Chapter I

Introduction
1.0. Infertility

Infertility is “the inability of a sexually active, non-contraception couples to achieve pregnancy in one year” [1]. Approximately 13-18% of couples in the reproductive age group worldwide are affected by infertility regardless of race and ethnic group [2]. Recent growth of the Indian population has been unprecedented; it stands currently over one billion and is expected to touch 2 billion by 2035 with an average growth rate of 2% [3]. Albeit curtailing population growth is a major national concern, a substantial number of infertile couples in our population have an equally great concern, that of having a child. Although, infertility is not life threatening, it is usually a traumatic condition associated with social stigma, resulting in intense societal and parental pressure to attain biological parenthood [4].

1.1. Classification

Infertility is classified into primary and secondary, based on the ability to achieve a pregnancy regardless of the outcome. Infertility is either primary (67-71%) when no pregnancy has ever occurred or secondary (29-33%), where there has been a pregnancy, irrespective of the outcome [5]. About one in ten couples are affected with infertility for various possible reasons; both sexes contribute equally to this problem (40% each), while remaining 20% of infertile couples are childless due to unknown etiology. Female factors include ovulation disorder, tubal damage, endometriosis, hyperprolactinemia, reproductive tract disease, ectopic pregnancy and unsafe abortions [6].

1.2. Causes of male infertility

It was assumed that most reproductive problems could be attributed to the female partner; but research in recent years has demonstrated that 50% of cases are associated with male factor infertility. Male infertility is a multifactorial syndrome encompassing a wide variety of disorders. Among infertile men, ~70% of the underlying etiological cause(s) can be detected
while the remaining 30% are referred to as unknown (idiopathic), could be congenital or acquired [9]. The distribution of infertility is shown in Figure 1.1.

**Figure 1.1. Distribution of infertility**

![Distribution of infertility](image)

The etiology of male factor infertility is associated with genetic conditions, such as consequence of a developmental disorder [10] and non-genetic factors including genital infections, structural abnormalities of the male genital tract, varicoceles, congenital absence of unilateral/bilateral vas deferens (CABUD/CABVD), chronic illness, hypogonadotrophic hypogonadism, previous scrotal or inguinal surgery, medications, exposure to chemicals, life style and environment [11].

A large proportion of male infertility is associated with either systemic defect such as diabetes, obesity, immunological factors or with imbalance in levels of gonadal steroids, trophic hormones [testosterone, dihydrotestosterone, follicle stimulating hormone (FSH), leuteinizing hormone (LH), and androgen receptor] [12].

Genetic factor accounts for ~10-15% of severe male infertility, including single gene defects, inherited disease, interactions of multiple genes, chromosomal aberrations, microdeletion on the Y chromosome and mutations or polymorphisms [13]. The frequency of genetic factors associated with male-factor infertility, is depicted in Figure 1.2.

Further, there appears to be a geographical variation in the prevalence of male infertility amongst infertile couples. This is as high as 59% in France, 26-32% in the UK and Kashmir Valley in
India, and about 36% in South Africa, Indonesia and Finland [17]. Thus, available reports indicate that ethnic, climatic, occupational factors influence on mean sperm concentration, counts and quality of spermatozoa and male infertility.

1.3. Lifestyle factors

The lifestyle and environmental factors include lack of exercise, smoking, alcohol consumption, substance abuse, air pollution, phthalates, pesticides, heavy metal exposure and poor nutrition that might affect gamete and embryo development, reduced sperm quality leading to infertility [18].

Figure 1.2. Contribution of genetic factors with male-factor infertility

![Graph showing contribution of genetic factors with male-factor infertility]

Source: [14, 15, 16].

An altered environmental milieu due to oxidative stress has shown to affect the spermatogenic process as well as the quality and quantity of sperm. Many factors are reported for an enhanced oxidative stress due to accumulation of reactive oxygen species (ROS). Obesity produces oxidative stress as adipose tissue releases pro-inflammatory cytokines that increase leukocyte production of ROS [19]. In addition, the accumulation of adipose tissue within the groin region results in heating of the testicle which has been linked with oxidative stress and decreased
sperm quality [20]. Excessive exercise activity has been associated with oxidative stress; since muscle aerobic metabolism is known to create a large amount of ROS in humans and rodents [21]. Studies have shown that cigarette smoking in infertile men is correlated with impaired sperm motility, fertilization capacity and sperm membrane oxidation [22].

Similarly, excessive alcohol consumption causes an increase in systemic oxidative stress as ethanol stimulates the production of ROS. Many alcohol abusers have diets deficient in protective antioxidants. It was also suggested that the presence of oxidative stress within the testicle could be due to significant reduction in plasma testosterone, increase in serum lipid peroxidation byproducts and a drop in antioxidants [23]. However, no study to date has directly examined the correlation between alcohol intake and sperm oxidative damage.

1.4. Environmental factors
Consistent with life style, several environmental pollutants have been associated with testicular oxidative stress. Significantly increased frequency of sperm with abnormal morphology and reduced motility was observed in infertile men with medium and high exposure to air pollution which may result in sperm DNA damage and thereby increase the rates of male-mediated infertility, miscarriage, and other adverse reproductive outcomes [24]. Phthalates are used as plasticizers, employed in a wide range of food packaging and personal care products. Exposure to phthalates can take place through food consumption, dermal absorption or inhalation have been linked with impaired spermatogenesis and increased sperm DNA damage [25]. Oral administration of phthalate esters to rats have shown an increase of ROS generation within the testis and a decrease in antioxidant levels, culminating in impaired spermatogenesis [26].

Pesticides such as lindane, methoxychlor and herbicide dioxin-TCDD have all been linked with testicular oxidative stress in rodent models [27]. The commonly used preservative sulfur dioxide has also been shown to produce testicular oxidative stress in laboratory animals [28]. Heavy metal exposure has been conclusively linked with an increase in testicular oxidative
stress and a resultant increase in sperm DNA oxidation [29]. The increase in infertility and miscarriage observed in the partners of welders and battery/paint factory workers may be due to oxidative damage to sperm DNA initiated by the inhalation of metal fumes [30].

