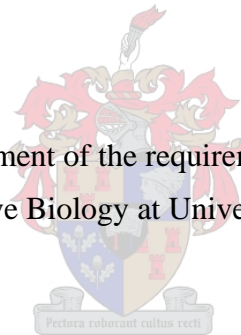# THE INFLUENCE OF SEX CHROMOSOMES ON THE OUTCOME OF HUMAN EMBRYO DEVELOPMENT.

KIMENTHRA RAJA

Thesis presented in partial fulfillment of the requirements for the degree of Master of Science in Reproductive Biology at University of Stellenbosch.
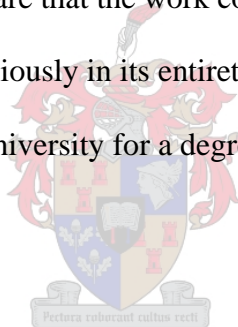
Promoter: Prof Daniel R Franken

Co –Promoter: Dr Aldo E Esterhuizen

December 2005

DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own

work and that I have not previously in its entirety or in part submitted it at any

university for a degree.

Signature...Date...

SUMMARY

CHAPTER 1 presents comprehensive background information regarding all aspects addressed in this thesis. Special attention was given to literature on paternal influences on embryonic development, the role of sperm RNA, sperm chromatin and sperm functional aspects i.e. morphology and acrosomal status and size. The experimental design and all relevant methods used during the study as well as the material that were used are presented in **CHAPTER 2.** The results of the different techniques and evaluations are provided in **CHAPTER 3**. It was found that 70% of the embryos that showed no developmental potential were Y-chromosome bearing embryos. The sperm selection process for ICSI based on the approach of choosing the "best looking" spermatozoon in the ejaculate seem to provide cells that can be classified as normal based on the length width ratio set by the WHO for normal cells. The chromatin packaging quality of the sperm correlated significantly and negatively with the percentage normal cells in the ejaculates. **CHAPTER 4** comprises of a general discussion of the results and short summary of the major findings during the project. The discussion section focused on the paternal influence on the embryonic development and provided a suggestion for future research that can possibly lead to the use of X-chromosome bearing sperm in case of severe male factor cases. **CHAPTER 5** contains the bibliographical information of the study.

OPSOMMING

HOOFSTUK 1 bied 'n volledige literatuu oorsig van alle aspekte wat moontlik 'n rol spell tydens embrio ontwikkeling. Daar word ook spediale aandag geskenk aan literatuur wat handel oor die vaderlike invloed, sperm RNS, kromatienpakking en funksionele aspekte soos spermsel morfologie, akrosoom status en grootte. **HOOFSTUK 2** verskaf die eksperimentele studie ontwerp en all metodes asook materiaal wat tydens die projek gebruik is. Die resultate word in **HOOFSTUK 3** aangebied. Daar is gevind dat 70% van die embrios wat nie verder ontwikkel het nie, - kromosomm draende embrios was. Dit blyk ook dat die sperm seleksie metode wat tydens die ICSI prosedure gebruik is, naamlik die "mees geskikte" sperm, voldoende was. Die ICSI geselekteerde sperms het voldoen aan die Wêreldgesondheids Organisasie se lengte:breedte riglyne vir normale spermatozoa. Kromatienpakkings kwaliteir het betekenisvol en negatief gekorreleer met die persentasie normal spermatozoa. In **HOOFSTUK 4** word die werk en resultate bespreek. Verder is daar ook 'n kort opsomming van die belangrikstre afleidings en toekoms ontwikkelings. 'n Voledige bibliografiese lys word in **HOOFSTUK 5** aangebied.

DEDICATION

I proudly dedicate this thesis to my loving family.

