Department of Production Animal Medicine
Faculty of Veterinary Medicine
University of Helsinki
Finland

IMPAIRED SPERMATOGENESIS IN FINNISH BOARS AND BULLS

Christine Kopp-Kuhlman

ACADEMIC DISSERTATION

Supervisors

Professor Magnus Andersson
Department of Production Animal Medicine
Faculty of Veterinary Medicine
University of Helsinki
Finland

Professor Antti Sukura
Department of Veterinary Biosciences
Faculty of Veterinary Medicine
University of Helsinki
Finland

Reviewers

Professor Dr. Dagmar Waberski
Unit for Reproductive Medicine
University of Veterinary Medicine
Hannover
Germany

Docent Matti Vierula
Department of Physiology
Faculty of Medicine
University of Turku
Finland

Opponent

Professor Lennart Söderquist
Division of Comparative Reproduction, Obstetrics and Udder Health, Department of Clinical Sciences
Faculty of Veterinary Medicine and Animal Science
Swedish University of Agricultural Sciences
Uppsala
Sweden

Chairman

Professor Magnus Andersson
Department of Production Animal Medicine
Faculty of Veterinary Medicine
University of Helsinki
Finland

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Contents

Abstract

Maintenance of breeding efficiency and high semen quality is essential for reproductive success in farm animals. Early recognition of possible inheritable factors causing infertility requires constant attention. This thesis focuses on describing different manifestations of impaired spermatogenesis, their impact on fertility and partly also their incidence in populations.

The reasons for spermatogenic failure are various. An interruption of germ cell differentiation, spermatogenic arrest, can lead to infertility. The incidence of azoospermia was investigated in the 1996–2005 survey of Finnish AI and farm breeding boars. We focused on the diagnosis, testicular morphometry and the possible reasons for the condition. The incidence of azoospermia was significantly higher in Yorkshire boars than in the Landrace breed. The most common diagnosis in Yorkshire boars was germ cell arrest at the primary spermatocyte level. The second most frequent diagnosis in Yorkshire boars was segmental aplasia of the Wolffian ducts with idiopathic epididymal obstruction. Other reasons for azoospermia were infrequent.

Certain sperm defects are associated with impaired fertility in humans and also in farm animals. In the second study we investigated the incidence of two relatively well-defined specific sperm defects in Finnish Yorkshire and Landrace boars during the same survey, the immotile short-tail sperm (ISTS) defect and the knobbed acrosome (KA) defect. In the Finnish Yorkshire boars the inherited ISTS defect, and the probably inherited KA defect, were important causes of infertility during 1996–2005. The ISTS defect was found in 7.6% and the KA defect in 0.8% of the Yorkshire boars. No Landrace boars were diagnosed with either of these two defects.

A significant and inverse relationship between morphology and sperm aneuploidy has been described in men. In the third study we described a new sterilizing sperm defect in an oligoasthenoteratozoospermic bull. Because of its morphological characteristics this defect was termed the multinuclear-multiflagellar sperm (MNMFS) defect. The number of Sertoli cells in the seminiferous tubuli was highly increased in the MNMFS bull compared with the number in normal bulls.

In the following two studies we used a combined approach of fluorescence *in situ* hybridization (FISH), flow cytometry and morphometric studies to provide information on the cytogenetic background of macrocephalic bull spermatozoa. We described cellular features of diploid spermatozoa and compared the failures in the first and second meiotic divisions.

Spermatogonial stem cell transplantation is a relatively new technique in the field of male infertility. In the last study we describe how the transplantation of testicular cells was used to determine whether spermatogonia derived from donor animals are able to colonize and produce motile spermatozoa in immune-competent unrelated boars suffering the ISTS defect. Transplantation resulted in complete focal spermatogenesis, indicated by the appearance of motile spermatozoa and confirmed by genotyping.

List of original publications

This thesis is based on the following original publications referred to in the text by their Roman numerals:

I   Kopp C, Ijäs R, Flyckt A, Taponen J, Parvinen M, Andersson M. Morphometric evaluations of testicular tissues from azoospermic boars in Finnish Yorkshire and Landrace breeds. Theriogenology 2008; 15; 70(7): 1129-1135.

II   Kopp C, Sironen A, Ijäs R, Taponen J, Vilkki, J, Sukura, A, Andersson, M. Infertile boars with knobbed and immotile short-tail sperm defects in the Finnish Yorkshire breed. Reproduction in Domestic Animals 2008; 43(6): 690-695.

III   Kopp C, Sukura A, Tuunainen E, Gustavsson I, Parvinen M, Andersson M. Multinuclear-multiflagellar sperm defect in a Bull – a new Sterilizing Sperm Defect. Reproduction in Domestic Animals 2007; 42: 208-213.

IV   Revay T, Kopp C, Flyckt A, Taponen J, Ijäs R, Nagy S, Kovacs A, Rens W, Rath D, Hidas A, Taylor JF, Andersson M. Diploid spermatozoa caused by failure of the second meiotic division in a bull. Theriogenology 2010; 73: 421-428. Epub 2009 Dec 2.

V   Revay T, Nagy S, Kopp C, Flyckt A, Rens W, Rath D, Hidas A, Kovacs A, Johannisson A, Rodriguez-Martinez H, Andersson M. Macrocephaly in bull spermatozoa is associated with nuclear vacuoles, diploidy and alteration of chromatin condensation. Cytogenetic and Genome Research 2009; 126(1-2): 202-209. Epub 2009 Dec 9.

VI   Mikkola M, Sironen A, Kopp C, Taponen J, Sukura A, Vilkki J, Katila T, Andersson M. Transplantation of normal boar testicular cells resulted in complete focal spermatogenesis in a boar affected by the immotile short-tail sperm defect. Reproduction in Domestic Animals 2006; 41: 124-128.

Abbreviations

AI	artificial insemination
FISH	fluorescence *in situ* hybridization
M1	first meiotic division
M2	second meiotic division
KA	knobbed acrosome
MNMFS	multinuclear-multiflagellar sperm defect
DNA	deoxyribonucleic acid
ISTS	immotile short-tail sperm defect
L	Landrace
Y	Yorkshire
TEM	transmission electron microscopy
CFTR	cystic fibrosis transmembrane conductance regulator
CBAVD	congenital bilateral absence of the vas deferens
CUAVD	unilateral absence of the vas deferens
ICSI	intracytoplasmic sperm injection
MMR	DNA Mismatch Repair genes

1. Introduction

Spermatogenesis is a long process of cell differentiation in which spermatogonia proceed through series of mitotic and meiotic cell divisions, and complex cytological transformations lead to the formation of haploid spermatozoa (Garcia-Gil et al. 2002). This process requires the expression and precise coordination of a multitude of genes (Kostova et al. 2007). Successful spermatogenesis requires normal testicular development and formation of the extratesticular ductal system (Thielemans et al. 1998). Before ejaculation, spermatozoa liberated from the germinal epithelium must pass through the rete testis, the efferent ducts, the epididymis, the vas deferens, the ejaculatory duct, and the urethra (Meschede et al. 1998).

The seminal ducts are mostly understood to refer to parts of the male genital track derived from the mesonephric (Wolffian) ducts; the epididymides, the vasa deferentia, the ejaculatory ducts and the seminal vesicles. The seminal ducts form a mechanical passageway for germ cells. Azoospermia, due to an obstruction of the genital tract, is one of the possible pathophysiological mechanisms that causes infertility. The obstruction of the seminal ducts may be congenital or acquired (Meschede et al. 1998). Earlier studies suggest that idiopathic azoospermia often have a genetic basis (Sadeghi-Nejad and Farrokhi 2006). A strong association between mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene and various subtypes of obstructive azoospermia has been established (Meschede et al. 1998). Congenital bilateral absence of the vas deferens (CBAVD) in humans is mostly caused by homozygosity or composite heterozygosity in the CFTR gene (Sadeghi-Nejad and Farrokhi 2006).

The most common chromosomal anomaly in azoospermic men is Klinefelter syndrome (XXY). Both maternal and paternal origins are described for this syndrome (Sadeghi-Nejad and Farrokhi 2006). Almost all non-mosaic XXY males are azoospermic, while mosaic XXY/XY males may have the capacity for producing spermatozoa in variable numbers (Egozcue et al. 2000; Sadeghi-Nejad and Farrokhi 2006). Klinefelter's syndrome with azoospermia and testicular hypoplasia has been diagnosed also in boars (Mäkinen et al. 1998).

The reasons for spermatogenic failure are various, and an interruption of germ cell differentiation, spermatogenic arrest, can lead to subfertility or infertility. This condition may be associated with altered hormone secretion or genetic abnormalities. Several studies have described cases of spermatogenic arrest in men, mice, rats and bulls (Moura and Erickson 2001).

Azoospermic boars with two descended testicles can have normal libido but no spermatozoa in the ejaculate. The reasons for azoospermia in these males can be congenital or acquired. In this thesis one of the aims was to survey boars with azoospermia in two Finnish pig breeds and to focus on the diagnosis and possible reasons for the condition. Ejaculates of 2048 boars were collected in the period 1996–2005. All boars were intended for use in breeding and had two descended testes. The most frequent diagnoses of azoospermia were arrested spermatogenesis at the pachytene spermatocyte stage and segmental aplasia of the Wolffian ducts. One of the boars with "Sertoli cell only" was cytogenetically diagnosed with Klinefelter's syndrome.

Specific sperm defects have been associated with male infertility for many years (Donald and Hancock 1953; Blom 1976). Inherited or presumably inherited specific sperm defects in humans include globozoospermia, where spermatozoa lack an acrosome (Aitken et al. 1990; Moretti et al. 2005), and the immotile cilia syndrome, where dynein arms are affected (Afzelius 1976). In bulls, boars, stallions, rams and dogs accordingly reported defects are the knobbed acrosome (KA) defect (Hurtgen and Johnson 1982; Toyama and Itoh 1993; Soderquist 1998; Chenoweth 2005; Santos et al. 2006) and phenotypically different sperm tail defects affecting the length of flagella in humans, bulls, stallions and boars (Ross et al. 1973; Vierula et al. 1987; Hrudka et al. 1991; Andersson et al. 2000; Sukura et al. 2002).

In the period 1996 to 2006, two specific sperm defects with strong negative associations with fertility were observed in Finnish breeding boars - the KA defect and the immotile short-tail sperm (ISTS) defect. In this thesis the incidence of these two specific sperm defects and their effect on fertility and testicular histology in two pig breeds were examined.

The treatment of human infertility using modern techniques, especially intracytoplasmic sperm injection (ICSI), has increased the interest in sperm defects, since the technique allows evaluation of the fertilizing potential of even severely abnormal sperm (In't Veld et al. 1997; Kahraman et al. 1999; Tempest and Griffin 2004; Machev et al. 2005). The risk of producing chromosomally abnormal embryos or offspring and embryonic death or abortion was evaluated for different human sperm defects (Kahraman et al. 1999). Moreover, mutant mouse models of specific genes have markedly increased our knowledge of the origin of specific sperm defects (Adham et al. 2001; Yao et al. 2002). In cattle breeding, superior sires and breeding lines have a decided impact on the genetics of future generations and certain genes are enhanced in the population to produce more profitable offspring. This may cause an increase in certain recessive genes, resulting in male sterility, embryonic death, abortions or malformations in calves (Donald and Hancock 1953; Shanks and Robinson 1989; Agerholm et al. 2001). This thesis describes a new sperm defect in a bull; the multinuclear-multiflagellar sperm (MNMFS) defect.

Diploidy, tetraploidy and aneuploidy of macrocephalic spermatozoa are mainly described in humans. In these described cases, the first (M1), the second (M2), or both the meiotic divisions are affected. Continuous presence of a large proportion of morphologically abnormal spermatozoa is often related to genetic abnormalities in animals (Barth and Oko 1989). The exact analysis of chromosomal content of spermatozoa became possible with the use of fluorescence *in situ* hybridization (FISH). In this thesis, both M1 and M2 types of meiotic disorders are described in bulls using flow cytometry and FISH.

Spermatogonial stem cell transplantation is a relatively new technique in the field of male infertility. The first successful transplantation was performed in mice in 1994 (Brinster and Avarbock 1994; Brinster and Zimmerman 1994). In large domestic animals, germline stem cells were transplanted in pigs (Honaramooz et al. 2002), goats (Honaramooz et al. 2003 a,b) and cattle (Izadyar et al. 2003). At the end of this thesis a description is provided of how the transplantation of testicular cells was used to determine whether spermatogonia derived from donor animals can colonize and produce motile spermatozoa in immune

competent unrelated Finnish Yorkshire boars with the hereditary ISTS defect (Andersson et al. 2000; Sironen et al. 2002; Sukura et al. 2002).

