CHAPTER 1

Introduction

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Subfertility, defined as one year of unprotected intercourse without conception, affects 10-15% of couples (Hull et al., 1985; De Kretser 1997; Snick et al., 1997; Evers 2002). According to the World Health Organization (WHO) in 47% of subfertile couples semen parameters are decreased (WHO 1987). However, the prognostic value of decreased semen parameters is limited in establishing the contribution of the male partner, as female parameters also contribute to the couple's fertility, and sperm measurements that discriminate between fertile and subfertile men are not well defined (Bonde et al., 1998; Guzik et al., 2001). Therefore, the exact prevalence of male subfertility is not known. Nevertheless, azoospernia and severe oligoasthenoteratozoospernia are significant aspects of male subfertility.

Male subfertility can be categorized as due to pre-testicular, testicular and post-testicular factors (De Kretser 1997). Hyperprolactinemia and hypogonadotropic hypogonadism are among the pre-testicular disorders. Epididymal or vasal obstruction and sexual dysfunctions are among the post-testicular factors. A relatively common cause in clinical practice is azoospernia resulting from vasectomy. Congenital bilateral absence of the vasa deferentia (CBAVD) is one of the symptoms in cystic fibrosis but can also be present without the respiratory component (Chillon et al., 1995; De Braekeleer and Ferec 1996).

Testicular dysfunction results in reduced semen parameters due to impaired spermatogenesis. The etiologic factors that cause impaired spermatogenesis can be divided in acquired and congenital factors. Acquired testicular failure can be due to chemotherapy, drugs, irradiation, torsion and orchitis (De Kretser 1997). Cryptorchidism is one of the congenital factors that can result in impaired spermatogenesis (Chilvers et al., 1986; Lee et al., 1996). In addition, several genetic causes have been identified in men with subfertility due to testicular failure. Numerical chromosomal abnormalities and structural chromosomal abnormalities have been found in patients with azoo- or oligozoospernia (Tuerlings et al., 1998; Hargreave, 2000; Dohle et al., 2002). Among the numerical chromosomal abnormalities the Klinefelter syndrome, which are men with a 47XXY phenotype who are in general subfertile, is most common. Also, five classes of Y chromosome deletions, AZFa (Azoospermia Factor a), P5/proximal-P1, P5/distal-P1, P4/distal-P1 and b2/b4 (AZFc) deletions, cause spermatogenic failure (Reijo et al., 1995; Vogt et al., 1996; Kremer et al., 1997; Repping et al., 2002 and 2003).

Male subfertility mostly occurs as an isolated disorder in otherwise healthy men but can also be among the symptoms of a complex disorder or a monogenic disorder. In Online Mendelian Inheritance in Man (OMIM), a genetic database of monogenic disorders, conditions associated with male subfertility are reported. In most syndromes relevant in this context, male subfertility is due to hypogonadotropic hypogonadism, cryptorchidism,
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delayed puberty, or male pseudohermaphroditism and ambiguous genitalia. Monogenic disorders are rare but as a group they may contribute to the total frequency of male subfertility (Meschede and Horst, 1997). Despite the known aetiologic factors listed above, in up to 40% of subfertile men the aetiology is unknown and subfertility in these men is classified as idiopathic (De Kretser, 1997). Idiopathic impaired spermatogenesis, in otherwise healthy subfertile men, is the phenotype under study in this thesis.

There are hardly any means to directly treat male subfertility. Treatment with a dopamine-agonist can be successful in men with hyperprolactinemia. Men with hypogonadotrophic hypogonadism can be treated with gonadotrophins. Surgical correction of the vasa deferentia can be successful after vasectomy although anti sperm auto antibodies may have been generated that still cause subfertility (Alexander and Anderson, 1979; Clayton and Moore, 2001).

In general, however, treatment of male subfertility is not possible. The best option for these men is to try and achieve a pregnancy by assisted reproductive techniques (ART). At present, intrauterine insemination (IUI) is the first line treatment for moderate male factor subfertility (Ombelet et al., 2003). In vitro fertilization (IVF) combined with intracytoplasmic sperm injection (ICSI) is the only therapeutic option for couples with subfertility due to severely decreased sperm counts (Palermo et al., 1992; Tournaye, 2000; Campbell and Irvine, 2000). Surgically retrieved sperm cells from the epididymis or the testis are used for ICSI in men with azoospermia (Palermo et al., 1999; Tournaye, 1999).