ACKNOWLEDGEMENTS

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AIMS OF THE STUDY

This multi-step study aimed to investigate the possible relationship between gender of discarded embryo's i.e. embryos that did not develop further after fertilization and

(i)   morphological appearance/shape of the sperm cell injected into the oocyte during ICSI therapy,

(ii)   the percent normal sperm cells in the ejaculate of the patient,

(iii)   acrosome size and status,

(iv)   chromatin packaging quality of the sperm population

CHAPTER 1

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1.   LITERATURE REVIEW

1.1   Introduction

Semen analysis is the basic and most commonly used test for predicting fertility; however, the standard measurements of sperm concentration, percentage motility, and morphology may not reveal subtle sperm defects. In this context, sperm chromatin abnormalities have been studied extensively in the past decade as a cause for male infertility. (Sakkas *et al.*, 1999).
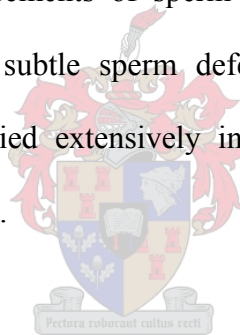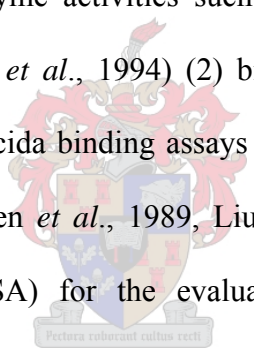
The first successful pregnancies after the intracytoplasmic injection of a single spermatozoon into an oocyte (ICSI) were rapidly followed by the widespread use of this novel technique for the treatment of male factor infertility (Palermo *et al.,* 1992, Palermo *et al.*, 1993). This has stimulated great interest in the indications, the application, the efficacy and also the safety of ICSI. The profound success of intracytoplasmic sperm injection as a treatment has transformed the perspective of male infertility (Glover and Barratt 1999, Brown *et al.,* .1999).

Unfortunately, and somewhat remarkably, the explosion of interest in clinical male infertility has not been accompanied by significant advances in our understanding of the

cellular and biochemical lesions that cause sperm dysfunction (Barrat and St John, 1999). However, there has been an increasing recognition of the significant contribution of male factor defects in infertility. In fact, in many studies, male factor infertility is regarded as the common most single cause of infertility (Irvine, 1998). Consequently, a wide variety of diagnostic tests have been developed in an effort to underline the cause of fertilization failure.

Categories of diagnostic assays that are usually recommended include: (1) tests that examine defective sperm functions indirectly through the use of biochemical tests i.e., measurement of the generation of reactive oxygen species or evidence of peroxidative damage, measurement of enzyme activities such as creatine phosphokinase and others (Aitken *et al*., 1989, Huszar *et al*., 1994) (2) bioassays of gamete interaction i.e., the homologous sperm-zona pellucida binding assays and induced-acrosome reaction scoring (Burkman *et al*., 1988, Franken *et al*., 1989, Liu *et al*., 1989); and (3) computer-aided sperm motion analysis (CASA) for the evaluation of sperm motion characteristics (ESHRE, 1996; WHO, 1999).

To bypass the various steps at which fertilization failure could occur, several micro-manipulation techniques were introduced for example, chemical zona drilling (Gordon and Talansky, 1986), partial zona dissection (Malter and Cohen, 1989; Vanderswalmen *et al.,* 1992) and subzonal insemination (Ng *et al.,* 1988, Fishel *et al.,* 1990, 1992; Gordts *et al*., 1993). Intracytoplasmic sperm injection (ICSI) however, has developed as a radical form of micro assisted fertilization (Fischel *et al.,* 1992; Van Steirteghem *et al.,* 1993). The successful implementation of ICSI has provided a unique means to allow couples diagnosed with male infertility to achieve their reproductive goals.

Although ICSI has become the preferred treatment of men with various degrees of sperm anomalies, it may carry a risk of transmission of chromosomal/genetic disease (Bonduelle, *et al.*, 1999).