2. Review of the literature

2.1.Spermatogenesis and meiosis in a male

Spermatogenesis is the process of sperm cell development. Rounded immature sperm cells undergo subsequent mitotic and meiotic divisions and a morphologic change to produce spermatozoa. The process is shown in following figures (Fig. 1 and 2).
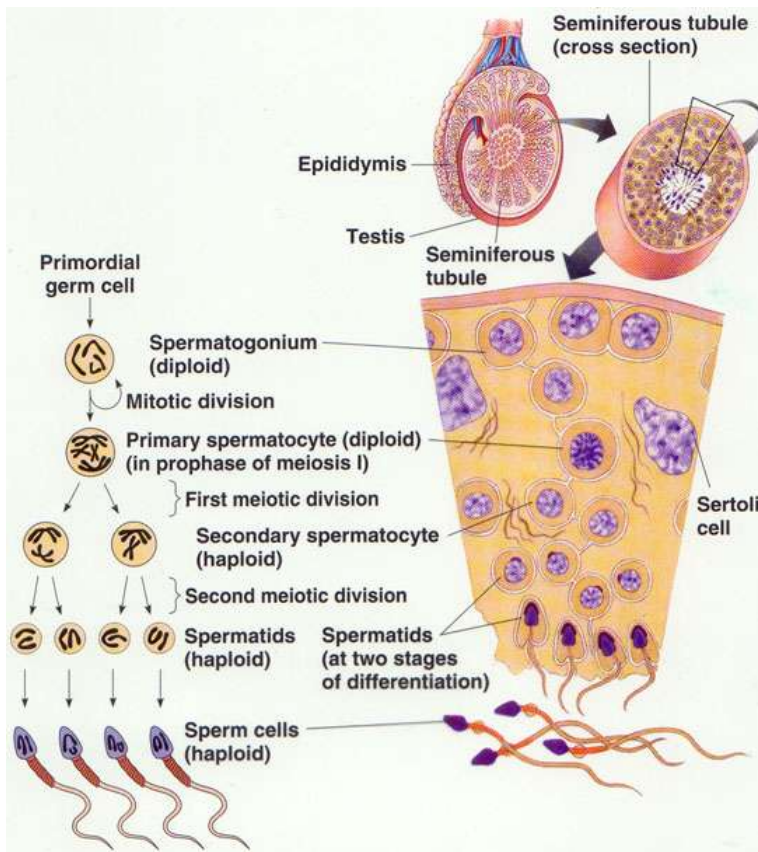

Figure 1. The stages of spermatogenesis are illustrated on the right.
On the left a diagram summarizing the stages of meiosis during spermatogenesis.
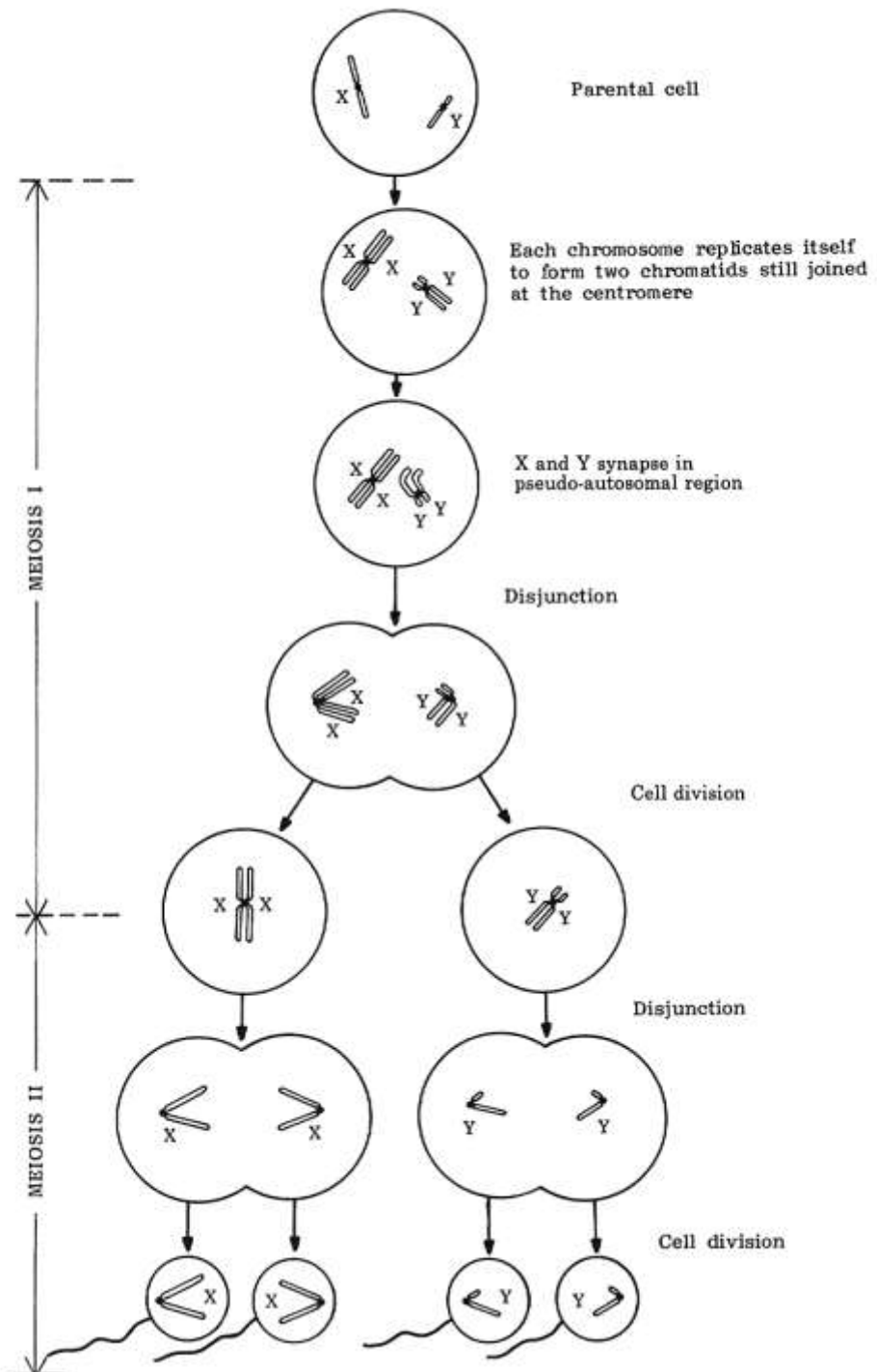*(Figure http://iceteazegeg.wordpress.com/2009/02/25/gametogenesis/spermatogenesis/)*

Figure 2 . Gametogenesis in a male. (Figure. Nicholas 2003, page 9)

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2.2 Azoospermia

Azoospermia (the absence of spermatozoa from the ejaculate) can be divided into pre-testicular, testicular and post-testicular azoospermia. Pre-testicular includes all types of azoospermia, whether congenital, acquired or idiopathic (Sharif 2000). Pre-testicular reasons for azoospermia have seldom been reported in farm animals. Testicular azoospermia includes testicular disorders, such as Klinefelter's syndrome (Maduro and Lamb 2002; Mau-Holzman 2005; Wattendorf and Muenke 2005) and arrested spermatogenesis (Moura and Erickson 2001; Kostova et al. 2007). Post-testicular azoospermia includes absence or occlusions of the efferent ductules, epididymis, vas deferens, ejaculatory duct and urethra (Sharif 2000).

2.2.1 Klinefelter's syndrome

The most common cytological anomaly in azoospermic men is 47,XXY, Klinefelter's syndrome, representing 82.5% of all sex chromosome abnormalities. Both maternal and paternal origins have been described. The typical Klinefelter male has spermatogenic and androgenic failure, but there is great variability. Klinefelter males can be of pure 47,XXY karyotype or a mosaic 46,XY/47,XXY (Sadeghi-Nejad and Farrokhi 2006). The usual cause of the uneven set is non-disjunction during mitosis at an early stage of embryo development. Since each of the cell lines has come from a single source, the individuals are termed mosaics (Nicholas 2003). Most of the non-mosaic XXY males are azoospermic, while mosaics may have the capacity for a minimal amount of spermatogenesis (Sadeghi-Nejad and Farrokhi 2006). The extra X chromosome does not permit the survival of germ cells in the testis, resulting in azoospermia due to germ cell aplasia. However, approximately half of mosaic men may have some degree of spermatogenesis. Klinefelter's syndrome may be associated with hypogonadism and testes are usually scrotal but small (Thielemans et al. 1998). Histology shows minor seminiferous hyalinisation and small tubules (Dunn et al. 1980; Thielemans et al. 1998).

Klinefelter's syndrome with azoospermia and testicular hypoplasia has been described also in boars, bulls, cats, dogs, horses and sheep (Hancock and Daker 1981; Dunn et. al 1980; Mäkinen et al. 1998; Nicholas 2003; Slota et al. 2003). In domestic animals XXY males are generally not recognizable until they are karyotyped (Nicholas 2003).

2.2.2 Spermatogenic arrest

An interruption of germ cell differentiation, spermatogenic arrest, leads to infertility and may be associated with either altered hormone secretion or genetic abnormalities. Spermatogenic arrest has been described in men, mice rats and bulls (Moura and Erickson 2001). In spermatogenesis, a series of mitotic and meiotic cell divisions leads to the production of haploid germ cells and postmeiotic male germ cells differentiate into mature spermatozoa. This process involves marked structural and biochemical changes, including nuclear DNA compaction and acrosome formation, and requires expression and precise coordination of numerous genes (Kostova et al. 2007; Nantel et al. 1996). Dysfunction of

genetic factors is associated with disturbed spermatogenesis and is suspected to be a frequent cause of male infertility.

Moura and Erikson (2001) did not discover deficiency in gonadotropin or steroid secretion in their study of testicular development, histology and hormone profiles in three young bulls with spermatogenic arrest. This points towards other abnormalities, including impaired gonadal responses to those hormones, secretion of intratesticular factors or genetic defects. Among the candidate genes with abnormal function associated with male infertility is the human BOULE gene, which is a possible fundamental mediator of meiotic transition. BOULE protein has been found in the cytoplasm of primary spermatocytes, and its absence has been associated with spermatogenic arrest at the meiotic stage, independent of the factors causing infertility (Kostova et al. 2007). Not only humans and primates, but also lower mammals such as mice, display analogous BOULE organization. BOULE may be considered as a candidate marker for the prediction of complete spermatogenesis (Kostova et al. 2007). In human and knockout mouse lines DNA Mismatch Repair (MMR) genes have been described to contribute to crossover events during meiotic recombination (Terribas et al. 2010). This suggests that MMR family gene expression may be altered in infertile males with spermatogenic failure.

2.2.3 Segmental aplasia of Wolffian ducts

Wolffian ducts develop into a system of connected organs between the testis and the prostate in a male. In the mature male, the functions of this system are to store and mature sperm and provide accessory semen fluid. Obstruction of the Wolffian duct results in the absence or atrophy of the vas deferens, epididymis, seminal vesicles and the ejaculatory ducts (Casals et al. 2000). Azoospermia due to an obstruction of the genital tract is one of the possible pathophysiological mechanisms causing infertility. Congenital bilateral absence of the vas deferens (CBAVD) is found in 6% of obstructive azoospermia in men. CBAVD in humans mostly results from homozygosity or composite heterozygosity for mutations in the cystic fibrosis transmembrane regulator (CFTR) gene (Sadeghi-Nejad and Farrokhi 2006). Composite heterozygosity is common, there being mostly one severe and one mild mutation or two mild mutations (Wang et al. 2002). Congenital absence of the vas deferens (CAVD) is a heterogeneous disorder, mostly due to mutations in the CFTR gene. It has been shown that otherwise healthy men with CBAVD frequently had CFTR gene mutations (Wang et al. 2002). Patients with CBAVD and unilateral absence of the vas deferens (CUAVD) might have a different etiology (Casals et al. 2000). The CFTR protein acts as a membrane-bound chloride channel and therefore CFTR mutation qualifies as one of the ion channel diseases (Ackerman and Clapham 1997).

2.3. Sperm defects causing subfertility and/or sterility

Teratozoospermia is characterized by the presence of spermatozoa with abnormal morphology. Sperm abnormalities have long been associated with infertility and sterility in most species studied. In general, sperm structure plays a substantial role in fertilization and pregnancy outcome. The causes of sperm defects may be environmental, genetic, or a combination of both (Chenoweth 2005). Spermatogenesis is a complex system of cellular differentiation that includes mitotic stem cell proliferation, meiosis and finally remodelling of haploid spermatids to produce mature spermatozoa (Gerton and Millette 1984). Sperm development includes the formation of an acrosome, the translocation of the nucleus-acrosome to the cell surface, the condensation and elongation of the nucleus as somatic histones are replaced by arginine-, lysine-rich protamines, generation of a motile flagellar apparatus, consisting of a microtubulus structure "9+2" axoneme and associated accessory structures, removal of the residual cytosolic bodies, and release of mature sperm into the lumen of the seminiferous tubule (Gerton and Millette 1984; Kato et al. 2004).

