sperm injection (ICSI) in patients with non-obstructive azoospermia.
Objective: To categorize histopathological patterns of testicular biopsy specimens from Azoospermic infertile males to Modified Johnson scoring system.
Methodology: Testicular biopsies from male patients with history of infertility and azoospermia were included in this study. All biopsies were received in 10% Bovian fixative and routinely processed and stained with Haematoxylin and Eosin (H and E). Clinical data was obtained and recorded on a proforma. All cases were examined microscopically and various histological patterns were evaluated and categorized according to the histopathological patterns and Modified Johnson scoring was performed.
Results: A total 32 cases of testicular biopsies from Azoospermic males were evaluated. Most frequent pattern found was of Seminiferous tubule hyalinization (34.375%), further categorized as Johnson score 1. The second most frequent was Germ cell maturation arrest (25%), further categorized as Johnson score 8-3.The third common was Sertoli cell only syndrome (18.75%), further categorized as Johnson score 2. There were 3 cases that showed mixed pattern and 2 case showed discordant pattern.
Conclusion: This study outlines the different histopathological patterns of testicular biopsies in cases of male infertility in our setup and identifies seminiferous tubule hyalinization as the most common pathological finding amongst various histopathological patterns.

Key Words: Testicular biopsy, hypospermatogenesis, male infertility

INTRODUCTION

Infertility is defined as inability to conceive after one year of unprotected intercourse. [1] Male infertility is one of the important causes of infertility among couples and it contributes to significant number of infertility cases. [2] It is estimated that around 1% of total male population and 10% of men seeking infertility treatment are affected by testicular failure. [3] Evaluation by testicular biopsy in cases of azoospermia provides reliable information regarding spermatogenesis which can further be categorized into various histopathological patterns. [4] This provides valuable information to the clinician for the purpose of prognosis and treatment. [5]

Diagnostic testicular biopsy is used to determine testicular histopathological patterns and to foresee the possibility of

Original Article

Immature germ cells in semen – Correlation with total sperm count and sperm motility

ABSTRACT

Background: Current data regarding infertility suggests that male factor contributes up to 30% of the total cases of infertility. Semen analysis reveals the presence of spermatozoa as well as a number of non-sperm cells, presently being mentioned in routine semen report as "round cells" without further differentiating them into leucocytes or immature germ cells.

Aim: The aim of this work was to study a simple, cost-effective, and convenient method for differentiating the round cells in semen into immature germ cells and leucocytes and correlating them with total sperm counts and motility.

Materials and Methods: Semen samples from 120 males, who had come for investigation for infertility, were collected, semen parameters recorded, and stained smears studied for different round cells. Statistical analysis of the data was done to correlate total sperm counts and sperm motility with the occurrence of immature germ cells and leucocytes. The average shedding of immature germ cells in different groups with normal and low sperm counts was compared. The clinical significance of "round cells" in semen and their differentiation into leucocytes and immature germ cells are discussed.

Conclusions: Round cells in semen can be differentiated into immature germ cells and leucocytes using simple staining methods. The differential counts mentioned in a semen report give valuable and clinically relevant information. In this study, we observed a negative correlation between total count and immature germ cells, as well as sperm motility and shedding of immature germ cells. The latter was statistically significant with a *P* value 0.000.

Key words: Immature germ cells; round cells; semen; total sperm count.

Introduction

Reproduction is the fundamental biological process of life essential for the continuation of the species. It depends on the fertility status of the male and female partners involved. As the prevalence of infertility is increasing, the brunt of infertility management is felt by the medical field. Worldwide surveys have shown that almost one in every seven couples faces problems of infertility. In India, the infertility rate is 9% of the reproductive population, which means almost 12 to 18 million couples visit infertility clinics annually for treatment. Of the total infertility cases, 50% are due to the male factors. Hence, in andrology, rapid advances with a lot of sophisticated and expensive newer diagnostic methods are emerging. This requires a very sophisticated infrastructure and trained personnel which may not be available at peripheral medical centers. Further this battery of investigations leads to a huge financial burden on the couple as well as the society which is beyond the reach of the economically compromised population in a developing country like India. In such a scenario, semen analysis forms the basic, cost-effective, and non-invasive investigation for screening of such a large number of patients.