Recent prospective studies have shown a correlation of psychological stress with reduction in sperm quality mediated by an increase in seminal plasma ROS generation and antioxidant protection [31]. Injury to testicles may also have issues with sperm production. Varicocele is a varicosity or varicose vein in the testicle that damages the blood flow to the testes, slowing or stopping the sperm production.

1.5. Infections
Post-testicular genital tract infection and inflammation (e.g., combined inflammation of the epididymis, testis or the prostate gland), like prostatitis, result in leukocytospermia, have been associated with increased levels of ROS and subsequent sperm DNA damage [32]. Bacteria responsible for prostate infection may originate from the urinary tract or can be sexually transmitted. Typical non-sexually transmitted pathogens include Streptococci (S. viridans and S. pyogenes), coagulase-negative Staphylococci (S. epidermidis and S. haemolyticus), gram-negative bacteria (Escherchia coli, Proteus mirabilis) and atypical mycoplasma strains (Ureaplasma urealyticum, Mycoplasma hominis) [33]. All these pathogens create an acute inflammatory response with an influx of leukocytes into the genital tract and result in the increase in ROS production [34] and have high degrees of sperm oxidative pathology [35]. Several studies have reported the influence of current or past Chlamydial infection, viral pathogens such as Cytomegalovirus, Herpes Simplex Virus, and Epstein-Barr virus with initiation or increase in oxidative damage to sperm. Herpes simplex DNA is found in 4–50% of infertile men’s semen. The IgM antibodies being associated with a 10-fold increase in the rate of leukospermia and oxidative stress [36].
1.6. Immunological causes

Immunological factor operates at every stage of human reproductive process; may be due to elevated level of antisperm antibodies caused by infective or auto-immune diseases of male reproductive system. Antisperm antibodies can impair fertilization in various ways; interfering with sperm motility by immobilizing or agglutinating the sperm, disturb transport of the spermatozoa, zygote development by impairing early cleavage, or even damaging the implantation process [37].

1.7. Biochemical factors/hormones

Spermatogenesis is hormonally regulated [38], problems that can affect male fertility are associated with (i) disorders involving hypothalamic secretion or action; (ii) pituitary development disorders; (iii) primary disorders of pituitary LH, FSH secretion or action. As LH and FSH are trophic hormones for the testes and ovaries, reduced secretion of these gonadotrophins (hypogonadotrophism) results in malfunctioning of the sex organs (hypogonadism) [39]. Most often, impaired male fertility arises from direct testicular injury to the germ cells or the supporting somatic (Sertoli) or steroidogenic (Leydig) cells, following testicular injury. The release of hypothalamic GnRH stimulates pituitary gonadotrophin secretion: the resultant LH predominantly acts on Leydig cells to promote steroidogenesis, whilst FSH acts on Sertoli cells. Testosterone, acting as an androgen, is important in the negative feedback control of both gonadotrophins, although hypothalamic aromatization to estradiol also increases the degree of inhibition. Many other hormones, including leptin, growth hormone and insulin-like growth factor-1 (IGF-1) may also play a role in spermatogenesis [40].

1.8. Sperm disorders

Research over the past few years has clearly demonstrated that infertile men with normal somatic karyotypes have an increased risk of producing aneuploid sperm [41]. Most of the human aneuploidy is considered to be of germ-cell origin arising from errors in maternal
and paternal meiotic chromosomal segregation. In turn, aneuploid sperm might result in an abnormal offspring with major or minor congenital malformations, severe dysmorphogenesis, chromosomally abnormal fetuses and pregnancy loss [42, 43]. The chances of live birth and conception rate drastically decreases when DNA fragmentation index is >30% [44]. Evenson et al. (2002) had reported that the spontaneous abortion rate as about 1.7 fold higher in sperms containing fragmented DNA [45]. The most common causes of male infertility are related to problems with the sperm maturation, abnormal morphology, unable to move properly (progressive motility) which occurs at the different stages of spermatogenesis.

There are various reasons where the volume of the semen sample may be reduced. Majority of the secretion might be from prostate or seminal vesicles that reflect abnormalities in sex gland with an obstruction in the reproductive tract. Hematospermia is a condition, where the presence of red blood cell count (RBC) in semen, may appear brown in color. Large volumes of semen are found in association with varicocele or after relatively long periods of sexual abstinence. The pH of the semen sample ranges between 7.2 and 8.0 that are determined by acidic and alkaline secretions of the prostate and seminal vesicles. If the pH exceeds 8.0, the semen sample is suspected to have infection due to decreased secretion of acidic products by the prostate, such as citric acid or occlusion of seminal vesicles.

1.8.1. Causes of sperm DNA damage
The causes of sperm DNA damage have been attributed to intra or extratesticular factors which were clearly associated with male infertility. Sperm DNA is unique and regulates timely maturation of the zygote. Damage to sperm DNA may occur due to life style or environmental factors. Different theories have been proposed to explain the origin of DNA damage in spermatozoa; either it could occur at the time, during the result of DNA packing and/or transition of histone to protamine complex at different stages of spermiogenesis. Maturation of spermatozoa is a unique process involving a series of meiotic and mitotic changes in cytoplasmic architecture. Topological rearrangements, alteration in transcription replacement of somatic cell-like histones with transition proteins, and the final addition of protamines leading to a
highly organized and condensed chromatin protects the genetic integrity during transport of the paternal genome through the male and female reproductive tracts [46]. Infertile men manifest various nuclear alterations in chromosomes, including an abnormal chromatin structure, microdeletions, aneuploidies and DNA strand breaks (single and double). Depending upon the count and motility of spermatozoa, the semen sample is classified as normozoospermia, oligozoospermia, asthenozoospermia, teratozoospermia, oligoasthenoteratozoospermia, azoospermia and globozoospermia as shown in Table 1.1.