1.2 Chromosomal/Genetic disorders of human gametes

As embryos must be implanted in the uterus in a time-sensitive manner, rapid analysis is required either by polymerase chain reaction (PCR) or fluorescence in-situ hybridization (FISH). For single gene studies, PCR is used to amplify specific DNA fragments that can then be analyzed for a mutation using standard analytic methods, such as restriction enzyme digestion or single-strand conformational polymorphism. At least 30 different diseases have been diagnosed by this technique (Table 1.1, Figures 1.1 &1.2)

Table 1.1

Inherited conditions for which Pre-implantation Genetic Diagnosis (PGD) has been reported (Shanine *et al.*, 2005)

X-linked	Autosomal dominant	Autosomal recessive
Agammaglobulinemia	Central core disease	Adrenogental syndrome
Alport syndrome	Charcot-Marie-Tooth disease	B-thamassemia
Duchenne muscular dystrophe	Crouson syndrome	CDGIC
Hemophilia A	Familial adenomatous polyposis coli	Congenital adrenal hyperplasya
Identification of sex	Huntington's chorea	Cystic fibrosis
Fragile X syndrome	Li Fraumeni syndrome	Epidermolysis bullosa
Ocular albinism 1	Marfan syndrome	Gaucher's disease
Orthinine transcarbamylase deficiency	Mytotic dystrophe	Hyperinsulinenic hypoglycemia
Orofacial-digital syndrome type 1	Neurofibromatosis type 2	Lesch-Nyhan syndrome
Retininitis pigmentosa	Osteogenesis imperfecta I & IV	Medium chain acyl-CoA dehydrogenase deficiency
Severe combined immunodeficiency	Stickler syndrome	Plakophilin I
	Tuberous sclerosis	Rh blood typing
		Sickle cell disease
		Spinal muscular dystrophy
		Tay-Sachs disease