In a semen sample, apart from spermatozoa there may be a variable number of non-sperm cells. In a routine semen report, they are mentioned as "round cells" without further differentiating them into leucocytes or immature germ cells. This could be because of the difficulty in identifying and differentiating those cells in an unstained wet preparation.[1]

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DOI: 10.4103/0970-9371.117682	

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PAPERS / POSTERS PRESENTED

1. Histopathological Patterns of Testicular Biopsies in Azoospermic Infertile males. Paper presented in 5th International conference "Angiogenesis Research : Targeted Anti-Angiogenic Therapy on 26th - 27th October 2018

2. Correlation of Immature germ cells in semen in severe Oligospermic and Azoospermic males. Poster presented in National conference on Advances in Laboratory Medicine (NCALM-2019) on 31st March 2019.

Certificate
of Presentation

D. Y. PATIL EDUCATION SOCIETY • KOLHAPUR • KNOWLEDGE IS POWER

NCCS
National Centre for Cell Science, Pune

iNSERB DIA
Science & Engineering Research Board
Department of Science & Technology
Government of India

5th INTERNATIONAL CONFERENCE

"ANGIOGENESIS RESEARCH: TARGETED ANTI-ANGIOGENIC THERAPY"

This certificate is presented to

Mr./Miss./Dr. Anita R. Gune

has presented a poster/ oral presentation on Histopathological patterns of testicular biopsies in Azoospermic male infertility.

in **5th International Conference** on

"Angiogenesis Research: Targeted Anti-Angiogenic Therapy"

held on 26th-27th October, 2018 at **D. Y. Patil Education Society (Deemed to be University), Kolhapur, India.**

Prof. P. B. Behere	Prof. C. D. Lokhande	Dr. Indumathi S.	Dr. G. C. Kundu
Chairman	Director	Secretory	Chairman

Certificate

DEPARTMENT OF PATHOLOGY,
D. Y. PATIL EDUCATION SOCIETY (INSTITUTION DEEMED TO BE UNIVERSITY)
&
ASSOCIATON OF PRACTICING PATHOLOGIST OF INDIA (APPI)

NATIONAL CONFERENCE ON ADVANCES IN LABORATORY MEDICINE (NCALM-2019)

MMC / Accre. Cert / MED-0207 / 2013
CME Code MMC / MAC / 2019 / B-012494

This is to certify that

Dr./Mr./Mrs./Miss Dr. Anita Rahul Gune

has participated as **Speaker / Chairperson / Delegate** with **Poster Presentation** in National Conference on **31st March 2019.**

MMC has granted Two (2) credit points for the Delegates.

Dr. (Mrs.) S. S. More	Dr. Vipul Patel	Dr. R. S. Patil	Dr. R. M. Shinde
Convener	Co-Convenor, (APPI) Secretary	Organizing Secretary	MMC/MAO-00480/2014
NCALM - 2019	NCALM - 2019	NCALM - 2019	MMC Observer, NCALM-2019

CONFERENCES ATTENDED

1. Emerging Trends of Genetics in Clinical Practice. Conducted by Department of Medical Genetics, SBS, MGMIHS on 5th October 2020.

2. Challenges in Implementation of CBME Curriculum in Anatomy. Conducted by JSS Academy of Higher Education and Research. On 3rd October, 2020.

3. Drawing Software for Medical Teachers to create learning Resources. Conducted by Department of Anatomy, JIPMER, Pondicherry on 1st October, 2020.

4. Medical Education during covid pandemic. International seminar conducted by UNESCO in collaboration of Kolhapur-India Bioethics unit on 2nd October 2020.

5. BODY DONATION, EMBALMING & CADAVERIC DISSECTION : PERSPECTIVES IN COVID ERA. On 31st August 2020

6. Learning Objectives Vs. Learning Outcomes in Medical/Health Sciences Education. On 28th June 2020

7. National Cadaveric Workshop on Advanced Knee Arthroscopy course. Conducted by Department of Anatomy on 1st March 2020.

8. National Cadaveric Workshop on Basic Knee Arthroscopy course. Conducted by Department of Anatomy on 29th February 2020.

9. National Cadaveric Workshop on External DCR. Conducted by Department of Anatomy on 11th January 2020.

10. National 3D Cadaveric: Live and Hands on Workshop on Professor Jean Destaunaud's technique in Endoscopic Spine Surgery. Conducted by Department of Anatomy on 21st & 23rd December 2019.

11. National Cadaveric Workshop on Eyelid Reconstruction Course. Conducted by Department of Ophthalmology & Department of Anatomy on 7th November 2019.

12. National Cadaveric Workshop on Endonasal Lacrimal Procedures. Conducted by Department of Ophthalmology & Department of Anatomy on 3rd August 2019.