<table>
<thead>
<tr>
<th>Clinical finding</th>
<th>Terminology</th>
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<tbody>
<tr>
<td>Normozoospermia</td>
<td>Normal ejaculate</td>
</tr>
<tr>
<td>Oligozoospermia</td>
<td>( \leq 10 \text{ million sperm/ml of semen} )</td>
</tr>
<tr>
<td>Asthenozoospermia</td>
<td>( \geq 50% ) spermatozoa with low motility</td>
</tr>
<tr>
<td>Teratozoospermia</td>
<td>( \geq 50% ) sperm with abnormal morphology</td>
</tr>
<tr>
<td>Oligoasthenoteratozoospermia</td>
<td>Signifies disturbance of oligozoospermia, asthenozoospermia and teratozoospermia</td>
</tr>
<tr>
<td>Azoospermia</td>
<td>Complete absence of sperm</td>
</tr>
<tr>
<td>Aspermia</td>
<td>Ejaculation does not emit any sperm</td>
</tr>
<tr>
<td>Necrospermia</td>
<td>Non viable/dead sperm</td>
</tr>
<tr>
<td>Hematospermia</td>
<td>Red blood cells present in the semen</td>
</tr>
<tr>
<td>Pyospermia</td>
<td>White blood cells present in the semen</td>
</tr>
<tr>
<td>Globozoospermia</td>
<td>Round-headed sperm</td>
</tr>
</tbody>
</table>

1.8.2. Azoospermia

Azoospermia is absence of both spermatozoa and spermatogenic cells in semen sample; might be due to complete obstruction of seminal ducts, which is often accompanied by secretary
dysfunction of the gonads. The spermatozoa is found to be completely absent in the seminal fluid which causes male infertility. The prevalence of azoospermia in the general population has been estimated at 2%; it was also found to be as high as 10-20%. Testicular histology shows different degrees of spermatogenic alterations, ranging from tubular damage to hypospermatogenesis.

1.8.3. Oligoasthenoteratozoospermia (OAT)
Oligozoospermia is a condition where semen sample shows a decreased sperm concentration with significant abnormalities in sperm morphology and motility. Studies on infertile men with OAT have reported an increased frequency of chromosomal abnormalities in sperm. It has been previously suggested that oligozoospermia might be a greatest risk factor because quantitative problems in sperm production have been associated with pairing abnormalities in both autosomes and sex chromosomes, resulting in partial or complete meiotic arrest [11]; that can lead to aneuploidy in cells capable of completing spermatogenesis and meiotic arrest in other cells causing oligozoospermia. Oligozoospermia is categorized according to sperm count into those with severe, moderate or mild (ranging from ≤ 5-10 million sperm/ml).

1.8.4. Globozoospermia
Semen with round-head sperms are termed as globozoospermia, few studies have analyzed sperm aneuploidy in spermatozoa from globozoospermic patients. The acrosomeless globozoospermia seems to be the most severe type among sperm abnormalities. The association between this globozoospermia and numerical chromosomal abnormalities is unclear [48].

1.9. Genetic causes of male infertility
In addition to the above-discussed factors 15-30% of genetic factors have been reported as a cause in spermatogenesis failure. Genes control various physiological processes, such as hypothalamus-pituitary-gonadal axis, germ cell development and differentiation. Spermatogenesis is a complex process, and is influenced by the molecular mechanism of many genes. It has been estimated that over 10% of genes are involved in the regulation of human spermatogenesis [49]. Genetic causes in infertile men include numerical and structural
chromosomal abnormalities, (reciprocal and robertsonian translocations) [50, 51] deletions of the azoospermia factor region (AZF) of chromosome-Y [52], mutations or polymorphisms in the cystic fibrosis transmembrane conductance regulator (CFTR) gene commonly associated with congenital absence of vas deferens [53], mitochondrial DNA mutations, multifactorial and endocrine disorders may be of genetic origin [9]. The prevalence of genetic abnormality associated with abnormal spermiogram of male infertility has been shown in Table 1.2.

Table 1.2. Genetic abnormalites of male infertility

<table>
<thead>
<tr>
<th>Genetic abnormality</th>
<th>Prevalence (%)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Normozoospermia</td>
</tr>
<tr>
<td>Chromosomal abnormalities</td>
<td>5</td>
</tr>
<tr>
<td>Klinefelter syndrome</td>
<td>5</td>
</tr>
<tr>
<td>Robertsonian translocation</td>
<td>0.8</td>
</tr>
<tr>
<td>Y chromosome microdeletion</td>
<td>Less than 2%</td>
</tr>
<tr>
<td>AZFa deletion (sertoli cell-only syndrome)</td>
<td>0.5 –1.0</td>
</tr>
<tr>
<td>AZFb deletion (spermatogenic arrest)</td>
<td>0.5 -1.0</td>
</tr>
<tr>
<td>AZFc deletion (severe oligozoospermia to nonobstructive azoospermia)</td>
<td>0.5 -1.0</td>
</tr>
<tr>
<td>Partial AZFc deletion</td>
<td>3-5</td>
</tr>
</tbody>
</table>

Source: [54]
1.9.1. Chromosomal abnormalities (CA)

Chromosomal abnormalities pertain to presence or absence of whole chromosomes (numerical chromosomal abnormalities); gain or loss of an entire or segment of chromosome (structural chromosomal abnormalities) that result in aneuploidy which occurs during various genetic levels [55]. Aneuploidy is one of the most common forms of chromosomal abnormalities; it is responsible for a large portion of human morbidity and mortality, infertility and pregnancy loss. The origin of CA associated with male infertility arises from errors in maternal and paternal meiotic chromosomal segregation. They are of two types: i) karyotype alterations affecting cells of both somatic and germ cell-lines, and ii) meiotic abnormalities which can produce infertility, either by spermatogenetic arrest or formation of chromosomally unbalanced gametes. This might either lead to spontaneous abortions, offspring with mental deficiency [56] or congenital malformations [57]. The incidence of CA in male infertility is reported to be 2.1 to 19.6% worldwide [9], which is higher than in general population (0.5-1%) [58]; similarly, the sample size varies between 72 and 2600 infertile men [59]. Nevertheless, all of them point to an increasing percentage of CA concomitant with decreasing sperm count. In addition, the nature of CA differs depending on whether a patient has oligoasthenoteratozoospermia or azoospermia. Therefore, chromosomal errors are a pertinent area of research to determine the role of genetics in male factor infertility.