2.3.1 Immotile short-tail sperm defect

A reduced number of spermatozoa in the semen is a characteristic of the immotile short-tail sperm defect. The spermatozoa are immotile, with obvious malformations in the tail structure, including an abundance of proximal cytoplasmic droplets, short and coiled tails. The testicles of ISTS boars do not differ macroscopically from normal age and breed-matched animals and the libido of affected boars is normal (Andersson et al. 2000; Sukura et al. 2002). At the transmission electron microscopy (TEM) level significant characteristics of the spermatozoa are malformations of the midpiece with severe defects in the axonemal complex. The sperm heads develop normally, but the construction of the tail is clearly disorganized (Fig.3). This syndrome is not found in the structure or function of other ciliated cells in the affected animals (Andersson et al. 2000; Sukura et al. 2002).

The ISTS defect is transmitted in a recessive manner and is caused by an inserted Line-1 retrotransposon within an intron in the KPL2 gene in pig chromosome 16 (Sironen et al. 2007). All homozygous boars with this insertion have the specific sperm defect (Sironen et al. 2006).

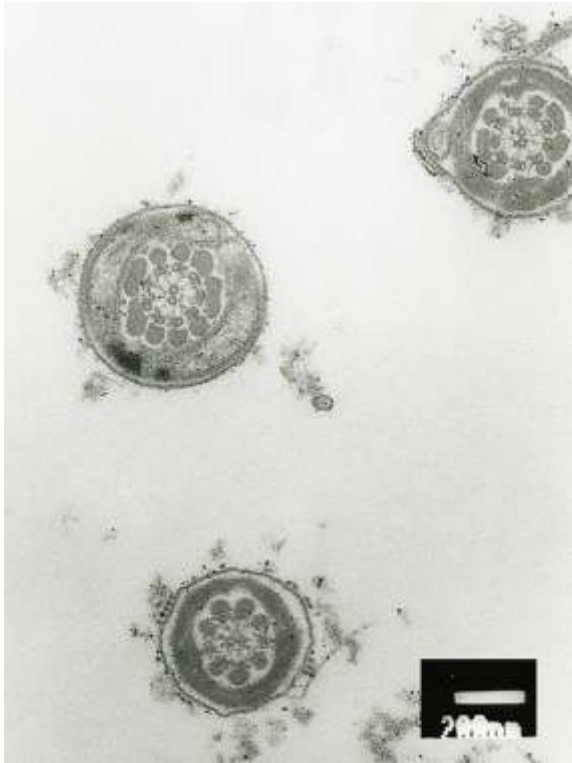*Figure 3. Thin section of the midpiece of boar spermatozoa. The bar represents 200 nm.*
Figure 1a) shows a cross section of tail and midpiece of normal spermatozoa.
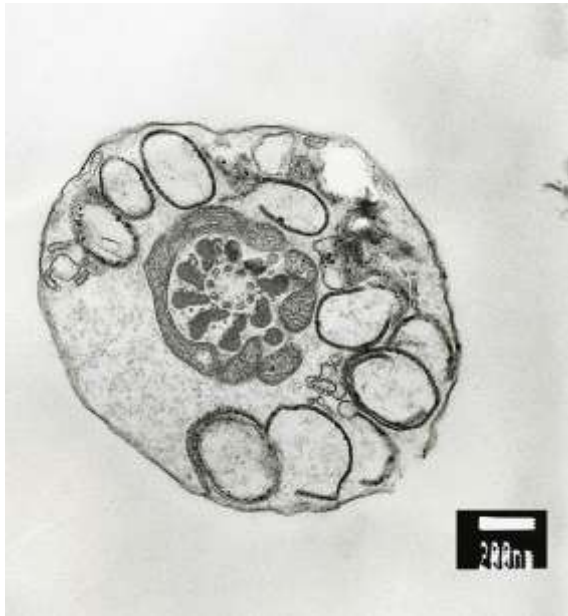*Note the presence of both central tubules.*

Figure 3 b) shows the cross section of the midpiece of
an immotile short tail sperm without the both central tubules.
Transmission electron microscopy and photographs by Maria Andersson.

2.3.2 Knobbed acrosome defect

The knobbed acrosome (KA) defect was first reported in a sterile Friesian bull and subsequently associated with an autosomal sex-linked recessive mode of genetic transmission in the breed (Chenoweth 2005). In dogs this defect has been described in the sperm of four closely related and significantly inbred males (Santos et al. 2006). The KA defect been associated with infertility in boars, stallions, rams and dogs (Hurtgen and Johnson 1982; Toyama and Itoh 1993; Soderquist 1998; Chenoweth 2005; Santos et al 2006).

KA in boars is characterized by knobs (spherical swellings of the acrosome) found at the apex of the spermatozoa. Two types of vacuoles were found in the swellings: a vacuole containing cell debris surrounded by 2 or 3 layers of membranes and a vacuole containing an amorphous material surrounded by a single membrane. The cell debris in the vacuoles was derived from the cytoplasm of the Sertoli cell. The origin of the amorphous material in the vacuoles is not known (Toyama and Itoh 1993). In the bull, the major manifestations of this defect are a refractile, thickened acrosomal apex and an indented sperm apex. Electron microscopy often reveals a cystic region containing vesicles with inclusions, abnormal fusion of acrosomal membranes and possibly a bending back, or abrupt termination, of sperm nuclear material (Chenoweth 2005). Two forms of the KA were observed in the dogs: a folded acrosome and a nipple-like extension at the apical end of the sperm. Frequently the knobs had a vesicular structure, filled with membranous material (Santos et al. 2006).

Elevated levels of knobbed acrosomes in semen may be caused by either genetic or environmental factors. When environmental, they are usually transient and associated with increased sperm abnormalities in general (Thundathil et al. 2000). In the boar and bull, the KA defect can occur as a consequence of testicular degeneration, but the proportion of affected spermatozoa in these cases is usually very low (Santos et al. 2006). A genetic cause is suspected when high proportions of sperm exhibit the KA defect without frequent numbers of other sperm abnormalities, and when the defect remains permanently at a high level (Thundathil et al. 2000).

2.4 Meiotic defects

Infertile males may show a particular defect in meiotic paring and recombination (Egozcue et al. 2000). The result of normal meiosis is that each sperm contains one member of each pair of chromosomes. Containing one half of the diploid number of chromosomes, gametes are haploid.

Meiosis is a process that includes two successive cell divisions, without DNA replication between them. In the first meiotic division (M1), which takes place in the primary spermatocytes, homologous chromosomes pair, and an exchange of genetic information may take place between them to produce new genetic combinations in the offspring. At the anaphase whole chromosomes migrate to the cell poles to produce haploid secondary spermatocytes, in which the chromosomes each have two chromatids. During the second meiotic division (M2), the chromatids migrate to the cell poles, to produce haploid spermatids. In these cells each chromosome has only one chromatid. After meiosis the spermatids change their shape and reorganize the location of their organelles to produce spermatozoa. Replication of the genetic material will only take place after fertilization. Any of these steps may go wrong, giving rise to different types of chromosome abnormalities such as diploidy (two copies of chromosomes) or aneuploidy (an abnormal number of chromosomes) (Egozcue et al. 2000). Spermatid differentiation can occur despite meiotic division abnormalities (Perrin et al. 2008), and since there is normally one disjunction in each stage of meiosis, there are two opportunities for non-disjunction during the formation of a germ cell (Nicholas 2003). Diploid spermatozoa may be produced as a result of defective meiotic division for example. Following the meiotic division pattern, XY-bearing spermatozoa originate from the first division (M1 diploids), and XX- or YY-bearing spermatozoa from the second meiotic division (M2 diploids).

2.4.1 Macrocephalic spermatozoa

Several investigators have reported an association between chromosomal aneuploids and morphological defects in spermatozoa heads (Viville et al. 2000; Devillard et al. 2002; Perrin et al. 2008). High frequencies of aneuploid and polyploid sperm have been found in men with teratozoospermia, which characterized by large-headed, multiple-tailed sperm (Perrin et al. 2008). Men presenting this type of teratozoospermia are considered to have the macrocephalic sperm head syndrome, also known as meiotic division deficiency (Escalier 2002; Perrin et al. 2008). Using FISH it has been shown that in these spermatozoa both first and second meiotic divisions may be associated with a failure in nuclear cleavage (Devillard et al. 2002; Perrin et al. 2008).

Since the discovery of the Y microdeletions in the 1970s, certain genes have formally been associated with impaired spermatogenesis in humans (Miyamoto et al. 2011). The first two detected were AURKC and SPATA16 in globozoospermia (Dam et al. 2007).

Typical hereditary macrocephalic spermatozoa in humans are characterized by a fourfold increase in the nuclear volume, irregular head shape, and four flagella. This phenotype is usually associated with more than 50% irregular macrocephalic sperm heads and tetraploidy, multiple tails, and severe male infertility or sterility (Nistal et al. 1977;

Escalier 1983; In't Veld et al. 1997; Pieters et al. 1998; Kahraman et al. 1999; Devillard et al. 2002; Dieterich et al. 2007; Dieterich et al. 2009). The cause for this syndrome was identified as homozygosity for a recessive mutation in the aurora kinase C gene (AURKC) (Dieterich et al. 2007). AURKC is expressed in the testes and is involved in chromosomal segregation and cytokinesis. Functional AURKC protein is necessary only for male meiotic cytokinesis and its absence does not impair oogenesis (Dieterich et al. 2009). Absence of AURKC causes production of large-headed multiflagellar polyploid spermatozoa (Dieterich et al. 2007).

Also other types of macrocephalic spermatozoa have been described, but they are less frequent (Dieterich et al. 2009). There are several reports of macrocephaly in sperm associated with diploidy (In't Veld et al. 1997; Bernardini et al. 1998; Weissenberg et al. 1998; Viville et al. 2000). In these cases the heads of the diploid spermatozoa are of more regular shape and they often have only one flagellum. Regular macrocephalic spermatozoa in humans have been subjected to sperm chromosome fluorescence *in situ* hybridization (sperm FISH), which reveals a failure of first or second meiotic divisions, giving rise to diploid spermatozoa. A case report from Yurov et al. (1996) describes an infertile man with 40% large-headed spermatozoa. The majority of these macrocephalic spermatozoa were contained in a diploid chromosomal content due to errors in first or second meiotic division, whereas the majority of normal-sized spermatozoa had a haploid content. Achard et al. (2007) describes a patient with 19% regular macronuclear spermatozoa and a prevalence of nondisjunction at the second meiotic division.

The association between specific sperm morphological abnormalities and sperm chromosomal abnormalities was described in a case report of three infertile men with severe oligoasthenospermia and total teratozoospermia (Lewis-Jones et al. 2003). Double-headed sperm, large-headed multinucleated sperm, and multiple tail deformities were prominent characteristics of all three patients. Large-headed sperm were thought to result from cytoplasmic retention or from the presence of multiple nuclei. The development of multiple tails has been associated with a failure of separation of cells during spermatogenesis or dysfunction of centriolar microtubules (Escalier 1983; Lewis-Jones et al. 2003). Spriggs et al. (1996) suggested that a recessive gene might predispose nondisjunction during cell division.

A combination of sperm abnormalities, rolled-head, nuclear crest and giant heads, have been observed to occur in bulls. Where such abnormalities occur in significant numbers, there have been suggestions of hereditary linkages (Barth and Oko 1989). It is also suggested that rolled sperm heads are often diploid and occasionally triploid or even tetraploid (Barth and Oko 1989; Chenoweth 2005). Effects of this sperm defect on fertility are unclear, but it is logical to assume that abnormal chromosome numbers would compromise fertility (Chenoweth 2005).

The rates of aneuploidy vary among cases and publications and probably different causes underlie this macrocephalic sperm phenotype because meiosis and spermatogenesis are complex processes involving numerous genes (Perrin et al. 2008).

2.5 Transplantation of testicular cells

Transplantation of testicular cells, also known as spermatogonial stem cell transplantation, is a relatively new technique that has been applied in research on male infertility to characterize defects in spermatogenesis. This technique has enabled diagnosis of conditions affecting germ cells (Boetter-Tong et al. 2000; Wistuba et al. 2002) and somatic cells (Mahato et al. 2000; Johnston et al. 2001). The first successful transplantation was done in mice in 1994 (Brinster and Avarbock 1994; Brinster and Zimmerman 1994). In large domestic animals, germline stem cells were transplanted a few years later in pigs (Honaramooz et al. 2002), goats (Honaramooz et al. 2003 a,b) and cattle (Izadyar et al. 2003). Also xenogenic transplantation between species (Clouthier et al. 1996), cryopreservation (Avarbock et al. 1996) and culture (Nagano et al. 1998) of spermatogonial cells have been successfully carried out and production of transgenic offspring following retroviral transmission of transgenes into germ cells has been reported (Nagano et al. 2001).

3. Aims of the study

Recognition of fertility-affecting failures of spermatogenesis is important for retaining good semen quality and productivity in farm animals.

The aims of this thesis are to describe different failures of spermatogenesis genetic origin that affect fertility and also partly to investigate their incidence in some populations.