13. National Cadaveric Workshop on Advanced Knee Arthroscopy course. Conducted by Department of Anatomy on 30th June 2019.

14. National Cadaveric Workshop on Basic Knee Arthroscopy course. Conducted by Department of Anatomy on 29th June 2019.

15. National Conference on Advances in Laboratory Medicine (NCALM-2019). Organized by Department of Pathology, D. Y. Patil Education Society (Institution of Deemed to be University), Kolhapur and APPI on 31st March 2019

16. National Hands on Advanced 3D Workshop on Laparoscopic Surgical / Gynecological Live & Cadaveric Workshop. Conducted by Department of Anatomy 7-9th December 2018

17. International 3D Cadaveric; LIVE and Hand's Workshop on Professor Jean Destaunaud's Technique in Endoscopic Spine Surgery. Conducted by Department of Anatomy 16-18th November 2018.

18. National Hands on Cadaveric Workshop on Advanced Shoulder Arthroscopy. Conducted by Department of Anatomy 14th October 2018.

19. National Hands on Cadaveric Workshop on Advanced Knee Arthroscopy. Conducted by Department of Anatomy 13th October 2018.

20. National Hands on India's First 3D Cadaveric and Live Workshop on Endoscopic Spine Surgery. Conducted by Department of Anatomy 17-19th August 2018

21. National Conference on Recent Updates on Nipah and Arbovirus-Indian Scenario. Organized by Department of Microbiology, D. Y. Patil Education Society (Deemed to be University), Kolhapur, 18th August 2018.

22. National Hands on Role of Yoga in stress management. Organized by Department of Physiology, D. Y. Patil Education Society (Deemed to be University), Kolhapur, on 22nd & 23rd March 2018.

23. 5th International Conference on Angiogenesis Research: Targeted Anti-Angiogenic Therapy. D. Y. Patil Education Society (Deemed to be University), Kolhapur on 26-27th Oct. 2018

24. 6th National Conference of Society of Clinical Anatomists (SOCA) 2017. Organized by Dept. of Anatomy, J. N. Medical College, KLE University, Belagavi, Karnataka. On 3rd & 4th June, 2017.

25. International Conference on Nanotechnology Addressing the Convergence of Material Science, Biotechnology and Medical Science. Organized by Department of CIR, D. Y. Patil Education Society (Deemed to be University), Kolhapur, on 9-11th November 2017.

26. 5th National Conference of Society of Clinical Anatomists (SOCA) 2016. Organized by Dept. of Anatomy, SGRDIMSAR, Vallah, Amritsar. On 9th & 10th April, 2016.

27. 3rd State Conference of Regional Chapter of Anatomy (Maharashtra) MAHACON- III 2016, Organized by Dept. of Anatomy, D. Y. Patil Medical College, Kolhapur on 22nd & 23rd September 2016.

28. National Workshop on "Stem Cell, Molecular Biology & Bioinformatics" (SMB - 2016) Organized by Department of Stem Cell & Regenerative Medicine Center D. Y. Patil University, Kolhapur on 9th & 10th July 2016.

29. 2nd State Conference of Regional Chapter of Anatomy (Maharashtra) MAHACON- III 2016, Organized by Dept. of Anatomy, Dr. D. Y. Patil Medical College and Research Centre, Pimpri, Pune on 29th & 30th January 2016.

30. National Conference on Convergence of Stem Cell and Medical Nanotechnology. Organized by Department of CIR, D. Y. Patil Education Society (Deemed to be University), Kolhapur, on 2nd & 3rd September 2015.

31. Event on "Annual Meet on Advanced Research (AMAR-2015) Organized by D.Y. Patil University, Kolhapur. On 17th March, 2015.

32. 25th Annual Conference of the West Zone Urological Society of India (WZ-USICON 2015) Goa on 10th – 13th September 2015

33. DYPU Anveshan 2015. Organized by Center for Interdisciplinary Research D. Y. Patil University, Kolhapur on 29th December 2015.

34. National Conference on "Recent Advances in Clinical Embryology RACE – 2014" Organized by Dept. of Anatomy, D. Y. Patil Medical College, Kolhapur on 18th & 19th January 2014.