1.9.2. Numerical chromosomal abnormality

Aneuploidy involves both autosomes and sex chromosomes that occur in around 4% of pregnancies [60]. However, it is estimated that up to 60% of conceptions are aneuploid and aborted spontaneously, even before a pregnancy is clinically recognized. It is also evident that loss (monosomy) of a chromosome is much more detrimental than gain (trisomy) of a chromosome. Monosomy X is the only non-mosaic condition that is compatible with life, and is largely attributed to X-chromosome inactivation. The most common numerical CA is paternal in origin and associated with sex chromosome and the incidence is reported to be 1:1000 live births [61]. Autosomal aneuploidies are much less frequent i.e., 1:7000 in trisomy 18 and
1:10000 for trisomy 13; [62] duplications and/or deficiencies are observed occasionally that result from the abnormal segregation of a structural rearrangement in a carrier father or mother [63]. Numerical chromosome errors are a frequent cause of male infertility. The incidence is inversely proportional to the number of sperms. The reported frequency of sex and autosomal CA in infertile men are shown in Table 1.3.

<table>
<thead>
<tr>
<th>Frequency (%)</th>
<th>Country</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.00</td>
<td>Turkey</td>
<td>[64]</td>
</tr>
<tr>
<td>4.20</td>
<td>France</td>
<td>[65]</td>
</tr>
<tr>
<td>25.80</td>
<td>Germany</td>
<td>[66]</td>
</tr>
<tr>
<td>11.40</td>
<td>Germany</td>
<td>[67]</td>
</tr>
<tr>
<td>10.60</td>
<td>Netherlands</td>
<td>[68]</td>
</tr>
<tr>
<td>10.20</td>
<td>India</td>
<td>[69]</td>
</tr>
<tr>
<td>4.40</td>
<td>India</td>
<td>[70]</td>
</tr>
<tr>
<td>15.50</td>
<td>Iran</td>
<td>[59]</td>
</tr>
<tr>
<td>11.20</td>
<td>Turkey</td>
<td>[71]</td>
</tr>
<tr>
<td>8.50</td>
<td>China</td>
<td>[72]</td>
</tr>
<tr>
<td>11.74</td>
<td>Turkey</td>
<td>[73]</td>
</tr>
<tr>
<td>18.30</td>
<td>Kuwait</td>
<td>[14]</td>
</tr>
</tbody>
</table>

### 1.9.3. Sex chromosome abnormalities

Sex chromosome abnormalities are the most frequent chromosome-related cause of infertility, arise *de novo* and are associated with an increased rate of sperm chromosome aneuploidy among infertile men [63]. The frequency of karyotype alterations is 7% and it inversely correlates with sperm count; it has been reported that 17.5%, 13.7% and 4.6% of chromosomal alterations are observed in severe oligozoospermia, azoospermia and oligozoospermic men respectively [83].
1.9.4. 47,XYY

The XYY karyotype occurs amongst 1:1000 live male births, that results from non-disjunction during cell division at a post-zygotic stage of mitosis which receives an extra Y chromosome, producing a 47,XYY karyotype. In a majority of cases the phenotypic features remain normal, which hampers further management based on the specific requirements associated with an etiology and produces 46,XY/47,XYY mosaics. El-Dahtory and Elsheikha (2009) investigated four cases of infertile men (4 out of 132) with azoospermia or severe oligozoospermia showed karyotype of 47,XYY [75]. Men with a 47,XYY karyotype are generally fertile and there is no evidence of transmission of the extra Y chromosome to their progeny because the supernumerary Y-chromosome is eliminated during meiosis; and some XYY germ cells can complete meiosis and produce mature aneuploid sperm.

1.9.5. Klinefelter syndrome

Klinefelter syndrome is the most common sex CA occurring at a rate of 1:500 live male births. Phenotypically, patients present with increased height, decreased intelligence quotient, obesity, diabetes mellitus, small firm testes, decreased Leydig cell function and sperm production, an increased risk of leukemia, extragonadal germ cell tumors, and breast cancer. Etiology is related to paternal or maternal sex chromosomal nondisjunction during meiosis which result in 90% and 10% of patients with nonmosaic 47,XXY and mosaic 46,XY/47,XXY karyotype. It has been reported that primordial 47,XXY germ cells enter meiosis theoretically; 50% of the spermatozoa will carry a 24,XX or 24,XY karyotype, and 50% will have a normal 23,X or 23,Y karyotype. However, based on the reproductive outcomes of men and women with comparable numerical chromosome abnormalities (47,XXY or 47,XXX) it can be extrapolated that the Klinefelter male, who reproduces spontaneously has a chance of having a child with three sex chromosomes of less than 1%. This relative low risk is due to natural selection in favor of normal euploid sperm. Previously, it was believed that Klinefelter syndrome is sterile and has been estimated that 25% of non-mosaic patients to have sperm in their ejaculate [69, 76] but men with the mosaic form of the disease may have residual spermatogenesis in their
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Seminiferous tubules. These patients may try to achieve pregnancy using intra cytoplasmic sperm injection as they have large numbers of aneuploid gametes and are at a risk of producing offspring with CA [77].

1.9.6. Structural chromosomal aberrations

Structural CA include the rearrangement, translocation and deletions of single genes that are known to be present in ~15% of azoospermic subjects. The rate of congenital chromosomal abnormalities in newborns is found to be 0.5% [78, 79].

1.9.7. Reciprocal translocation

Reciprocal translocation is defined as the exchange of chromosomal material between the arms of two heterologous chromosomes, thus changing the order, but not the amount of genetic material. Chromosomes 12, 22 and Y are involved more often than expected on the basis of their relative lengths. A balanced reciprocal Y; autosome translocation has been demonstrated between almost every autosome carriers of balanced translocations in infertiles are at high risk, either leads to recurrent spontaneous abortions or an affected offspring that are found in 0.2% of the neonatal population [65, 80]; 0.6% of infertile couples [67]; 2 to 3.2% with severe male factor infertility [81] and 9.2% of fertile couples experiencing recurrent miscarriages [82].

1.9.8. Robertsonian translocation

These chromosomal translocations are mainly observed in D (13, 14, 15) and G group chromosomes (21, 22 and Y). Robertsonian translocations are one of the most common structural chromosomal aberrations observed in humans with an incidence of 1.23:1000 live births of which ~50% are de novo; [83] this has been shown that the frequencies are nine times higher in infertile men [84] more common in oligozoospermic and azoospermic men [58] with rates of 1.6 and 0.09%, respectively [67]. In general, carriers are phenotypically normal but are at increased risk for spontaneous abortions, are chromosomally unbalanced and lack gamete
production [76]. The D/D translocation is the most frequent type, including a high predominance of 13;14 translocation. These translocations originate through centric fusion of the long arms of the acrocentric chromosomes with the translocated chromosome bearing either one or two centromeres with a simultaneous loss of both short arms resulting in a balanced karyotype of 45 chromosomes. Whilst the chromosomes pair during meiosis, translocated chromosomes and their homologues appear as trivalent can lead to reduced fertility [85]. Malsegregation of these abnormal chromosomes could result in trisomy or monosomy of complete chromosomes [86].