The specific aims of the research were to:
1) Survey boars with azoospermia in two pig breeds and diagnose conditions by studying histological findings (I)
2) Examine the incidence of knobbed and immotile short-tail sperm defects in two pig breeds (II)
3) Describe a new sperm defect in a bull (III)
4) Describe cellular features of diploid spermatozoa (IV, V)
5) Determine whether spermatogonia derived from donor animals can colonize and produce motile spermatozoa in immotile short-tail sperm defect boars (VI)

4. Materials and methods

Basic information about the materials and methods are presented in this section and in Table 1. For details, see the original publications.

4.1 Animals and semen sample collection

In 1996–2005 semen samples from 2048 boars (1097 Yorkshire, 951 Landrace) were collected (studies I and II). All boars were intended for use in artificial insemination or natural breeding. The specimens originated mainly from two boar stations (88% of all samples) and were from candidates for use in AI. Some semen samples were collected at boar test stations (9%) and the rest were from farm breeding boars associated with a possible boar-related fertility disturbance on the farm (3%). The age of the boars at the collection varied between 9 and 14 months.

Two Ayrshire bulls and a Finncattle bull were initially selected to become AI bulls (III, IV, and V). The bulls were transported to an AI centre for semen collection and freezing. Routine semen evaluation revealed abnormal semen quality. The control animals were corresponding AI Ayrshire bulls showing normal sperm quality.

In study VI spermatogonial cells were harvested from the excised testes of donor boars, which were six normal pre-pubertal crossbreds (Yorkshire–Finnish Landrace) 2–5 weeks of age. Recipient boars were two Yorkshire boars affected by the hereditary immotile short-tail sperm defect (ISTS). The ages of recipient boars were 12 and 22 months at the time of transplantation. Management of the animals throughout the experiment was approved by the University of Helsinki Ethics Committee for animal experimentation.

4.2 Testicular histology (I-III)

Testicular histology was evaluated in studies I–III. Fresh testes were sent from the abattoir to the laboratory. Histological specimens were taken immediately upon arrival of the material and fixed in Bouin's fixative and embedded in paraffin. Sections (5 μm) were cut and stained with haematoxylin and eosin (HE). In study III, in addition, the Sertoli cells were detected by their vimentin expression (mouse monoclonal, code M0725; Dako, Glostrup, Denmark or UltraVision detection system; LabVision, Fremont, CA, USA).

For morphometry, 20 randomly chosen HE stained tissue sections from each animal were used (I-III). The total tissue area and the areas of the seminiferous tubules and interstitium were recorded. To analyze the absolute cross-sectional value of each seminiferous tubule, the area and the shortest and longest axes of the tubule were recorded. Spermatogenic and Sertoli cells were identified according to Garcia-Gil et al. (2002), and their numbers were assessed per unit tubule area as groups of spermatogonia, spermatocytes, round spermatids, elongated spermatids and Sertoli cells (Fig.4). The number of Sertoli cells was counted from the vimentin stained tissue sections (III). The data were presented as numbers of nuclei per unit total tubule area.

The morphometry of testicular tissue was examined using digitalized light microscopy views of testicular samples and imaging software (3.0 Image Analysis Software, Soft Imaging Systems GmbH, Muenster, Germany) coupled to a digital camera (Color View 12, Soft Imaging Systems GmbH). Morphological parameters of testicular tissue were manually measured with the software.
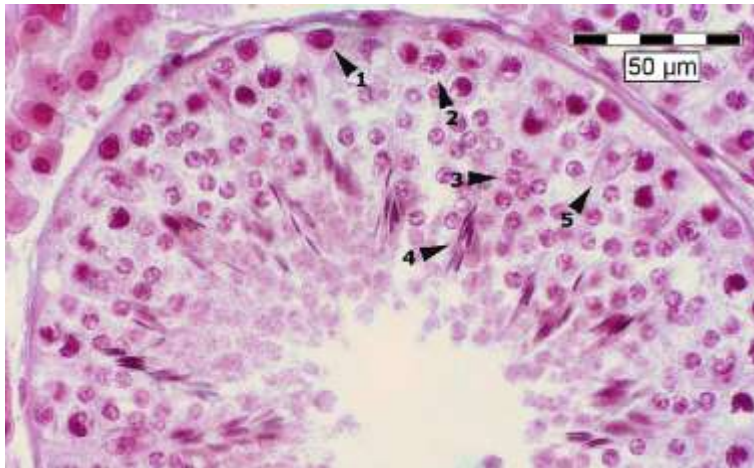

Figure 4.
Normal seminiferous tubule
1. Spermatogonia 2. Spermatocytes 3. Round spermatid 4. Elongated spermatids
5. Sertoli cells

4.3 Sperm morphology and morphometry (II-V)

Sperm morphology and morphometry were essential features of studies II-V. An overview of methods used during each study is presented here.

In study II the ejaculated semen samples were diluted with 1:1 MR-A (Kubus, Madrid, Spain) prior to making a thin semen smear. The smears were stained using a Giemsa staining method (Watson 1975). 100 spermatozoa per smear were examined. In some cases the testicles and epididymides were sent from the slaughterhouse to the laboratory for further studies, and semen samples were collected from cauda epididymides and diluted 1:10 with MR-A extender prior to making a semen smear. For morphological examination, 100 spermatozoa were examined under a light microscope using Blom (1983) classification. Boars with more than 20% specific sperm defects were considered to have these sperm defects. For these boars a new semen smear was prepared 2 months later.

Fresh semen smears were prepared in study III, for morphological and morphometric analyses of spermatozoa. The sperm concentration was measured with a hemocytometer (Bürker counting chamber; VWR International, Haasrode, Belgium). Two ejaculates were combined because of the very low number of spermatozoa in the ejaculates.

The semen sample was centrifuged and the supernatant fluid was removed. The sample was diluted 1:1 in BTS solution (Beltsville Thawing Solution, Kubus S.A., Madrid, Spain). Two age- and breed-matched normal bulls served as controls. Semen samples of the control bulls were diluted 1:3 with BTS. Semen samples were stained with Giemsa according to Watson (1975). The rest of the semen sample of the case bull was mixed 1:2 with a formol–saline solution (Hancock 1957). For fluorescence staining, spermatozoa were stained with Hoechst 33342 (Molecular Probes Europe BV, Leiden, The Netherlands) to visualize the number of nuclei. Hoechst solution was added to the formol-saline-diluted semen sample.

A total of 200 Giemsa-stained spermatozoa from the case bull were compared with spermatozoa from two normal fertile bulls using the Blom (1983) classification. For more specific morphology studies, a total of 50 spermatozoa were divided into groups according to the morphology of the head and tail. In 50 randomly chosen spermatozoa, the area of the head was measured. In another 50 spermatozoa, the morphology of sperm heads and the number of tails were assessed. The tail lengths were measured from 30 spermatozoa and compared with 30 spermatozoa from two normal bulls.

The morphology of midpieces was evaluated from 100 spermatozoa in formol–saline solution. In addition, 50 Hoechst-stained spermatozoa were analyzed for the number of nuclei and sperm tails simultaneously, according to de Leeuw et al. (1991). The spermatozoa were divided into the four groups according to the morphology of head and tail according to Kahraman et al. (1999): (1) amorphous heads with irregular shape, (2) multinuclear heads without excessive cytoplasm attached to flagella, (3) pinheads with no head structure and (4) underdeveloped spermatozoa with coiled flagella.

Spermatozoa of the case bull were examined also by using transmission electron microscopy. Two ejaculates were combined and then centrifuged and washed in phosphate-buffered saline, fixed with 2.5% glutaraldehyde in sodium cacodylate buffer (0.15 M) and post-fixed with 1% osmium tetroxide in the same buffer. The samples were dehydrated in acetone and embedded in Epon (Epon Research Industries, Burlington, VT, USA). The ultrathin sections were stained with uranyl acetate and lead citrate. Observations were made with a Jeol Jem 100 S electron microscope.

In studies IV and V semen straws were thawed prior to making semen smears for sperm morphology studies. The smears were air-dried overnight and stained with Giemsa according to Watson (1975). For morphological evaluation, spermatozoa were examined under a light microscope.

In study IV the examined spermatozoa scored were categorized into one of the following five groups: normal-sized sperm heads without nuclear crest, normal-sized sperm heads with nuclear crest, large-headed sperm without nuclear crest, large-headed sperm with nuclear crest, and underdeveloped small sperm heads. The proportions of nuclear-crested spermatozoa and visible double tailed and macrocephalic spermatozoa were evaluated from unsorted spermatozoa, flow cytometrically separated haploid and diploid sorted spermatozoa, and from two normal control bulls. For each group, 200 spermatozoa were evaluated. Sperm morphology was also examined on wet mounts by differential interference contrast microscopy (DIC; Leica DMRXA microscope, Leica Microsystems

GmbH, Wetzlar, Germany) at x 1000 magnification (Barth and Oko 1989) to visualize the presence of nuclear crests. Two straws of semen were thawed in a water bath and pooled, diluted 10X in 0.9% NaCl, and a sample of diluted sperm was analyzed under DIC.

The projected sperm head area was measured from 200 spermatozoa from the case bull and from two fertile control bulls. In addition, the projected sperm head area was also measured from haploid and diploid fractions of flow cytometrically separated spermatozoa. The area of each spermatozoon was analyzed from digitalized light microscopy views with imaging software (Cell^P Soft Imaging Systems GmbH, Muenster, Germany) coupled to a digital camera (Olympus DP70; Olympus Corporation, Tokyo, Japan). The projected sperm head area was manually measured from the images of Giemsa-stained spermatozoa with the Cell^P software using a pen tablet (Wacom CTE-440; Wacom Co. Ltd., Saitama, Japan). The proportion of spermatozoa with a nuclear crest was estimated from the unsorted spermatozoa and from the flow cytometrically separated haploid and diploid fractions. In addition, normal-sized (projected area) spermatozoa in the diploid sort were evaluated for the presence of a nuclear crest. Spermatozoa with a projected area of ≥ 52 mm^2 were considered macrocephalic (Giemsa-stained, measured post thaw). In each of these studies, 200 spermatozoa were evaluated for each fraction.

In study V projected sperm head area was measured manually according to study IV. Spermatozoa clearly larger than the average-sized spermatozoa were considered macrocephalic. The ratio of nuclear vacuoles was examined on wet mounts by differential interference contrast microscopy (DIC, Leica DMRXA microscope) at x 1000 magnification. In each sample 200 spermatozoa with a recognizable sperm head (normal, large or small) were counted. A total of 4 categories were distinguished: normal and abnormal (large, crested, small) sperm heads with or without nuclear vacuoles.

4.4 Transplantation of testicular cells (VI)

Spermatogonial stem cells were harvested from the excised testes of six normal pre-pubertal crossbred piglets 2-5 weeks old for transplantation. Donor cells were transplanted into the testes of two boars affected by the hereditary ISTS defect. Five weeks before the spermatogonial transplantations, the recipients were given busulfan (Myleran, Glaxo Wellcome, Middlesex, UK) to suppress endogenous spermatogenesis. Both boars received methylprednisolone acetate (Depo-Medrol 40 mg/ml; Pharmacia Animal Health AB, Helsingborg, Sweden) 23 days after the initiation of busulfan treatment to prevent possible effects of thrombocytopenia. The transplantation procedure was performed under general anaesthesia. The scrotal area was prepared for aseptic transplantation, an 18-gauge 80-mm long needle (Intraflon, Vycon, France) was connected to a 25 cm long tube (BD Connecta™ Plus 3, Becton Dickinson Infusion Therapy AB, Helsingborg, Sweden) and a 20 ml syringe (once, Codan Medical Aps, Rodby, Denmark) was inserted through the scrotal skin into the rete testis using ultrasound guidance (Aloka SSD-500, Aloka Co., Ltd, Tokyo, Japan, equipped with a 5-MHz linear array transducer), and the cell suspension was injected into the rete testis. The total number of cells transplanted into a volume of 10–15 ml per testis was approximately 350 x 10^6.

The recipient's ejaculates were collected once in every second week and analysed for the presence of motile spermatozoa, beginning 1 day before the first busulfan dose was administered and ending at the termination of the experiment. Spermatozoa manually selected under a microscope from a frozen aliquot of ejaculate collected 27 weeks after transplantation were genotyped.

4.5 Complementary methods

In addition to above described, complementary methods were used in the research for this thesis. These methods are represented in a table (Table 1).