These assisted reproductive techniques (ART) place a heavy burden on the normal fertile female partners of the subfertile men. These women have to undergo controlled ovarian hyperstimulation and, in case of IVF and ICSI, follicle aspiration, which both are potentially risky procedures. Another negative side effect accompanying these treatments is a high rate of multiple pregnancies with more complications compared to singleton pregnancies (Lambert, 2002).

Above all, there has been major concern about the rate of birth defects of the ICSI offspring (Bowen et al., 1998; Meschede et al., 1998; Johnson, 1998; te Velde et al., 1998; Campbell and Irvine, 2002). Several follow up studies have been published in the past years. Fortunately, most of these studies report no increase in the incidence of major birth defects after ICSI (Palermo et al., 1996; Wennerholm et al., 2000; Sutcliffe et al., 2001; Bonduelle et al., 2002 and 2003). Only one follow up study reported a twofold higher risk of a major birth defect in ICSI children as in naturally conceived infants (Hansen et al., 2002).

Recently, a higher incidence of the imprinting disorders Beckwith Wiedemann and Angelman syndrome has been reported in children conceived by ICSI (Cox et al., 2002; DeBaun et al., 2003; Maher et al., 2003; Orstavik et al., 2003). Imprinting is a mechanism
in which gene expression depends on the parental origin of the allele. It has been suggested that the use of ICSI could increase the risk for imprinting disorders (Maher et al., 2003; Devroey and Van Steirteghem, 2004). However, the exact risk is not yet clear as this association is only based on case reports and uncontrolled cohorts.

In addition, Y chromosome deletions are transmitted to sons by ICSI and therefore these boys are likely to be subfertile in adulthood (Kamischke et al., 1999; Page et al., 1999). If other yet unknown genetic aberrations play a role in impaired spermatogenesis, these genetic abnormalities might also be transmitted to the next generation by ICSI with a possible negative impact on fertility. It is therefore quite obvious that further research into the genetic causes of male subfertility due to impaired spermatogenesis is needed.

Before the writing of this thesis, there were some indications that idiopathic impaired spermatogenesis has a genetic background. Several families with multiple subfertile male family members had been described in which a genetic defect segregates through the family, indicating that impaired spermatogenesis can be a heritable condition. (Chaganti and German, 1979; Leonard et al., 1979; Shabtai et al., 1980, Cantu et al., 1981; Rivera et al., 1984; Meschede et al., 1994; Chang et al., 1999; Saut et al., 2000; Rolf et al., 2002; Tuerlings et al., 2002).

Familial clustering of male subfertility had been observed in a case control study, which could be explained by an autosomal recessive mode of inheritance in the majority of cases (Lilford et al., 1994). In this study, conclusions were based on a significantly increased number of subfertile brothers of men with reduced sperm counts as compared to fertile controls. These findings were supported by a study describing that men with an ICSI indication had fewer siblings than fertile controls (Meschede et al., 2000). The fertility status of a couple, however, is not exclusively based on semen parameters as female factors play an important role as well. In both studies, the control group consisted of fertile men of whom no semen parameters were available. Reporting bias might have influenced the data found in the case control study, as fertile controls are probably less informed about fertility problems among their relatives, than men with reduced semen parameters (Van der Avoort et al., 2003). Therefore, these studies are not sufficient to conclude that male subfertility due to impaired spermatogenesis clusters in a quantitative amount. Thus we explored to what extent spermatogenic failure has a familial background and which mode of inheritance might be involved. In addition, during our research, we came across a unique family with five brothers who were infertile due to azoo- or very severe oligozoospermia. In this family, we performed a detailed genetic evaluation to identify whether there is a single genetic cause of the infertility.

In addition to family studies, genetic studies in several different animal models like the yeast S. cerevisiae, the worm C. elegans, the fly Drosophila and the mouse have provided evidence
for existence of hundreds of X chromosomal and autosomal genes that can mutate to male sterile alleles (Hackstein et al., 2000; Venables and Cooke, 2000). Therefore, it is very suggestive that yet unknown autosomal genes are involved in idiopathic impaired spermatogenesis.

To identify genes that are not yet known as disease causing genes in humans, several methods have been described. As the pathogenesis of impaired spermatogenesis and the biochemical functions of genes that might be involved are not known at this moment, functional cloning strategies can not be used. Population based mapping studies, like linkage analysis and association analysis, have been described as promising approaches to detect disease-causing genes (Strachan and Read 1999). In this thesis we evaluated whether these methods are proper tools in studying idiopathic impaired spermatogenesis.