1.9.9. Marker chromosome
Marker chromosomes are defined as unidentified structurally abnormal chromosomes and their consequences may range from being harmless to detrimental. Most of the marker chromosomes are supernumerary marker chromosomes (SMCs) that occur in addition to the 46 normal chromosomes or as constituents of the 46 chromosomes by replacing normal chromosomes [74]. The prevalence of SMCs at birth is estimated at 0.6-1.5:1,000 newborns [87]. About 80% of SMCs arise de novo and more frequently with advanced maternal age [88, 89], infertile men; without clinical features present a karyotype of 47,XY,+mar.

1.9.10. Variant chromosome
Chromosomal alterations include minor or “normal” polymorphic chromosomal variants of heterochromatin regions in addition to major chromosomal abnormalities. The term “variant” represents the deviations from type of chromosome morphology, and “heteromorphic” designates the homologous chromosomes with arms of different length or variable bands [90]. Heterochromatin are of two types: facultative and constitutive. Facultative heterochromatin is a reversible form, not rich in satellite DNA and becomes transcriptionally active in some phases of the cell cycle. Constitutive heterochromatin remains transcriptionally inert during the entire cell cycle [91]. Though, several reports on male infertility had discussed about the presence of chromosomal variants, most authors state that a person carrying a variant chromosome do
not have an increased risk of having spontaneous abortion, abnormal offspring, or any other reproductive problem.

1.10. The Y chromosome

Male spermatogenesis involves different genes regulating mitotic and meiotic cell divisions, as well as subsequent differentiation to spermatozoa [92] of which some are responsible for spermatogenesis, development and maintenance of male gonads [11, 93, 94]. The Y chromosome is male-specific, 60 megabases (Mb) in size, consisting of 80 different proteins and 60 million nucleotides, but has the least number of genes compared to any other chromosome.

**Figure 1.3. Genes located on chromosome Y**

Twenty seven genes on the Y chromosome were identified, nine are located on the Yp (short arm) and the remaining 18 on the Yq (long arm). Skaletsky *et al.*, (2003) had reported the complete sequence of the euchromatin segment (23 Mb), in which male specific region of the Y is designated as *MSY*. Figure 1.3. shows the diagrammatic view of some genes present on
chromosome-Y [95]. Despite intense efforts, only a few human “spermatogenic genes” have been identified but their precise function remains unknown. It has been reported that in certain ethnic group, men with specific haplotype might have lower sperm concentration; the frequency of haplotype (II) is more common in azoospermic men compared with normal men [96].

1.10.1. Y chromosomal polymorphism

Y chromosome polymorphisms have been preferentially seen in azoospermia and severe oligozoospermia. The variation in the length of Y chromosome is usually due to the distal part of the long arm that is known to contain heterochromatin. The incidence of Yqh+ in infertile men is reported to be 4.5-7.9% [97]. Nagvenkar et al., 2005 had reported the occurrence of long (Yqh+) and short (Yqh-) as 3.4 and 27.3 per cent respectively [69]. Minocherhomji et al., (2008) found Yqh+ variant in 26.9% (102 of 380) infertile men, but other authors have not reported any association between an increased risk of pregnancy loss and Yqh- variant in carriers [98].

1.10.2. Inversion

The structural rearrangements in infertile men were found to be 0.1%. Inversion occurs when two chromosomes break on the same chromosome and join after 180 degrees rotation [99]. During meiosis, chromosomes are forced to form inversion loops which enables the homologous chromosomes to pair. The formation of these inversion loops can influence fertility due to the mechanism and time limitations. Pericentric inversion of Y chromosome are reported to be more prevalent among infertile men than in newborns.

1.10.3. Yq microdeletion

Germ cell development is under the control of several genes located on autosomes and Y chromosome in mammals [100]. The Y chromosome is the smallest chromosome that contains
many genes that are critical for the development of male gonads and spermatogenesis. The variation present on the Y chromosome and deletions of large segments of the chromosome involving multiple genes make it difficult to determine the exact cause of certain infertile phenotypes in men. The prevalence of Y chromosome microdeletions in infertile men is estimated to be 1:2000 to 1:3000. The frequency in azoospermia or severe oligozoospermia men is about 5-12%, although there is a marked difference reported in different world regions.

Tiepolo and Zuffardi (1976) had hypothesized a correlation between Yq microdeletions and male infertility. These deletions were clustered in interval 5 and 6 of the Y chromosome was defined as “Azoospermia Factor” (AZF), individuals observed with its terminal deletions were azoospermic [116]. Based on the molecular results, Y chromosome has been subdivided into seven deletion intervals from A-G [117]. Three regions were defined: AZFa, AZFb and AZFc, which map on the long arm (Yq) from the centromere to the telomere. A fourth region, named AZFd, located between AZFb and AZFc was also reported [118]. Y-chromosomal genes involved in spermatogenesis mostly reside on the AZFa, b and c regions.