Table 1. Complementary methods used in the research for this thesis

Method	Description	Reference, manufacturer
Single-nucleotide polymorphism analysis	Study IV	Illumina BovineSNP50 BeadChip, Illumina, San Diego, CA, USA
Genetic studies of the ISTS and the KA defect	Study II	Sironen et al. 2002, 2006; Alanko 1985; US Pig Gene Mapping Coordination Program; Finnzymes; Amersham Biosciences
Karyotyping	Studies I, III and IV	Gustavsson et al. 1983; Seabright 1971; Dutrillaux et al. 1973; Iannuzzi 1990; Moorhead et al. 1960
Flow cytometry	Studies IV and V	MoFlo, Daco-Cytomation Inc., Fort Collins, CO, USA Coherent, Paladin Compact 355-2000 Coherent Inc., Santa Clara. USA
Fluorescence *in situ* hybridization	Studies IV and V	Rens et al. 2001; Goldamer et al. 1997; Revay et al. 2003
Fertility trial	Study IV	
Sperm chromatin structure assay and degree of DNA maturation	Study V	Evenson and Jost 2000; Becton Dickinson, Immunochemistry Systems; Innova 90, Coherent; Becton Dickinson; DeNovo Software

5. Results

5.1 Azoospermic boars in Finnish Yorkshire and Landrace breeds (I)

Azoospermia was diagnosed in 16 of the 1097 Yorkshire boars (Y, 1.5%) and in 2 of 951 Landrace boars (L, 0.2%). The distribution of azoospermic boars into different diagnostic groups was as follows: pre-testicular 0Y/1L, testicular 9Y/1L and post-testicular (obstructive) azoospermia 7Y/0L. Of the Yorkshire boars, 8 were afflicted with arrested spermatogenesis at the pachytene spermatocyte stage (spermatocyte arrest), 7 with segmental aplasia and 1 with cytogenetically diagnosed Klinefelter syndrome. Of the Landrace boars, one was affected with severe testicular hypoplasia, but had a normal karyotype. A Landrace boar was diagnosed with arrested spermatogenesis at around the spermatid stage (spermatid arrest) (Table 2). The morphometric histology results differed statistically between normal boars and azoospermic boars. Typical histological findings were as follows: lower proportion of seminiferous tubules in tissue sections, a smaller tubular diameter, less round spermatids and absent elongated spermatids per unit tubular area compared with normal boar samples.

Table 2. Azoospermia in Yorkshire (Y) and Landrace (L) boars, diagnosis and testicular weight

Diagnostic group	Diagnosis	Number of boars affected (mean single testicle weight, weight compared with control testes and breed Y/L)
Pre-testicular azoospermia	Testicular hypoplasia	1 (26 g, 6 %, L)
Testicular azoospermia	Klinefelter's syndrome (XXY)	1 (135 g, 33 %, Y)
	Spermatocyte arrest	8 (187 g, 46 %, Y)
	Spermatid arrest	1 (203 g, 49 %, L)
Post-testicular azoospermia	Segmental aplasia (type [1]CUAVD)	7 (337 g, 82 %, Y)
Normal controls		8 (410 g, 100 %, 2L + 6Y)

[1]CUAVD, congenital unilateral absence of the vas deferens and idiopathic epididymal obstruction

5.2 Knobbed and immotile sperm defects in the Finnish Yorkshire boars (II)

Of the 1097 Yorkshire boars, 9 (0.8%) were afflicted with the KA defect and 83 (7.6%) with the ISTS defect (Fig. 5). None of the 951 Landrace boars had either of the defects. Fertility data were available from two artificial insemination (AI) boars and six farm breeding boars affected with the KA defect and for 40 ISTS-affected farm breeding boars. Boars possessing the KA defect showed a low fertility when the proportion of knobbed spermatozoa exceeded 25% (Table 3). AI boars with 25-30% knobbed spermatozoa had a poor non-return rate (on average 47% compared with 85% for normal control boars) and produced small litters, on average 2.5 piglets less than other boars of the same breed. Breeding boars with 45% to 81% knobbed spermatozoa (n=6) did not produce any litters. ISTS boars were shown to be completely sterile. All of the 40 ISTS boars were bred to several sows, but produced no litters. In studies of testicular histology the boars with the KA defect had a smaller diameter seminiferous tubules (p<0.05) and a lower number of Sertoli cells (p<0.05) than controls. Boars with ISTS had a significantly lower number of elongated spermatids (p<0.05) and also produced, on average, only 12% of the sperm of normal boars.
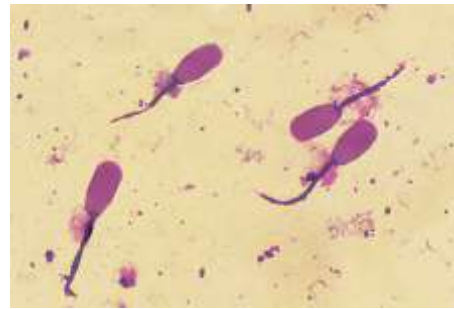

Figure 5. Giemsa stain.
Spermatozoa from boars affected with
a) knobbed acrosome (KA) defect

b) immotile short-tail sperm (ISTS) defect

Table 3. Knobbed acrosome (KA) defect in Yorkshire boars

		Non-return rate (60 days)	Mean litter size (number of litters)	Spermatozoa % with the KA defect
Boars selected for use in AI	Boar 1	44%	9.5 (37)	27
	Boar 2	50%	8.3 (35)	26
	Boar 3	Not used in AI	-	47
Farm breeding boars	Boar 4			45
	Boar 5	No pregnancies	-	65
	Boar 6	in this group		64
	Boar 7	(5-37 sows		81
	Boar 8	bred/boar)		70
	Boar 9			60

AI, artificial insemination

5.3 Multinuclear-multiflagellar sperm defect in a bull (III)

Testicular weight of the case bull was within normal limits, but the sperm concentration was extremely low compared with the normal control bulls. The karyotype was normal. All ejaculates contained only morphologically abnormal spermatozoa. Semen analysis revealed severe oligoasthenoteratozoospermia. All spermatozoa were immotile and 60% had multiple tails (Fig. 6). The proportion of major sperm defects was 100%. No acrosomes were detected in any of the spermatozoa. A midpiece with a mitochondrial helix was found in only 5% of the analyzed spermatozoa. In transmission electron microscopy (TEM), spermatids and spermatozoa showed multinucleation (Fig. 8). A positive correlation was found between the number of nuclei and the number of flagella ($p<0.05$) (Table 4). Morphometry of testicular tissue revealed no significant difference in the proportion of seminiferous tubules, but the absolute cross-sectional area of the tubules was reduced ($p<0.05$). The proportion of spermatogonia in seminiferous tubules was comparable with controls, but the numbers of spermatocytes and round spermatids were clearly reduced and only few elongated spermatids were observed. The number of Sertoli cells was significantly increased, in HE staining ($p<0.01$) and in vimentin staining ($p<0.001$) compared with control bulls (Fig.7).

Figure 6. Giemsa stained spermatozoa.
a) Multiflagellar sperm cell and medusa cell (right).
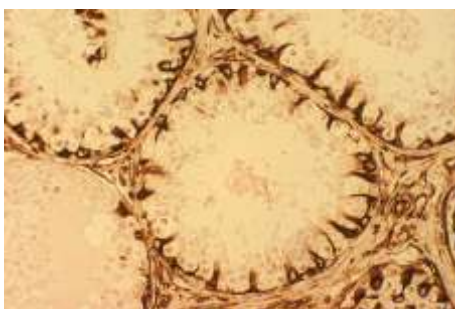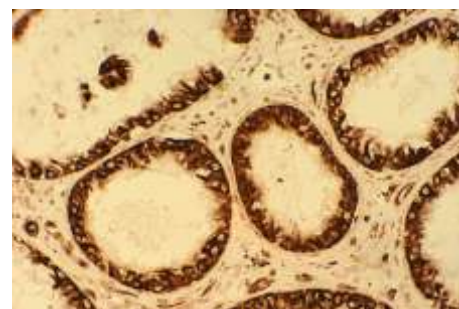
b) Multinuclear sperm cell with double tail.

Figure 7. Vimentin stained testicular tissue.
a) Normal control bull.

b) Multinuclear-multiflagellar sperm defect.
Observe the high number of Sertoli cells.
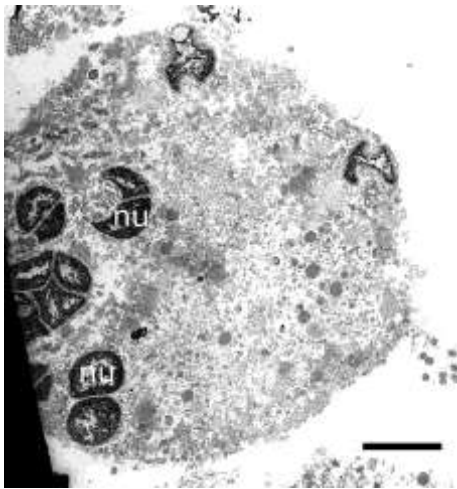
Figure 8. Cross-section of multinuclear sperm cell
Multiple nuclei visible (nu). Scale bar 10 µm.
Transmission electron microscopy and photograph by Antti Sukura.

Table 4. Simultaneous evaluation of the number of nuclei and sperm tails in spermatozoa from the case bull (n = 50 Hoechst-stained spermatozoa)

Head	Number of nuclei	Number of flagella
Amorphous (n=32)	1-9	1-11
Multinuclear heads without cytoplasm (n=6)	2-4	2-5
Pinhead (n=9)	0	1-7
Underdeveloped (n=3)	1	1

5.4 Meiotic disorders in bull spermatozoa (IV, V)

Abnormal spermiogram of the Finn cattle bull was investigated in study IV. It showed a normal sperm concentration, an initial sperm motility of 40%, and a post-thaw motility of 20%. In morphological evaluation 23% of the unsorted spermatozoa had a large head area, differing from the normal size, and the proportion of nuclear crests was 14% (Fig. 9). Nuclear crests were found in spermatozoa with a normal head size and in spermatozoa with large head size. Flow cytometric sorting revealed the initial sperm population consisted of haploid and diploid spermatozoa. The proportion of nuclear crests was highly concentrated (58%) in the diploid sort compared with the haploid sort (2%). The bull had a normal karyotype (60XY) with no detectable abnormality.

No difference was detected between the 50 000 SNP genotypes scored in the haploid and diploid fractions. In the fertility trial, 6 of the 12 inseminated cows were pregnant 2 months after insemination. The sperm FISH chromosome study was done on the flow cytometrically sorted diploid sort. The majority of cells carried XX or YY chromosomes and XY-bearing spermatozoa were very rare (approx 0.7% in the diploid fraction). In scoring more than 1000 randomly sampled spermatozoa 32% showed the YY diploid signal and 18% the Y signal. This result proves that the sperm diploidy in this bull was caused by an incomplete partitioning of sister chromatids during the second meiotic division (M2).
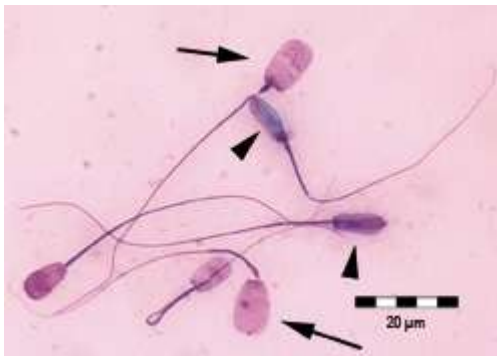

Figure 9.
Abnormal morphology of bovine spermatozoa.
Giemsa stain.
Diploid spermatozoa (arrows).
Nuclear crests (arrow heads).

In study V two bulls (A and B) were investigated that had abnormal spermiograms with frequent macrocephaly, double tails and nuclear vacuoles. The proportion of immature spermatozoa was 9.54% in A and 7.75% in B, compared with 0.47% in the control bull. Nuclear fragmentation was shown in 7.1% and 31% in A and B, compared to 2% in the control bull. Bull B was investigated in a previous study and proven to M2 diploid. Bull A, an Ayrshire bull, showing normal sperm concentration, had 60% morphologically normal spermatozoa and 17% macrocephalic spermatozoa, where 15% had nuclear crests. The initial sperm motility was 60% and the post thaw motility 40%. Although the sperm morphology was below the acceptable limits of 70% normal spermatozoa conventionally

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applied in Finland, the bull was used for 3404 artificial inseminations in total (1727 first inseminations). The non-return rate (60 days) was 3.2% below the average for this breed. In this study large headed spermatozoa of bull A were investigated with FISH using an X-Y painting probe set. 7.5% of the spermatozoa showed the XY diploid signal. This result indicates a meiotic arrest in the first division (M1).

5.5 Transplantation of testicular cells in a boar (VI)

Before the spermatogonial transfer, no motile sperm were observed in recipients' semen samples. Morphological analyses revealed that 0% and 37% of the sperm had normal tail lengths for boars 1 and 2 respectively. Mean sperm tail length in a boar was 17.8 μm (range 11.3-36.1) compared with 48 μm (range 46.5-48.3) in control boars (p<0.001). Thirteen weeks after the transfer of spermatogonial cells, the first motile spermatozoa were detected in boar 1 ejaculates. The boar produced donor-derived progressively motile spermatozoa in every ejaculate collected every second week between 13 and 29 weeks after the transfer. Spermatozoa manually selected under a microscope from an ejaculate collected 27 weeks after transplantation were genotyped. In two of the 20 vials the donor-derived genotype was visible. The estimated number of motile donor-derived spermatozoa was from 5 000 to 10 000 per ejaculate. Between weeks 31 and 51 after transfer, no motile spermatozoa were detected in the ejaculates. On week 54, motile spermatozoa reappeared and remained until the boar was killed 59 weeks after transfer. No motile spermatozoa were observed in boar 2 within 22 weeks of transfer, thus, colonization and subsequent sperm production were considered unsuccessful.