Figure 1.1 Diagram illustrating autosomal dominant conditions. http://www.nlm.nih.gov/medlineplus/ency/article/002049.html.	Figure 1.2 Diagram illustrating autosomal recessive conditions http://www.nlm.nih.gov/medlineplus/ency/article/002052.
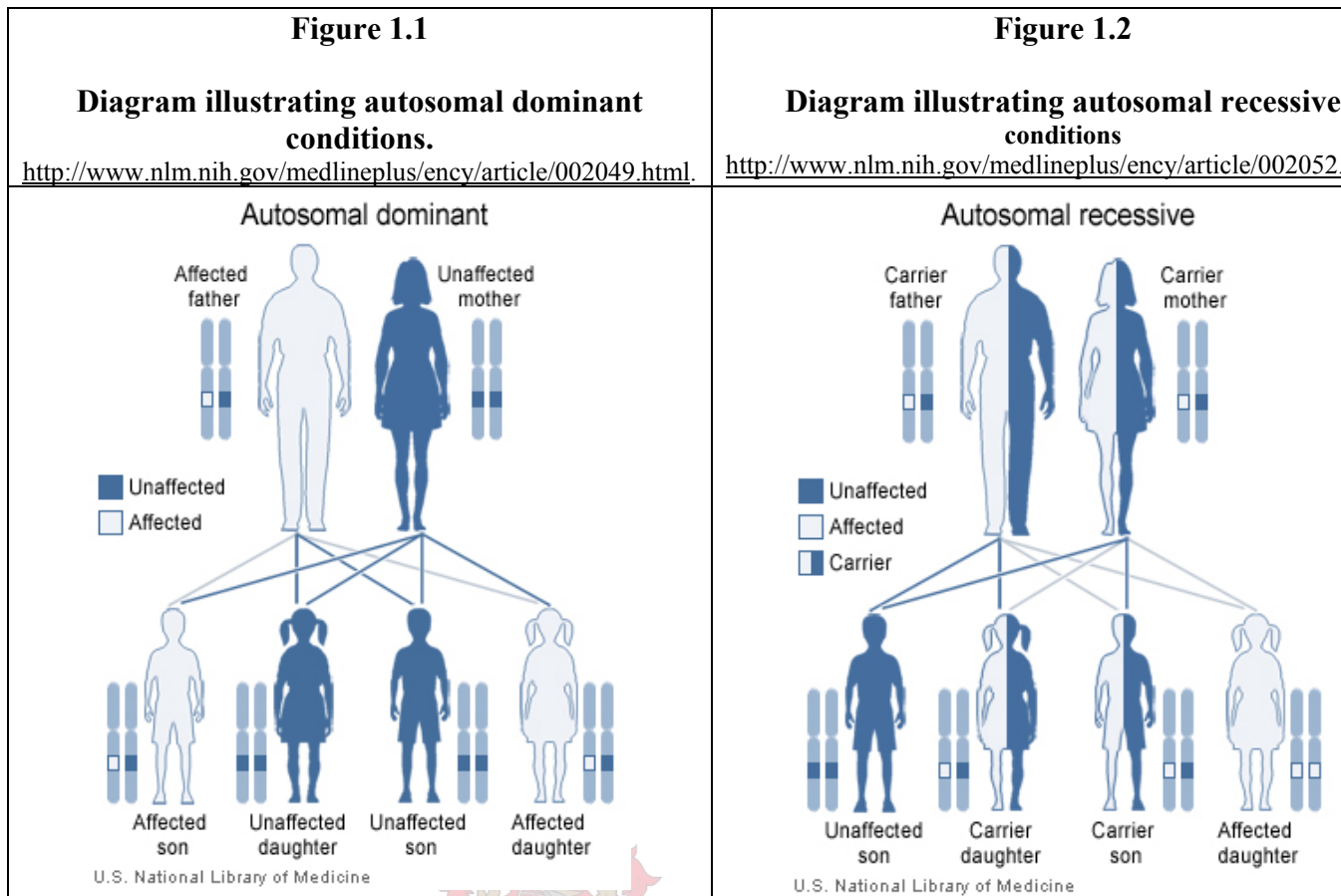
In the case of autosomal dominant genes (Figure 1.1), a single abnormal gene on one of the autosomal chromosomes (one of the first 22 "non-sex" chromosomes) from either parent can cause the disease. One of the parents will have the disease (since it is dominant) in this mode of inheritance and that person is called the CARRIER. Only one parent must be a carrier in order for the child to inherit the disease.

Two mutated copies of the gene are present in each cell when a person has an autosomal recessive disorder. An affected person usually has unaffected parents who each carry a single copy of the mutated gene (and are referred to as carriers) (Figure 1.2). Autosomal recessive disorders are typically not seen in every generation of an affected family

1.2.1 Oocytes

The first attempts to analyse the chromosomal content of human female gametes were made in the early 1970s (Chandly, 1971), and led to limited data because of the paucity of materials and the inadequacy of the procedure used. The years to follow brought a resurgence of interest in this field, because of the development of human IVF techniques which made oocytes unfertilized *in vitro* available for cytogenetic analysis (Jagiello *et al*., 1976, Gutierrez-Mateo *et al*., 2005).

Today it is believed that early embryonic wastage caused by chromosome aberrations is thought to be one of the single most important factors which contribute to the low fertility rate in humans (Baçhe *et al*., 1999). Some evidence suggests that there is a negative selection against some chromosome abnormalities during the first stages of embryonic development (Boué *et at*., 1985). This may explain the fact that the rate of aneuploidies in cleavage- stage embryos (Munné *et al*., 1995a; Márquez *et aI*., 2000) is much higher than that found in spontaneous abortions and liveborns (Hassold and Hunt, 2001).

Chromosome abnormalities have been associated with maternal age, stimulation protocols, oocyte aging, increasing follicular harvesting and spindal disruption and subsequent loss or scattering of chromosomes (Martini et al., 1997, Wall et a., 1996). A number of studies have described the DNA status of the unfertilized oocyte in an attempt to explain the etiology of partial and total fertilization failure.