In the AZFa region, USP9Y is an important functional gene involves in spermatogenesis. The main candidate gene in the AZFb region is the RBMY (RNA binding motif) gene family, which has a restricted expression in the testis [119]. The AZFc region also has a specific expression in the testis and therefore, has been shown to play a crucial role [120], which represents the most frequently deleted region in infertile men. The prevalence of Yq microdeletions among infertile men from different regions are given in Table 1.4. Several genes located on critical region Yq which is defined as a chromosomal deletion that spans but not large enough to be detected using conventional cytogenetic methods and Polymerase Chain Reaction (PCR) is an important screening tool.
Table 1.4. Prevalence of Yq microdeletions in infertile men

<table>
<thead>
<tr>
<th>Country</th>
<th>Prevalence (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>India</td>
<td>5.0</td>
<td>[7]</td>
</tr>
<tr>
<td>India</td>
<td>3.33</td>
<td>[101]</td>
</tr>
<tr>
<td>Kuwait</td>
<td>7.14</td>
<td>[14]</td>
</tr>
<tr>
<td>Pakistan</td>
<td>17.6</td>
<td>[102]</td>
</tr>
<tr>
<td>Chinese</td>
<td>6.4</td>
<td>[72]</td>
</tr>
<tr>
<td>Seria</td>
<td>15.6</td>
<td>[103]</td>
</tr>
<tr>
<td>Kuwait</td>
<td>10.4</td>
<td>[104]</td>
</tr>
<tr>
<td>Russia</td>
<td>7.5</td>
<td>[105]</td>
</tr>
<tr>
<td>Saudi Arabia</td>
<td>3.2</td>
<td>[106]</td>
</tr>
<tr>
<td>Croatian</td>
<td>0.95</td>
<td>[107]</td>
</tr>
<tr>
<td>Turkey</td>
<td>9.1</td>
<td>[108]</td>
</tr>
<tr>
<td>Egypt</td>
<td>12</td>
<td>[109]</td>
</tr>
<tr>
<td>Slovenia</td>
<td>4.4</td>
<td>[110]</td>
</tr>
<tr>
<td>Belgium</td>
<td>3.9</td>
<td>[111]</td>
</tr>
<tr>
<td>Israel</td>
<td>6</td>
<td>[112]</td>
</tr>
<tr>
<td>Korea</td>
<td>20</td>
<td>[113]</td>
</tr>
<tr>
<td>United States</td>
<td>7.7</td>
<td>[114]</td>
</tr>
<tr>
<td>United States</td>
<td>17.2</td>
<td>[115]</td>
</tr>
</tbody>
</table>

1.10.3.1. Azoospermia factor a (AZFa)

The AZFa spans around 400-600kb of DNA and is located in the proximal portion of deletion interval 5. The two main protein encoding genes located in the AZFa region are USP9Y and DBY also called as DDX3Y [121]. Deletions in the AZFa region that remove both of these genes cause Sertoli cell–only syndrome, a condition characterized by the presence of complete Sertoli cells in the testes but a lack of spermatozoa in the ejaculate DBY, has a probable role in infertility because it is localized in the testis and is involved in the development of pre-meiotic germ cells [122]. The Lardone et al., (2007) had studied transcriptional activity of several genes on AZF region and found that men with Sertoli cell–only syndrome had shown reduced levels of DBY transcripts, suggesting that DBY may play an important role in spermatogenesis [123]. The USP9Y is also involved in spermatogenesis, as shortening or deletion of the USP9Y causes azoospermia [124] or oligoasthenoteratozoospermia [125]. These findings suggests that DBY
gene has a more critical role in spermatogenesis than the USP9Y gene. Further research must be performed to determine the exact role of these genes in fertility to develop more targeted Y chromosome screening practices for infertile men.

1.10.3.2. Azoospermia factor b (AZFb)

The AZFa spans around 1-3Mb of DNA and is located on the distal portion of deletion interval 5 to the proximal end of deletion interval 6. Deletions of the AZFb region cause arrest of spermatogenesis at the primary spermatocyte stage indicating that the region is essential for fertility [122]. The protein coding genes in the AZFb region are RBMY and PRY located on the ampliconic region. RBMY1 codes for an RNA binding protein, [126] which is a testis-specific splicing factor expressed in the nuclei of spermatogonia, spermatocytes, and spermatids [52]. Lavery et al. (2007) had a expression of RBMY1 in azoospermic men. PRY genes are involved in the regulation of apoptosis, an essential process that removes abnormal sperm from the population of spermatozoa [127]. The deletions of RBMY and PRY genes were studied in patients presenting with hypospermatogenesis, if both of them were removed, spermatogenesis was arrested completely, indicating that these are the major genes involved in fertility [119].

1.10.3.3. Azoospermia factor c (AZFc)

A deletion in the AZFc region produces a wide range of phenotypes, many of which are associated with low sperm concentration due to reduced spermatogenesis [52]. The AZFc spans about 3.5Mb of euchromatin and is located at the distal part of deletion interval 6 of chromosome Y. The first identified gene in the in the AZFc was “Deletion in Azoospermia Factor” (DAZ) encodes RNA-binding proteins that are expressed in the germ cells. Deletion in AZFc has been reported in approximately 12% and 6% of non-obstructive azoospermia and severe oligozoospermia cases [128]. Studies demonstrate that AZFa and AZFb regions are merely needed to initiate spermatogenesis but without the AZFc region, spermatogenesis will not be completely normal [77]. The complete deletions of the AZFc region may occur in two different ways: either as a result of a previous deletion within the AZFc or spontaneously from a normal AZFc region.
1.10.4. Sex determining region on Y (SRY) gene

Genetic analysis of cases with abnormal sexual development, presenting with chromosomal abnormalities like translocations, deletions or duplications, has resulted in the identification of many genes playing a role in sex determination [129]. The presence or absence of Y chromosome determines the sex in mammals [130]. SRY is thought to direct the sex-determination pathway towards male development [131]. SOX9 and DAX1 have recently been proposed to function downstream to SRY gene in male sex-determination pathway [132]. A karyotype of 46,XX male syndrome is a rare disorder with a frequency of 1:20 000–25,000 men, exist in three medical categories: (a) 46,XX males with normal genitalia; (b) ambiguous genitalia; and (c) true hermaphrodites with ovarian and testicular tissues [133]. An increasing number of reports suggest that the male phenotype can develop even in the absence of SRY gene [134] and apparently there are no other reports on Y-chromosome sequences. According to the presence or absence of the Y-chromosome sequences, approximately 90% of the cases carry varying amount of the Y sequences due to a recombination between X and Y chromosomes, whereas 10% do not have any Y-chromosome sequences. Most of the 46,XX men with SRY have normal genitalia, whereas most SRY-negative cases have ambiguous genitalia [135].

The testes differentiation pathway is active in sex determination and the absence of the signals results in ovary development. Except a few studies, the etiology of development in 46,XX men (SRY-negative) remains unexplained [136]. The development of the testis and normal male genitals in 46,XX (SRY-negative) males provides an indication to the autosomal or X-linked genes during the sex-determining pathway. Genetic analysis of these cases may help to study new gene(s) involved in the sex-determining pathway [137].