6. Discussion

6.1 General discussion

The common subject for the studies in this thesis was impaired spermatogenesis caused by genetic factors resulting in reproductive failure. Some of the genetic reasons are known, but several remained at least partly unresolved during the study.

Genetic defects are usually more easily observed in spermatozoa than in oocytes. One reason for this is the specialized morphology and complex formation of spermatozoa that requires activation of several genes and transcription factors (Sassone-Corsi 2002).

The treatment of human infertility by intracytoplasmic sperm injection (ICSI) has increased the interest in sperm defects since the technique allows evaluation of the fertilizing potential of severely abnormal spermatozoa (In't Veld et al. 1997; Kahraman et al. 1999; Tempest and Griffin 2004; Machev et al. 2005). Before the use of intracytoplasmic sperm injection (ICSI), an understanding of the genetic etiology of a patient-specific disorder is critical for proper counseling and decision-making (Sadeghi-Nejad and Farrokhi 2006). Mutant mouse models of specific genes have markedly increased our knowledge of the origin of specific sperm defects. The risk for producing chromosomally abnormal embryos followed by embryonic death, abortion or chromosomally abnormal offspring has been evaluated for different human sperm defects (Kahraman et al. 1999).

Also in domestic animals, some sperm defects are associated with impaired fertility or even sterility, while other sperm defects affect fertility only moderately when recommended doses of sperm are used in artificial insemination (AI) (Hancock 1953; Andersson et al. 1990). Identification of inherited sperm defects is crucial also in farm animals, artificial insemination (AI) boars and bulls and farm breeding boars, for maintenance of high semen quality and good fertility. In cattle breeding, superior sires and breeding lines have a significant impact on the genetics of future generations and certain genes are enhanced in the population. This fact may cause an increase in certain recessive genes, resulting in male sterility, embryonic death, stillbirths or malformations in calves (Donald and Hancock 1953; Shanks and Robinson 1989; Agerholm et al. 2001).

Research on human infertility is extremely active and therefore many of the references in this thesis are from the field of human infertility. The final goal of the research may be different, but many of the methods are common. Maintenance of breeding efficiency and a high semen quality in farm animals is economically essential. Therefore the aim is mainly the recognition of different factors causing subfertility or infertility and detecting their possible inheritance.

In this thesis the use of combined modern techniques provided valuable and previously unknown information about the cytogenetic background of different spermatogenic failures. Even so, many questions are open for further studies.

6.2 Impaired spermatogenesis in two pig breeds

Azoospermia in boars was one of the main topics of this thesis. In Finland pigs used for breeding are mainly of Landrece or Yorkshire breeds. In the survey of 2048 Finnish AI and farm breeding boars during 1996–2005 the incidence of azoospermia was significantly higher in boars of Yorkshire breed (1.5%) than in those of Landrace breed (0.2%) (I).

The etiology for azoospermia in these two breeds was different. The most common diagnosis in Yorkshire boars was germ cell arrest at the primary spermatocyte level. The reason for this defect was unknown at the time of writing the manuscript, but later results suggest that the defect maps to chromosome 15 (Sironen et al. 2010). Histologically our findings were quite similar to the Jackson Laboratory knockout mouse strain repro 23 with arrested spermatogenesis and defect in chromosome 7 (JAX Reproductive Mutagenesis Program). Some other features of reproductive mutant mice (Bannister et al. 2004) also resemble spermatocyte arrest in boars. The second most frequent diagnosis affecting Yorkshire boars was segmental aplasia of the Wolffian ducts with idiopathic epididymal obstruction. In humans a mutation in the CFTR gene is a potential cause of this condition (Mickle et al. 1995; Meshede et al. 1998). In boars the cause has not been studied. The remaining reasons for azoospermia were all infrequent, one of each occurring: Klinefelter syndrome in a Yorkshire boar, and in Landrace boars spermatogenic arrest, subtype spermatid arrest, and probable hypogonadotrophic hypogonadism with an obstruction between testicles and epididymides. The finding of different etiologies for azoospermia in these two pig breeds indicates that the reasons for azoospermia are mainly genetic and thus warrant further research.

Also the incidence of two relatively well-defined specific sperm defects, immotile short-tail sperm (ISTS) defect and knobbed acrosome (KA) defect were investigated in Finnish Yorkshire and Landrace boars in 1996-2005 (II). The ISTS defect was found in 7.6%, and the KA defect in 0.8%, of the Yorkshire boars. None of the Landrace boars was diagnosed with either of the defects. Both sperm defects occurred only in Yorkshire boars, indicating that these sperm defects are inherited. The KA defect is known to be caused by a single autosomal recessive gene in bulls (Hancock 1953; Hafez and Hafez 2000). In 2006 Santos et al. (2006) described the KA defect in four closely related and significantly inbred dogs. Already in 1985 it was suggested that the KA defect is also inherited recessively in the Finnish Yorkshire breed (Alanko 1985). A recessive manner of transmission is expected based on the pedigrees of affected boars (Alanko 1984, 1985; Sironen et al. 2002). In the present study, the results of homozygosity mapping of this defect were not statistically significant, but they may indicate the most probable positions of the KA defect-associated chromosomal segments. The ISTS defect is already known to be caused by an insertion within an intron in the KPL2 gene in pig chromosome 16 (Sironen et al. 2006), and even a molecular test for this defect is available. Currently all Finnish Yorkshire AI boars are now tested for this defect.

In the survey the prevalence of impaired spermatogenesis and the aetiology for the conditions in these two breeds were different. The reason for this remains unclear, but the influence of genetic variation is probable.

6.3. Meiotic defects in AI bulls

Sperm diploidy, aneuploidy, and polyploidy have primarily been described in humans. In these cases, the first, the second, or both meiotic divisions are affected. A significant and inverse relationship between morphology and sperm aneuploidy has been described in men (Martin et al. 2003). Diploidy is the most frequent sperm chromosome anomaly in infertile males, originating by either meiotic mutations or by a compromised testicular environment (Egozcue et al. 2000). The exact analysis of chromosomal content is possible with the use of fluorescence *in situ* hybridization (FISH). The commercial availability of probes in particular has facilitated the use of this technique. FISH has proven invaluable when assessing aneuploidy rates of spermatozoa in men (Tempest et al. 2009). This technique has now been extrapolated to cattle. We used FISH analysis of chromosomal constitution in morphologically abnormal bovine spermatozoa in two case studies (IV, V). In both studies diploidy was proven in macrocephalic spermatozoa. FISH also provided more information about the cytogenetic background by making it possible to discriminate between M1 and M2 diploid spermatozoa.

We also described a new oligoasthenoteratozoospermic defect in a bull (III). Because of the morphological characteristics of this defect it was named the multinuclear-multiflagellar sperm (MNMFS) defect. Meiosis and the spermiogenesis were severely affected and all spermatozoa in the ejaculate were abnormal. As far as we know, comparable sperm defects have not been previously described in cattle. Morphologically similar sperm defects have been described in a knockout mouse strain homozygous for the $Hrb^{-/-}$ gene (Kang-Decker et al. 2001; Juneja and van Deursen 2005).

6.4 Economic aspects

One of the economic cornerstones for the success of farms producing piglets and of dairy farms is effective reproduction in breeding animals.

All of the disorders described in this thesis are associated with reproductive failure. Most of the disorders are associated with sterility, although some are less detrimental. One of the disorders is associated with subfertility and lowered litter size in its mild form. In another study the high sperm diploidy rate in a bull was associated with a calving rate of less than half the average for normal bulls. To conclude, high sperm quality is essential for effective reproduction, and early detection of disorders can have long-term effects on productivity.

7. Conclusions

I) Based on our findings, causes for azoospermia in Finnish Yorkshire and Landrace boars were different, indicating that the reasons for azoospermia are mainly genetic and in populations of different breeds different frequencies of azoospermia can be expected. The aetiology for azoospermia in boars remains obscure and warrants further research.

II) We conclude that in the Finnish Yorkshire boars the probably inherited KA defect and the inherited immotile short-tail sperm (ISTS) defect were important reasons for infertility during the years 1996-2005.

III) In this study we described a new sperm defect in the oligoasthenoteratozoospermic bull intended for use in artificial insemination (AI); the multinuclear-multiflagellar sperm (MNMFS) defect.

IV) The diploidy in examined macrocephalic bull spermatozoa was caused by an incomplete partitioning of sister chromatids during the second meiotic division (M2) associated with a failure in nuclear cleavage.

V) A combined approach of fluorescence *in situ* hybridization (FISH) and flow cytometry provided more information about the cytogenetic background of macrocephalic bull spermatozoa and led to the description and comparison of a bull bearing M1 (the first meiotic division) and another bull bearing M2 (the second meiotic division) diploid spermatozoa.

VI) The transplantation of testicular cells can result in donor-derived spermatogenesis and sperm production in recipient boars.

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References

Achard, V., Paulmyer-Lacroix, O., Mercier, G., Porcu, G., Saias-Magnan, J., Metzler-Guillemain, C. & Guichaoua, M.R. 2007, "Reproductive failure in patients with various percentages of macronuclear spermatozoa: high level of aneuploid and polyploid spermatozoa", *Journal of Andrology,* vol. 28, no. 4, pp. 600-606.

Ackerman, M.J. & Clapham, D.E. 1997, "Ion channels--basic science and clinical disease", *The New England Journal of Medicine,* vol. 336, no. 22, pp. 1575-1586.

Adham, I.M., Nayernia, K., Burkhardt-Gottges, E., Topaloglu, O., Dixkens, C., Holstein, A.F. & Engel, W. 2001, "Teratozoospermia in mice lacking the transition protein 2 (Tnp2)", *Molecular Human Reproduction,* vol. 7, no. 6, pp. 513-520.

Afzelius, B.A. 1976, "A human syndrome caused by immotile cilia", *Science (New York, N.Y.),* vol. 193, no. 4250, pp. 317-319.

Agerholm, J.S., Bendixen, C., Andersen, O. & Arnbjerg, J. 2001, "Complex vertebral malformation in holstein calves", *Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc,* vol. 13, no. 4, pp. 283-289.

Aitken, R.J., Kerr, L., Bolton, V. & Hargreave, T. 1990, "Analysis of sperm function in globozoospermia: implications for the mechanism of sperm-zona interaction", *Fertility and Sterility,* vol. 54, no. 4, pp. 701-707.

Alanko, M., 1984: "The knobbed sperm defect in boars: fertility and inheritance of the defect". In*: 10th International Congresson Animal Reproduction and Artificial Insemination,*Vol. III, June 10–14, University at Urbana-Champaign, IL,3 pp.

Alanko M., 1985: "Über die angeborenen Kopfkappenverä nderungender Eberspermien und ihre Wirkung auf die Befruchtung der Ova bei der Sau " [Inherited acrosome defects of boar spermatozoa and their effect on ova fertilization in the sow]. *Tierärztliche Umschau* 40: 564–566.

Andersson, M., Peltoniemi, O.A.T., Makinen, A., Sukura, A., Rodriguez-Martinez, H. 2000 "The Hereditary `Short Tail' Sperm Defect - A New Reproductive Problem in Yorkshire Boars", *Reproduction in Domestic Animals*, vol. 35, no. 2, pp. 59-63(5)

Andersson, M., Vierula, M. & Alanko, M. 1990, "Three types of acrosomal aberrations of bull spermatozoa and their relation to fertility", *Acta Veterinaria Scandinavica,* vol. 31, no. 2, pp. 175-179.

Avarbock, M.R., Brinster, C.J. & Brinster, R.L. 1996, "Reconstitution of spermatogenesis from frozen spermatogonial stem cells", *Nature Medicine,* vol. 2, no. 6, pp. 693-696.

Bannister, L.A., Reinholdt, L.G., Munroe, R.J. & Schimenti, J.C. 2004, "Positional cloning and characterization of mouse mei8, a disrupted allele of the meiotic cohesin Rec8", *Genesis (New York, N.Y.: 2000),* vol. 40, no. 3, pp. 184-194.

Barth, A.D., Oko, R.J. 1989, "Abnormal Morphology of Bovine Spermatozoa", Iowa State University Press, Ames, Iowa, pp.130-136.

Bernardini, L., Martini, E., Geraedts, J.P., Hopman, A.H., Lanteri, S., Conte, N. & Capitanio, G.L. 1997, "Comparison of gonosomal aneuploidy in spermatozoa of normal fertile men and those with severe male factor detected by in-situ hybridization", *Molecular Human Reproduction,* vol. 3, no. 5, pp. 431-438.

Blom, E. 1983, "Pathological conditions in genital organs and sperm as a cause for the rejection of breeding bulls for import into and export from Denmark (an andrologic retrospective, 1958-1982)", *Nordisk Veterinaermedicin,* vol. 35, no. 3, pp. 105-130.