Abnormal female chromatin was detected among unfertilized IVF oocytes viz. a nucleus or clumped chromatin, instead of MII chromosomes, was detected. (Van Wissen et

al., 1992). The expulsion of the second polar body was in these cases not possible. A prevalence of 25-35% of chromosomal abnormalities was described in unfertilized oocytes (Van Wissen & Bomsell-Heinrich, 1994).

1.2.2 Spermatozoa

At the gene level, reproductive failure may be associated with cystic fibrosis mutations (in men presenting with obstructive azoospermia due to congenital absence of the vas deferens) and with Y-microdeletions (in men with severe oligozoospermia and non-obstructive azoospermia due to spermatogenic failure). Such abnormalities can also be detected by peripheral blood screening using PCR methodologies (conventional, nested, multiplex, fluorescent or quantitative PCR) (St. John, 1999).

Spermatozoa of infertile men have also been shown to contain various nuclear alterations. Some of them include an abnormal chromatin structure, aneuploidy, chromosomal microdeletions and DNA strand breaks (Sakkas, *et al*., 1999). Presently, various tests are available for detection of some of those anomalies, including the aniline blue staining, acridine orange, sperm chromatin structure assay (SCSA) and assessment of DNA damage or fragmentation (Evenson, *et al*., 1999; Barroso, *et al*., 2000).

1.3 Paternal influence on embryonic development

The question of whether there is a paternal effect on embryonic development and eventually the reproductive outcome has been studied in depth in animal models (Kola &Wilton, 2005, Lee & De Mayo, 2004).

Various animal models and even in *in vitro* studies in the human (Kruger *et al.,* 198; Oehninger *et al.,* 1996; Burello *et al.,* 2004; de Vos *et al.,* 2003) have indicated that abnormal spermatozoa can drastically affect fertilization, embryo development, implantation and fetal development.

The most profound paternal effect is observed in cases where the spermatozoa are considered abnormal and in particular when spermatozoa have been affected with drugs, such as cyclophosphamide, or irradiation. Parker *et al.,* (1999) have shown among men who worked under nuclear radiation exposure, a significant positive association between a father's annual summary radiation dosage and stillbirth risk.

The use of ICSI in the field of assisted reproduction has caused some concern over the paternal influence on the embryo quality and development. These concerns were mainly based on the invasive nature of the technique and also on the increased possibility of using an abnormal spermatozoon during the injection phase of the technique. The use of ICSI, and the subsequent use of possibly compromised spermatozoa, has also led to numerous publications suggesting a link to major birth defects and imprinting anomalies (Egozcue *et al.,* 1997; Bonduelle *et al.,* 1999).

Several studies are now focusing on identifying nuclear anomalies in human spermatozoa and linking them with poor reproductive outcomes (Egozcue *et al.,* 1997; Bonduelle *et al.,* 1999). The chromosomal anomalies linked to inheritance of a defective paternal genome can be identified; however problems arise when a subtle anomaly is linked to a paternal influence. One area of interest is that examining for an association between abnormal sperm nuclear DNA and reproductive outcome (Bonduelle *et al.,* 1999)

The use of chromosome specific DNA probes labeled with fluorochromes and especially the combination of several probes has been used to indirectly study the chromosome constitution of decondensed sperm nuclei by fluorescence in-situ hybridization (FISH), and has allowed this test to be included in the protocol of a study of infertile males (Egozcue *et al.,* 1997). There are now various mechanisms allowing the investigation of nuclear anomalies in spermatozoa, for example: the sperm chromatin structure assay, TUNEL labeling, *in situ* nick translation, numerous fluorochromes and the COMET assay.