1.10.5. SOX gene

Camptomelic dysplasia is a rare, often lethal, congenital osteochondrodysplasia, dominantly inherited, associated with male-to-female autosomal sex reversal in two-thirds of the affected
men. This is located on the X chromosome, expressed in the urogenital ridge, from which the
gonads develop, and this continues to be expressed in the adult gonads of both sexes [138].
Like the Y-located testis-determining gene SRY, SOX9 is a member of the SOX gene family of
transcription factors that share an amino acid sequence identity of 60% or more in their high-
mobility group (HMG) domain present in SRY [100]. The HMG domain is an 80 amino acid
DNA-binding and bending motif that characterizes, besides the SOX proteins, a whole class
of transcription factors, as these mutations are all heterozygous and appear to cause loss of
function of SOX9. In humans, a large deletion (including SOX3) of the X chromosome has
been reported in men with small testes had shown deletions [139]. Thus, SOX gene should
still be considered in future studies of male infertility, till other commonly affected genes are
identified.

1.11. Genotype-phenotype correlations of Yq microdeletion

Severe combined clinical and molecular studies have sought to define recurrently deleted regions
of Yq is to determine the incidence of microdeletions among azoospermic and oligozoospermic
men, and to correlate the size and position of the deletions that causes infertile phenotype.
The reported incidence of microdeletions in infertile men is reported to be 1–55% that varies
enormously and also depends on study design and sample size. The study populations for Yq
microdeletion includes: azoospermic, oligozoospermic, azoospermic/oligozoospermic patients
or normospermic men. Most clinical studies select individuals with idiopathic azoospermia
or oligozoospermia, although others include unselected infertile men with known or unknown
causes of infertility. Unfortunately, however, there is no general agreement on what constitutes
idiopathic infertility. Varicocele and history of cryptorchidism are considered idiopathic in some
studies and non-idiopathic in others. Another variable that affects Yq microdeletion frequency
is marker density or the position of markers. Despite these, it is possible that differences in
deletion frequency and/or localization, between studies, may reflect on genuine geographic
or ethnic differences, perhaps related to a particular Y chromosome haplogroup, the genetic
background, or environmental influences.
Vogt et al., (1996) originally proposed that AZFa deletions result in Sertoli-cell only syndrome (SCOS) type I, in which no spermatogonia develop, whereas deletions in AZFb cause spermatogenic arrest, usually at the spermatocyte stage, and deletions in AZFc are associated with a more variable phenotype, ranging from type II SCOS-absence of germ cells in most testis tubules to hypospermatogenesis-presence of all germ-cell types, albeit in reduced numbers.

In general, subsequent studies have supported these findings, but there have been exceptions: both AZFa and b deletions have been reported in oligozoospermic men [52]. Another problem in the definition of genotype/phenotype correlations is the variability of the phenotype in the same man, over time. In one study, an individual with an AZFc deletion showed a progressive decrease in sperm concentration, from severe oligozoospermia to azoospermia [140].

First, microdeletions are found almost exclusively in males affected by azoospermia, severe oligozoospermia or occasionally in patients with other abnormal andrological findings. Second, a higher frequency of Yq deletions is found in azoospermic men, compared with oligozoospermic men or in men with well-defined idiopathic infertility. Third, large deletions generally are associated with mild-severe spermatogenic defects. Finally, the frequency of AZFa deletions is reported to be 1–5% is generally associated with SCOS type I, whereas AZFc, AZFa and AZFb deletions, may be associated with a variety of spermatogenetic defects, including oligozoospermia [141].

1.12. Genes associated with male infertility

1.12.1. DNA polymerase gamma (POLG) gene

Mitochondrion has the genetic material that is capable of producing many essential components of the respiratory chain. The sperm mitochondrial DNA (mtDNA) is a small, circular DNA that is not bound to proteins, exhibits a high rate of mutation, which might affect the amount of energy for sperm motility because of aberrations in the mitochondrial sheath [142]. The inheritance of mtDNA is primarily maternal, paternal transmission of mutations have been reported but not more than 1% of inheritance. Sperm mitochondria plays an important role in
spermatozoa functionality; because of the high adenosine triphosphate (ATP) [143] alterations
to mtDNA may have consequences for normal fertilization or an impact on quality of sperm
production in male factor infertility. The nuclear enzyme involved in the elongation and repair
of mtDNA strands is DNA polymerase gamma (POLG), supposed to be the only polymerase
acting in the mitochondria. The catalytic subunit of POLG is encoded by the \textit{POLG} gene, 
which has been mapped to Yq15 (15q25). Studies have indicated an association between \textit{POLG}
polymorphisms or mutations in the mitochondrial genome and sperm dysfunction [144] such
as asthenozoospermia.

1.12.2. Sex hormone-binding globulin (\textit{SHBG}) gene
The \textit{SHBG} gene (located on chromosome 17) is shown to be involved in both delivering sex
hormones to target tissues and controls concentration of androgens in the testis. Androgens
play an essential role in sexual differentiation and spermatogenetic process; if androgen levels
are interrupted, fertility might decrease. Several studies have revealed that \textit{SHBG} alleles were
associated with altered levels of spermatogenesis and male infertility.

1.12.3. Follicle stimulating hormone receptor (\textit{FSHR}) gene
The FSH is an essential hormone necessary for the normal functioning of gonads which
mediates its function by binding with its receptor. The FSH receptor-\textit{FSHR} gene is located on
chromosome 2; it has been shown that partial deletions of the \textit{FSHR} gene might have slight
effects on spermatogenesis [145]. In addition, \textit{FSHR} Single-Nucleotide Polymorphism (SNP)
had been reported in the male infertility [76].

1.12.4. Deleted in azoospermia-like (\textit{DAZL}) gene
The autosomal homologue of the \textit{DAZL} gene is located on chromosome 3 and codes for the
RNA binding proteins that are involved in the regulation of protein expression and meiosis
[146]. Tung \textit{et al.}, in 2006 had identified four new mutations and haplotypes in the \textit{DAZL} gene
related to sperm count, but further studies are required to determine their effects [147] on male
factor infertility.
1.12.5. Methylene tetrahydrofolate reductase (MTHFR) gene

The MTHFR gene is located on the short arm of chromosome 1 [148]. It codes for an enzyme the tetrahydrofolate reductase, involved in folate metabolism, an essential factor in DNA methylation and spermatogenetic process. The polymorphism 677C/T causes the substitution of an alanine for a valine, which decreases the activity of the enzyme. The reduced activity of MTHFR can lead to the dysregulation of folic acid metabolism, causing errors in the methylation of genomic DNA and subsequent implications in spermatogenesis. Further studies are required to confirm the role of this gene on spermatogenesis [146].