Blom, E. 1976, "A sterilizing tail stump sperm defect in a Holstein-Friesian bull", *Nordisk Veterinaermedicin,* vol. 28, no. 6, pp. 295-298.

Boettger-Tong, H.L., Johnston, D.S., Russell, L.D., Griswold, M.D. & Bishop, C.E. 2000, "Juvenile spermatogonial depletion (jsd) mutant seminiferous tubules are capable of supporting transplanted spermatogenesis", *Biology of Reproduction,* vol. 63, no. 4, pp. 1185-1191.

Brinster, R.L. & Avarbock, M.R. 1994, "Germline transmission of donor haplotype following spermatogonial transplantation", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 91, no. 24, pp. 11303-11307.

Brinster, R.L. & Zimmermann, J.W. 1994, "Spermatogenesis following male germ-cell transplantation", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 91, no. 24, pp. 11298-11302.

Casals, T., Bassas, L., Egozcue, S., Ramos, M.D., Gimenez, J., Segura, A., Garcia, F., Carrera, M., Larriba, S., Sarquella, J. & Estivill, X. 2000, "Heterogeneity for mutations in the CFTR gene and clinical correlations in patients with congenital absence of the vas deferens", *Human Reproduction (Oxford, England),* vol. 15, no. 7, pp. 1476-1483.

Chenoweth, P.J. 2005, "Genetic sperm defects", *Theriogenology,* vol. 64, no. 3, pp. 457-468.

Clouthier, D.E., Avarbock, M.R., Maika, S.D., Hammer, R.E. & Brinster, R.L. 1996, "Rat spermatogenesis in mouse testis", *Nature,* vol. 381, no. 6581, pp. 418-421.

Dam, A.H., Koscinski, I., Kremer, J.A., Moutou, C., Jaeger, A.S., Oudakker, A.R., Tournaye, H., Charlet, N., Lagier-Tourenne, C., van Bokhoven, H. & Viville, S. 2007, "Homozygous mutation in SPATA16 is associated with male infertility in

human globozoospermia", *American Journal of Human Genetics,* vol. 81, no. 4, pp. 813-820.

de Leeuw, A.M., den Daas, J.H. & Woelders, H. 1991, "The fix vital stain method. Simultaneous determination of viability and acrosomal status of bovine spermatozoa", *Journal of Andrology,* vol. 12, no. 2, pp. 112-118.

Devillard, F., Metzler-Guillemain, C., Pelletier, R., DeRobertis, C., Bergues, U., Hennebicq, S., Guichaoua, M., Sele, B. & Rousseaux, S. 2002, "Polyploidy in large-headed sperm: FISH study of three cases", *Human Reproduction (Oxford, England),* vol. 17, no. 5, pp. 1292-1298.

Dieterich, K., Soto Rifo, R., Faure, A.K., Hennebicq, S., Ben Amar, B., Zahi, M., Perrin, J., Martinez, D., Sele, B., Jouk, P.S., Ohlmann, T., Rousseaux, S., Lunardi, J. & Ray, P.F. 2007, "Homozygous mutation of AURKC yields large-headed polyploid spermatozoa and causes male infertility", *Nature Genetics,* vol. 39, no. 5, pp. 661-665.

Dieterich, K., Zouari, R., Harbuz, R., Vialard, F., Martinez, D., Bellayou, H., Prisant, N., Zoghmar, A., Guichaoua, M.R., Koscinski, I., Kharouf, M., Noruzinia, M., Nadifi, S., Sefiani, A., Lornage, J., Zahi, M., Viville, S., Sele, B., Jouk, P.S., Jacob, M.C., Escalier, D., Nikas, Y., Hennebicq, S., Lunardi, J. & Ray, P.F. 2009, "The Aurora Kinase C c.144delC mutation causes meiosis I arrest in men and is frequent in the North African population", *Human Molecular Genetics,* vol. 18, no. 7, pp. 1301-1309.

Donald, H.P. & Hancock, J.L. 1953, "Evidence of gene-controlled sterility in bulls." *J. Agric Sci.,* vol. 43, pp. 178-181.

Dunn, H.O., Lein, D.H. & McEntee, K. 1980, "Testicular hypoplasia in a Hereford bull with 61,XXY karyotype: the bovine counterpart of human Klinefelter's syndrome", *The Cornell Veterinarian,* vol. 70, no. 2, pp. 137-146.

Dutrillaux, B., Laurent, L., Couturier, J., Lejeune, J. 1973, "Staining of human chromosomes with acridine orange after treatment with 5 C bromodeoxyuridine. *C.R. Acad. Sc.* (Paris);276:3179-181.

Egozcue, J., Blanco, J. & Vidal, F. 1997, "Chromosome studies in human sperm nuclei using fluorescence in-situ hybridization (FISH)", *Human Reproduction Update,* vol. 3, no. 5, pp. 441-452.

Egozcue, S., Blanco, J., Vendrell, J.M., Garcia, F., Veiga, A., Aran, B., Barri, P.N., Vidal, F. & Egozcue, J. 2000, "Human male infertility: chromosome anomalies, meiotic disorders, abnormal spermatozoa and recurrent abortion", *Human Reproduction Update,* vol. 6, no. 1, pp. 93-105.

Escalier, D. 2002, "Genetic approach to male meiotic division deficiency: the human macronuclear spermatozoa", *Molecular Human Reproduction,* vol. 8, no. 1, pp. 1-7.

Escalier, D. 1983, "Human spermatozoa with large heads and multiple flagella: a quantitative ultrastructural study of 6 cases", *Biology of the cell / under the auspices of the European Cell Biology Organization,* vol. 48, no. 1, pp. 65-74.

Evenson, D.P., Jost, L.K., Corzett, M. & Balhorn, R. 2000, "Characteristics of human sperm chromatin structure following an episode of influenza and high fever: a case study", *Journal of Andrology,* vol. 21, no. 5, pp. 739-746.

Garcia-Gil, N., Pinart, E., Sancho, S., Badia, E., Bassols, J., Kadar, E., Briz, M. & Bonet, S. 2002, "The cycle of the seminiferous epithelium in Landrace boars", *Animal Reproduction Science,* vol. 73, no. 3-4, pp. 211-225.

Gerton, G.L. & Millette, C.F. 1984, "Generation of flagella by cultured mouse spermatids", *The Journal of Cell Biology,* vol. 98, no. 2, pp. 619-628.

Goldammer, T., Brunner, R.M. & Schwerin, M. 1997, "Comparative analysis of Y chromosome structure in Bos taurus and B. indicus by FISH using region-specific, microdissected, and locus-specific DNA probes", *Cytogenetics and Cell Genetics,* vol. 77, no. 3-4, pp. 238-241.

Gustavsson, I., Settergren, I. & King, W.A. 1983, "Occurrence of two different reciprocal translocations in the same litter of domestic pigs", *Hereditas,* vol. 99, no. 2, pp. 257-267.

Hafez, E.S.E., Hafez, B. 2000: Reproduction in Farm Animals, 7th edn. Lippincott Williams & Wilkins, Philadelphia, PA.

Hancock, J.L. 1953: The spermatozoa of sterile bulls. J Exp Biol 30, 50–56.

Hancock, J.L. 1957, "The morphology of boar spermatozoa", *Journal.Royal Microscopical Society (Great Britain),* vol. 76, no. 3, pp. 84-97.

Hancock, J.L. & Daker, M.G. 1981, "Testicular hypoplasia in a boar with abnormal sex chromosome constitution (39 XXY)", *Journal of Reproduction and Fertility,* vol. 61, no. 2, pp. 395-397.

Honaramooz, A., Behboodi, E., Blash, S., Megee, S.O. & Dobrinski, I. 2003, "Germ cell transplantation in goats", *Molecular Reproduction and Development,* vol. 64, no. 4, pp. 422-428.

Honaramooz, A., Behboodi, E., Megee, S.O., Overton, S.A., Galantino-Homer, H., Echelard, Y. & Dobrinski, I. 2003, "Fertility and germline transmission of donor haplotype following germ cell transplantation in immunocompetent goats", *Biology of Reproduction,* vol. 69, no. 4, pp. 1260-1264.

Honaramooz, A., Megee, S.O. & Dobrinski, I. 2002, "Germ cell transplantation in pigs", *Biology of Reproduction,* vol. 66, no. 1, pp. 21-28.

Hrudka, F., Betsch, J.M. & Kenney, R.M. 1991, "Anomalies of centriolar derivatives manifest in spermatic flagella and respiratory cilia of the stallion", *Archives of Andrology,* vol. 27, no. 3, pp. 161-175.

Hurtgen, J.P. & Johnson, L.A. 1982, "Fertility of stallions with abnormalities of the sperm acrosome", *Journal of Reproduction and Fertility. Supplement,* vol. 32, pp. 15-20.

Iannuzzi, L. 1990, "An improved characterization of cattle chromosomes by means of high-resolution G- and R-band comparison", *The Journal of Heredity,* vol. 81, no. 1, pp. 80-83.

In't Veld, P.A., Broekmans, F.J., de France, H.F., Pearson, P.L., Pieters, M.H. & van Kooij, R.J. 1997, "Intracytoplasmic sperm injection (ICSI) and chromosomally abnormal spermatozoa", *Human Reproduction (Oxford, England),* vol. 12, no. 4, pp. 752-754.

Izadyar, F., Den Ouden, K., Stout, T.A., Stout, J., Coret, J., Lankveld, D.P., Spoormakers, T.J., Colenbrander, B., Oldenbroek, J.K., Van der Ploeg, K.D., Woelders, H., Kal, H.B. & De Rooij, D.G. 2003, "Autologous and homologous transplantation of bovine spermatogonial stem cells", *Reproduction (Cambridge, England),* vol. 126, no. 6, pp. 765-774.

J:92463 JAX Reproductive Mutagenesis Program, "Heritable mouse mutants from the Jackson Laboratory Genomics Mutagenesis Program, URL: http://reproductivegenomics.jax.org/mutants/G1-476-14.html" MGI Direct Data Submission 2004-7.

Johnston, D.S., Russell, L.D., Friel, P.J. & Griswold, M.D. 2001, "Murine germ cells do not require functional androgen receptors to complete spermatogenesis following spermatogonial stem cell transplantation", *Endocrinology,* vol. 142, no. 6, pp. 2405-2408.

Juneja, S.C. & van Deursen, J.M. 2005, "A mouse model of familial oligoasthenoteratozoospermia", *Human Reproduction (Oxford, England),* vol. 20, no. 4, pp. 881-893.

Kahraman, S., Akarsu, C., Cengiz, G., Dirican, K., Sozen, E., Can, B., Guven, C. & Vanderzwalmen, P. 1999, "Fertility of ejaculated and testicular megalohead spermatozoa with intracytoplasmic sperm injection", *Human Reproduction (Oxford, England),* vol. 14, no. 3, pp. 726-730.

Kahraman, S., Akarsu, C., Cengiz, G., Dirican, K., Sozen, E., Can, B., Guven, C. & Vanderzwalmen, P. 1999, "Fertility of ejaculated and testicular megalohead spermatozoa with intracytoplasmic sperm injection", *Human Reproduction (Oxford, England),* vol. 14, no. 3, pp. 726-730.

Kang-Decker, N., Mantchev, G.T., Juneja, S.C., McNiven, M.A. & van Deursen, J.M. 2001, "Lack of acrosome formation in Hrb-deficient mice", *Science (New York, N.Y.),* vol. 294, no. 5546, pp. 1531-1533.

Kato, A., Nagata, Y. & Todokoro, K. 2004, "Delta-tubulin is a component of intercellular bridges and both the early and mature perinuclear rings during spermatogenesis", *Developmental Biology,* vol. 269, no. 1, pp. 196-205.

Kostova, E., Yeung, C.H., Luetjens, C.M., Brune, M., Nieschlag, E. & Gromoll, J. 2007, "Association of three isoforms of the meiotic BOULE gene with spermatogenic failure in infertile men", *Molecular Human Reproduction,* vol. 13, no. 2, pp. 85-93.

Krawetz, S.A. 2005, "Paternal contribution: new insights and future challenges", *Nature reviews. Genetics,* vol. 6, no. 8, pp. 633-642.

Lewis-Jones, I., Aziz, N., Seshadri, S., Douglas, A. & Howard, P. 2003, "Sperm chromosomal abnormalities are linked to sperm morphologic deformities", *Fertility and Sterility,* vol. 79, no. 1, pp. 212-215.

Machev, N., Gosset, P. & Viville, S. 2005, "Chromosome abnormalities in sperm from infertile men with normal somatic karyotypes: teratozoospermia", *Cytogenetic and Genome Research,* vol. 111, no. 3-4, pp. 352-357.

Maduro, M.R. & Lamb, D.J. 2002, "Understanding new genetics of male infertility", *The Journal of Urology,* vol. 168, no. 5, pp. 2197-2205.