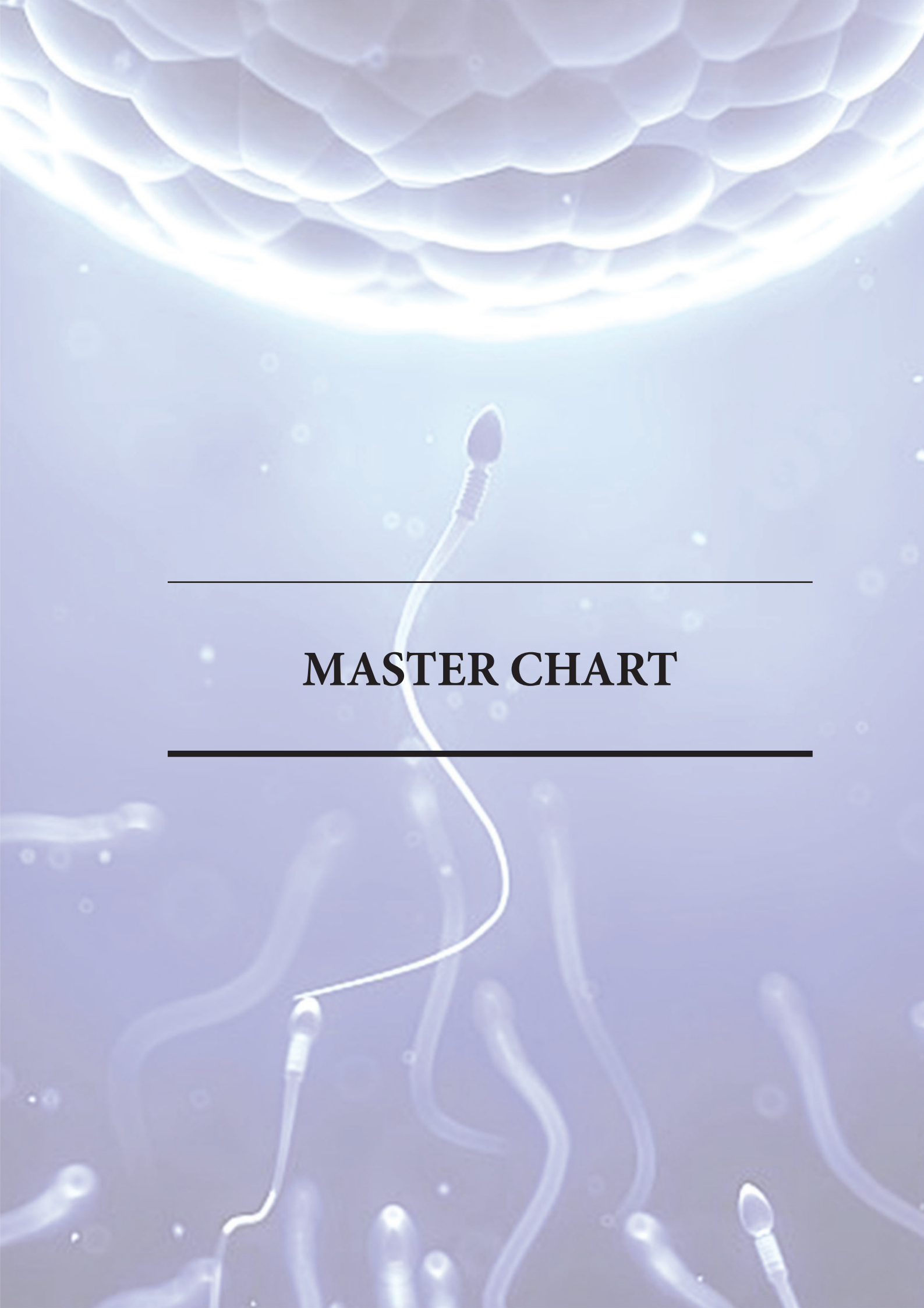
35. National Conference on "Electrophysiology & Neurology Trends in Electro Diagnosis & Research – ENTER - 2014" Organized by Dept. of Physiology, on 31st January & 1st February 2014.

36. National Conference on "Recent Advances in RNTCP & Approach to Multi Drug resistant Tuberculosis-2014" Organize by Dept. of Community Medicine & Dept. of Microbiology D. Y. Patil Medical College, Kolhapur on 14th & 15th February 2014.

37. USI – WEST ZONE MOSCOW MEETING Organized by urological Society of India at Hotel Radisson Slavyonskaya, Moscow on 11th June 2014.

38. Workshop on Renal Calculi 'SCISSORS TO LASERS' Organized By Kolhapur Urology Society, D. Y. Patil University, Kolhapur on 22nd & 23rd November 2014.

39. National Conference on "Nano-Pharmacology" NCNP-2014 Organized by Dept. of Pharmacology, D. Y. Patil University, Kolhapur. On 19th & 20th December 2014.



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