A more direct approach to identify disease-causing genes is screening of candidate genes for mutations in men with idiopathic impaired spermatogenesis. Based on genetically manipulated animal models and specific expression in testis, a large number of genes has been put forward as candidates. In this thesis we decided to focus on three candidate genes located on chromosomal region 11p15: the Zinc Finger (ZNF) genes 214 and 215 and the Heterogeneous Nuclear Ribonucleoprotein G-T (HNRNP G-T) gene. In addition we concentrated on the Protein C Inhibitor (PCI) gene, which is very likely to play a role in teratozoospermia as a recent study reported this specific subfertile phenotype in PCI knockout mice (Uhrin et al., 2000).

To study genes possibly involved in idiopathic severely impaired spermatogenesis we defined very stringent inclusion criteria for patient selection: otherwise healthy men who did not conceive a pregnancy within one year of unprotected intercourse, with a total sperm count of less than $20 \times 10^6$ spermatozoa in two consecutive semen samples, for which no known cause could be identified. Data were compared with a control group consisting of men recruited from the same population but with normozoospermia defined as a total sperm count of at least $40 \times 10^6$ spermatozoa with a progressive motility and normal morphology of at least 40%, in two consecutive semen samples. Patients and controls were included consecutively during the whole study period. All data generated in this thesis derived from this still growing study cohort.
Aim of the thesis

The aim of this thesis was:

1. To study whether male subfertility due to impaired spermatogenesis has a familial component and, in case there is, to study the mode of inheritance.
2. To establish the possible genetic basis of impaired spermatogenesis in a family with several subfertile brothers with severely reduced semen parameters.
3. To evaluate whether genetic epidemiology provides proper tools to identify genes involved in idiopathic impaired spermatogenesis.
4. To test whether chromosomal region 11p15 is associated with impaired spermatogenesis and to screen the ZNF214 and ZNF215 genes, which are located on this region, for mutations in men with spermatogenic failure.
5. To screen the candidate gene HNRNP G-T for mutations in men with idiopathic impaired spermatogenesis.
6. To screen the candidate gene PCI for mutations in men with idiopathic azoospermia or teratozoospermia.
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Outline of the thesis

In CHAPTER 2 we present the results of a case control study, comparing the prevalence of subfertility among relatives of patients with severe idiopathic impaired spermatogenesis with the prevalence of subfertility among relatives of patients with normal semen parameters. If permission was given, semen parameters of the subfertile brothers were collected in both groups. In addition, a segregation analysis was performed.

In CHAPTER 3 we report a unique family with five brothers who are infertile because of severely impaired spermatogenesis. To elucidate the possible genetic basis for the infertility in this family, we screened all five brothers as well as their parents and two other paternally related family members for known genetic causes of impaired spermatogenesis. In addition, results of an extensive analysis of the Y chromosome, X chromosomal linkage analysis, and mutation analysis of the mitochondrial DNA are presented.

In CHAPTER 4 we give an overview of the genetic basis of impaired spermatogenesis. Various genetic models that might be involved in male subfertility are described. In addition, we discuss the applicability of several methods, which potentially could identify genes that might play a role in impaired spermatogenesis.

In CHAPTER 5 we studied the involvement of two candidate genes, Zinc Finger 214 (ZNF214) and Zinc Finger 215 (ZNF215), located on chromosomal region 11p15 in idiopathic impaired spermatogenesis and impaired spermatogenesis due to cryptorchidism. We performed an association study based on allele and estimated haplotype frequencies of common single nucleotide polymorphisms in those genes. Additionally, both genes were screened for mutations.

In CHAPTER 6 we present the results of a mutation screen in the heterogeneous nuclear ribonucleoprotein G-T (HNRNP G-T) gene, also located on chromosomal region 11p15. The specific function of this gene is not known, but it is thought to play a role in cell-specific pre-mRNA splicing in germ cells. Therefore, we hypothesized that the HNRNP G-T gene might be involved in male subfertility and performed a mutation screen in patients with idiopathic impaired spermatogenesis.

In CHAPTER 7 we describe studies on the role of the Protein C inhibitor encoded by the PCI gene in human teratozoospermia and azoospermia. Several reasons why PCI might be involved in male subfertility are discussed. In this study we performed a mutation screen in men with severe teratozoospermia or idiopathic azoospermia. In addition, the PCI antigen
level is evaluated in semen plasma of patients in which a genetic aberration was found.

In CHAPTER 8 we summarize the results of the studies presented in this thesis and give implications for future work in the search for genetic factors involved in male subfertility.

References


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Page, D.C., Silber, S. and Brown, L.G. (1999) Men with infertility caused by AZFc deletion can produce sons by intracytoplasmic sperm injection, but are likely to transmit the deletion and infertility. Hum. Reprod., 14, 1722-1726.