Mahato, D., Goulding, E.H., Korach, K.S. & Eddy, E.M. 2000, "Spermatogenic cells do not require estrogen receptor-alpha for development or function", *Endocrinology,* vol. 141, no. 3, pp. 1273-1276.

Makinen, A., Andersson, M. & Nikunen, S. 1998, "Detection of the X chromosomes in a Klinefelter boar using a whole human X chromosome painting probe", *Animal Reproduction Science,* vol. 52, no. 4, pp. 317-323.

Martin, R.H., Rademaker, A.W., Greene, C., Ko, E., Hoang, T., Barclay, L. & Chernos, J. 2003, "A comparison of the frequency of sperm chromosome abnormalities in men with mild, moderate, and severe oligozoospermia", *Biology of Reproduction,* vol. 69, no. 2, pp. 535-539.

Mau-Holzmann, U.A. 2005, "Somatic chromosomal abnormalities in infertile men and women", *Cytogenetic and Genome research,* vol. 111, no. 3-4, pp. 317-336.

Meschede, D., Dworniczak, B., Nieschlag, E. & Horst, J. 1998, "Genetic diseases of the seminal ducts", *Biomedicine & Pharmacotherapy = Biomedecine & Pharmacotherapie,* vol. 52, no. 5, pp. 197-203.

Mickle, J., Milunsky, A., Amos, J.A. & Oates, R.D. 1995, "Congenital unilateral absence of the vas deferens: a heterogeneous disorder with two distinct subpopulations based

upon aetiology and mutational status of the cystic fibrosis gene", *Human Reproduction (Oxford, England),* vol. 10, no. 7, pp. 1728-1735.

Miyamoto, T., Tsujimura, A., Miyagawa, Y., Koh, E., Namiki, M., Horikawa, M., Saijo, Y. & Sengoku, K. 2011, "Single nucleotide polymorphism in the UBR2 gene may be a genetic risk factor for Japanese patients with azoospermia by meiotic arrest", *Journal of assisted reproduction and genetics,* [Epub ahead of print] .

Moorhead, P.S., Nowell, P.C., Mellman, W.J., Battips, D.M. & Hungerrford, D.A. 1960, "Chromosome preparations of leukocytes cultured from human peripheral blood", *Experimental Cell Research,* vol. 20, pp. 613-616.

Moretti, E., Collodel, G., Scapigliati, G., Cosci, I., Sartini, B. & Baccetti, B. 2005, "'Round head' sperm defect. Ultrastructural and meiotic segregation study", *Journal of submicroscopic cytology and pathology,* vol. 37, no. 3-4, pp. 297-303.

Moura, A.A. & Erickson, B.H. 2001, "Testicular development, histology, and hormone profiles in three yearling angus bulls with spermatogenic arrest", *Theriogenology,* vol. 55, no. 7, pp. 1469-1488.

Nagano, M., Avarbock, M.R., Leonida, E.B., Brinster, C.J. & Brinster, R.L. 1998, "Culture of mouse spermatogonial stem cells", *Tissue & cell,* vol. 30, no. 4, pp. 389-397.

Nagano, M., Brinster, C.J., Orwig, K.E., Ryu, B.Y., Avarbock, M.R. & Brinster, R.L. 2001, "Transgenic mice produced by retroviral transduction of male germ-line stem cells", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 98, no. 23, pp. 13090-13095.

Nantel, F., Monaco, L., Foulkes, N.S., Masquilier, D., LeMeur, M., Henriksen, K., Dierich, A., Parvinen, M. & Sassone-Corsi, P. 1996, "Spermiogenesis deficiency and germ-cell apoptosis in CREM-mutant mice", *Nature,* vol. 380, no. 6570, pp. 159-162.

Nicholas, F.W. 2003, *Introduction to veterinary genetics*, Blackwell Publishing Ltd, second edition, pp. 4-10, 93-96.

Nistal, M., Paniagua, R. & Herruzo, A. 1977, "Multi-tailed spermatozoa in a case with asthenospermia and teratospermia", *Virchows Archiv.B: Cell pathology,* vol. 26, no. 2, pp. 111-118.

Perrin, A., Morel, F., Moy, L., Colleu, D., Amice, V. & De Braekeleer, M. 2008, "Study of aneuploidy in large-headed, multiple-tailed spermatozoa: case report and review of the literature", *Fertility and sterility,* vol. 90, no. 4, pp. 1201.e13-1201.e17.

Pieters, M.H., Speed, R.M., de Boer, P., Vreeburg, J.T., Dohle, G. & In't Veld, P.A. 1998, "Evidence of disturbed meiosis in a man referred for intracytoplasmic sperm injection", *Lancet,* vol. 351, no. 9107, pp. 957.

Rens, W., Yang, F., Welch, G., Revell, S., O'Brien, P.C., Solanky, N., Johnson, L.A. & Ferguson Smith, M.A. 2001, "An X-Y paint set and sperm FISH protocol that can be used for validation of cattle sperm separation procedures", *Reproduction (Cambridge, England),* vol. 121, no. 4, pp. 541-546.

Revay, T., Kovacs, A., Presicce, G.A., Rens, W. & Gustavsson, I. 2003, "Detection of water buffalo sex chromosomes in spermatozoa by fluorescence in situ hybridization", *Reproduction in domestic animals = Zuchthygiene,* vol. 38, no. 5, pp. 377-379.

Ross, A., Christie, S. & Edmond, P. 1973, "Ultrastructural tail defects in the spermatozoa from two men attending a subfertility clinic", *Journal of reproduction and fertility,* vol. 32, no. 2, pp. 243-251.

Sadeghi-Nejad, H. & Farrokhi, F. 2006, "Genetics of azoospermia: current knowledge, clinical implications, and future directions. Part I", *Urology journal,* vol. 3, no. 4, pp. 193-203.

Santos, N.R., Krekeler, N., Schramme-Jossen, A. & Volkmann, D.H. 2006, "The knobbed acrosome defect in four closely related dogs", *Theriogenology,* vol. 66, no. 6-7, pp. 1626-1628.

Sassone-Corsi, P. 2002, "Unique chromatin remodeling and transcriptional regulation in spermatogenesis", *Science (New York, N.Y.),* vol. 296, no. 5576, pp. 2176-2178.

Seabright, M. 1971, "A rapid banding technique for human chromosomes", *Lancet,* vol. 2, no. 7731, pp. 971-972.

Shanks, R.D. & Robinson, J.L. 1989, "Embryonic mortality attributed to inherited deficiency of uridine monophosphate synthase", *Journal of dairy science,* vol. 72, no. 11, pp. 3035-3039.

Sharif, K. 2000, "Reclassification of azoospermia: the time has come?", *Human reproduction (Oxford, England),* vol. 15, no. 2, pp. 237-238.

Sironen, A., Vilkki, J., Bendixen, C. & Thomsen, B. 2007, "Infertile Finnish Yorkshire boars carry a full-length LINE-1 retrotransposon within the KPL2 gene", *Molecular genetics and genomics : MGG,* vol. 278, no. 4, pp. 385-391.

Sironen, A., Thomsen, B., Andersson, M., Ahola, V. & Vilkki, J. 2006, "An intronic insertion in KPL2 results in aberrant splicing and causes the immotile short-tail sperm defect in the pig", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 103, no. 13, pp. 5006-5011.

Sironen, A.I., Andersson, M., Uimari, P. & Vilkki, J. 2002, "Mapping of an immotile short tail sperm defect in the Finnish Yorkshire on porcine Chromosome 16", *Mammalian genome: official journal of the International Mammalian Genome Society,* vol. 13, no. 1, pp. 45-49.

Sironen, A., Uimari, P., Nagy, S., Paku, S., Andersson, M. & Vilkki, J. 2010, "Knobbed acrosome defect is associated with a region containing the genes STK17b and HECW2 on porcine chromosome 15", *BMC genomics,* vol. 11, pp. 699.

Slota, E., Kozubska-Sobocinska, A., Koscielny, M., Danielak-Czech, B. & Rejduch, B. 2003, "Detection of the XXY trisomy in a bull by using sex chromosome painting probes", *Journal of Applied Genetics,* vol. 44, no. 3, pp. 379-382.

Soderquist, L. 1998, "Reduced fertility after artificial insemination in a ram with a high incidence of knobbed acrosomes", *The Veterinary record,* vol. 143, no. 8, pp. 227-228.

Spriggs, E.L., Rademaker, A.W. & Martin, R.H. 1996, "Aneuploidy in human sperm: the use of multicolor FISH to test various theories of nondisjunction", *American Journal of Human Genetics,* vol. 58, no. 2, pp. 356-362.

Sukura, A., Makipaaa, R., Vierula, M., Rodriguez-Martinez, H., Sundback, P. & Andersson, M. 2002, "Hereditary sterilizing short-tail sperm defect in Finnish Yorkshire boars", *Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc,* vol. 14, no. 5, pp. 382-388.

Tempest, H.G. & Griffin, D.K. 2004, "The relationship between male infertility and increased levels of sperm disomy", *Cytogenetic and genome research,* vol. 107, no. 1-2, pp. 83-94.

Tempest, H.G., Ko, E., Rademaker, A., Chan, P., Robaire, B. & Martin, R.H. 2009, "Intra-individual and inter-individual variations in sperm aneuploidy frequencies in normal men", *Fertility and sterility,* vol. 91, no. 1, pp. 185-192.

Terribas, E., Bonache, S., Garcia-Arevalo, M., Sanchez, J., Franco, E., Bassas, L. & Larriba, S. 2010, "Changes in the Expression Profile of the Meiosis-Involved Mismatch Repair (MMR) Genes in Impaired Human Spermatogenesis", *Journal of andrology, .*

Thielemans, B.F., Spiessens, C., D'Hooghe, T., Vanderschueren, D. & Legius, E. 1998, "Genetic abnormalities and male infertility. A comprehensive review", *European journal of obstetrics, gynecology, and reproductive biology,* vol. 81, no. 2, pp. 217-225.

Thundathil, J., Meyer, R., Palasz, A.T., Barth, A.D. & Mapletoft, R.J. 2000, "Effect of the knobbed acrosome defect in bovine sperm on IVF and embryo production", *Theriogenology,* vol. 54, no. 6, pp. 921-934.

Toyama, Y. & Itoh, Y. 1993, "Ultrastructural features and pathogenesis of knobbed spermatozoa in a boar", *American Journal of Veterinary Research,* vol. 54, no. 5, pp. 743-749.

Vierula, M., Alanko, M., Andersson, M. & Vanha-Perttula, T. 1987, "Tail stump sperm defect in Ayrshire bulls: morphogenesis of the defect", *Andrologia,* vol. 19 Spec No, pp. 207-216.

Viville, S., Mollard, R., Bach, M.L., Falquet, C., Gerlinger, P. & Warter, S. 2000, "Do morphological anomalies reflect chromosomal aneuploidies?: case report", *Human reproduction (Oxford, England),* vol. 15, no. 12, pp. 2563-2566.

Wang, Z., Milunsky, J., Yamin, M., Maher, T., Oates, R. & Milunsky, A. 2002, "Analysis by mass spectrometry of 100 cystic fibrosis gene mutations in 92 patients with congenital bilateral absence of the vas deferens", *Human reproduction (Oxford, England),* vol. 17, no. 8, pp. 2066-2072.

Watson, P.F. 1975, "Use of a Giemsa stain to detect changes in acrosomes of frozen ram spermatozoa", *The Veterinary record,* vol. 97, no. 1, pp. 12-15.

Wattendorf, D.J. & Muenke, M. 2005, "Klinefelter syndrome", *American Family Physician,* vol. 72, no. 11, pp. 2259-2262.

Weissenberg, R., Aviram, A., Golan, R., Lewin, L.M., Levron, J., Madgar, I., Dor, J., Barkai, G. & Goldman, B. 1998, "Concurrent use of flow cytometry and fluorescence in-situ hybridization techniques for detecting faulty meiosis in a human sperm sample", *Molecular human reproduction,* vol. 4, no. 1, pp. 61-66.

Wistuba, J., Schlatt, S., Cantauw, C., von Schonfeldt, V., Nieschlag, E. & Behr, R. 2002, "Transplantation of wild-type spermatogonia leads to complete spermatogenesis in testes of cyclic 3',5'-adenosine monophosphate response element modulator-deficient mice", *Biology of Reproduction,* vol. 67, no. 4, pp. 1052-1057.

Yao, R., Ito, C., Natsume, Y., Sugitani, Y., Yamanaka, H., Kuretake, S., Yanagida, K., Sato, A., Toshimori, K. & Noda, T. 2002, "Lack of acrosome formation in mice lacking a Golgi protein, GOPC", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 99, no. 17, pp. 11211-11216.

Yurov, Y.B., Saias, M.J., Vorsanova, S.G., Erny, R., Soloviev, I.V., Sharonin, V.O., Guichaoua, M.R. & Luciani, J.M. 1996, "Rapid chromosomal analysis of germ-line cells by FISH: an investigation of an infertile male with large-headed spermatozoa", *Molecular Human Reproduction,* vol. 2, no. 9, pp. 665-668.

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