1.12.6. Insulin-like factor 3 (INSL3) gene

Mutations in the INSL3 gene (located on chromosome 19) and its receptor LGR8 gene (relaxin/insulin-like family peptide receptor 2) (located on chromosome 13) have been associated with cryptorchidism. The first phase of normal testicular descent is controlled by INSL3 [149] and may have a role in testicular dysgenesis syndrome (TDS), that are associated with hypospadias, testicular cancer, and infertility [150].

1.12.7. Estrogen receptor gene

Estrogen receptor genes (ESR1 and ESR2) have a possible involvement in fertility. Recent reports have identified the relationship between abnormal spermatogenesis and estrogen insufficiency. ESR1 gene is located on chromosome 6 this has several different polymorphisms. The promoter region of ESR1 has a variable number of tandem repeats, (TA)n polymorphism that is related to sperm output: a higher number of repeats on both alleles is correlated with lower levels of spermatogenesis. These genes have been studied for their role in male factor infertility, especially in relation to severe oligozoospermia, with varied results and that may be most likely due to gene interactions, environment factors and different ethnic groups [151].

1.12.8. Cystic fibrosis transmembrane conductance regulator (CFTR) gene

Congenital unilateral/bilateral absence of vas deferens is a frequent cause of azoospermia. About 1-2% male infertility is associated with mutations and polymorphisms in CFTR gene [152].
Polymorphisms outside and within the *CFTR* gene may affect transcription or function of the CFTR protein eg: F508del mutation and R347H, M470V polymorphism [76, 153]. Mutation analysis of the *CFTR* gene in CBAVD cases has shown that a large proportion of these sterile men carry a mutation in at least one of their *CFTR* genes. It has been suggested that the CFTR protein may also be involved in the process of spermatogenesis or sperm maturation apart from the development of epididymal glands and vas deferens.

### 1.12.9. Androgen receptor (*AR*) gene

Androgen insensitivity syndrome is alternatively called as the testicular feminization syndrome is a disease characterized by variable defects in virilization. It occurs due to loss of functional mutations in the androgen receptor gene that results in peripheral androgen resistance for development and maintenance of the male phenotype [154] and spermatogenesis [155]. It is a single copy gene that spans ~90Kb of genomic DNA and lies on the Xq11-12 region on long arm of X-chromosome., expressed in the testis and involves conversion of spermatocytes to round spermatids during spermatogenesis.

### 1.12.10. Kallman syndrome-1 sequence (*KAL1*) gene

Kallmann syndrome is another genetic condition that can cause male infertility and has both X-linked and autosomal genetic components, defined as idiopathic hypogonadotropic hypogonadism combined with anosmia or hyposmia. This disorder is caused by a defect in the migration of the GnRH neurons and characterized by low levels of sex steroids in combination with low to normal levels of FSH and LH [156]. This leads to the absence or low levels of sex steroids inhibit; stunt sexual development and spermatogenesis in men. *KAL1* gene is located on the short arm of the X chromosome, involved in the migration of the GnRH neurons, and codes for anosmin-1, a cell adhesion molecule.
1.13. Paternal age

There is a general belief that the fertility potential of older man is fairly well preserved. However, recent evidence support the concept that advanced paternal age is associated with an increase in sperm chromosomal aneuploidies. Moskovtsev et al., (2006) demonstrated that the rate of sperm with fragmented DNA doubled in men at the age of 45 years and above when compared to those less than 30 years old [157]. Siddighi et al., (2007) showed increased necrosis, DNA damage and apoptosis while rapid progression and total motility declined with advancing male age beginning as early as 35 years [158]. DNA damage in sperm is promutagenic, it does not impair fertilization or cleavage as paternal genome is transcriptionally inactive till two days after fertilization. However, once the paternal genome is active it results in poor blastocyst development, unequal cleavage, implantation failure or early fetal loss. Plastira et al., (2007) demonstrated that increased age in infertile patients was associated with an increase in sperm DNA fragmentation and poor chromatin packaging, as well as with a decline in semen volume, sperm morphology and motility [159]. Several studies have reported that sperm DNA damage increases with advancing age in both fertile and infertile men.

The scrutiny of literature suggests that the genetic basis of infertility is unequivocal and varying; it includes chromosomal abnormalities, mutations as well as polymorphisms and is multifactorial. It is very difficult to accurately assess the overall importance and contribution of individual genes and role to infertility. At present, with the advent of assisted reproductive technologies and the possibility of vertical iatrogenic transmission of genetic anomalies to the offspring, diagnosis of a genetic etiology is very significant, which helps to counsel these patients about the risk of an offspring being born with genetic anomalies. An association between male infertility and genetic abnormalities has been reported that there is an increased risk of embryos with chromosomal abnormalities, which could be one of the main reasons for implantation failure or recurrent spontaneous abortions.
Therefore, the aim of the study is to investigate the chromosomal aberrations, azoospermia factor (AZF), cystic fibrosis transmembrane regulatory (CFTR) gene mutations in the peripheral blood lymphocytes (PBL) of infertile men with abnormal sperm parameter and compare it with healthy control subjects to delineate the genetic causes of male infertility.

OBJECTIVES OF THE STUDY

• To study the prevalence of chromosomal abnormalities (CA) in the entire genome of infertile men with abnormal spermiogram (azoospermia (AZ); oligoasthenoteratozoospermia (OAT) severe oligoasthenoteratozoospermia (SOAT) globozoospermia and compare it with fertile controls.

• To characterize the most common forms of chromosomal abnormalities such as numerical (CA), structural (CA) and variants.

• To screen microdeletion in the AZFa (sY84, sY86), AZFb (sY127, sY134) and AZFc (sY254, sY255) genes on Y chromosome.

• To study M470V polymorphism in the CFTR gene in relevance to male infertility.
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Introduction