Two techniques of importance are; (a) The Sperm Chromatin Structure Assay (SCSA) and (b) TUNEL labeling (terminal deoxynucleotidyl transferase TdTI-mediated dUTP nick end-labeling). The SCSA has been used to show that anomalies in the sperm chromatin structure are linked to failure of patients to establish pregnancies (reviewed in Evenson *et al.,* 2002).
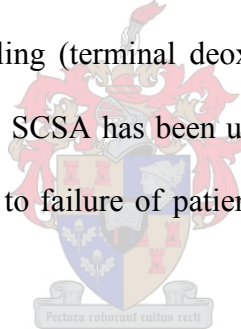
The SCSA relies on the fact that abnormal sperm chromatin has a greater susceptibility to the physical induction of partial DNA denaturation *in situ*. The extent of DNA denaturation following heat or acid treatment is determined by measuring the metachromatic shift from green fluorescence (Acridine Orange intercalated into double-stranded nucleic acid) to red fluorescence (Acridine Orange associated with single stranded DNA). (Drazynkiewicz *et al*., 1975). In clinical applications, the SCSA parameters not only distinguish fertile and infertile men but also are able to classify men according to the level of *in vivo* fertility (pregnancy initiated in less than 3 months), moderate fertility

(pregnancy initiated within 4–12 months), and no proven fertility (no pregnancy by 12 months).

The TUNEL technique, a process which identifies DNA breaks by labeling 3'-OH termini using exogenous terminal deoxynucleotidyl transferase, has been used to identify a population of spermatozoa in the ejaculate that are believed to be apoptotic (Sakkas *et al.,* 2002). This technique has also shown a plausible link to the ability of patients to achieve pregnancy (Tomlinson *et al.,* 2001).

1.4   Sperm RNA

Considering the shortage of cytoplasm, and the lack of any detectable protein synthesis in mature sperm heads, biologists had long assumed that sperm contributes little to an embryo. In contrast, the egg is abundant with molecules such as proteins and RNAs that nourish and direct the development of the embryo (Ainsworth, 2005). Studies now suggest that defects in sperm can disrupt embryo development even if the genes carried by the cells are perfectly normal (Loppen *et al.,* 2005). Evidence suggests that defective sperm can be a cause of a significant number of miscarriages (Ostermeier *et al.,* 2002). Studies on sperm from infertile men indicated the presence of 3000 different kinds of messenger RNA (Saunders *et al*., 2002; Ostermeier *et al*., 2002; Sutovsky *et al*., 2000).

This suggested that sperm could deliver RNAs that help direct an embryo's early development. Some biologists were unconvinced, arguing that the RNAs were simply non-functional remnants from the processes of sperm development. Earlier findings showed however that a specific package of RNAs is indeed transferred from sperm to egg

(Ostermeier *et al.,* 2004). Recently, Krawetz and his colleagues found that these include micro-RNAs, which do not code for proteins but are known to play a role in controlling gene activity (Ostermeier *et al*., 2004; Ostermeier *et al*., 2005).

Evidence exists suggesting that messenger RNAs help protect paternal genes that are needed soon after fertilization from being shut down as sperm mature. Normally, most of a sperm's DNA is tightly packed and protected by proteins called protamines. One possibility is that the RNAs could mask the genes that code for them and another possibility is that paternal RNAs, particularly micro-RNAs, might be involved in controlling imprinting, the differential activation of genes according to whether they are inherited from the mother or the father (Ostermeier *et al*., 2004; Ostermeier *et al*., 2005).

The new view of sperm as carriers of molecules crucial for early embryo development has thought provoking implications for reproductive medicine. Comparing the RNA profiles of fertile and infertile men might reveal causes of unexplained infertility. (Ostermeier *et al*., 2004; Ostermeier *et al*., 2005

Such studies may also raise questions about the wisdom of an *in vitro* fertilization technique called intracytoplasmic sperm injection, or ICSI, used to help men whose sperm do not fertilize their partner's eggs. ICSI involves injecting faulty or immature sperm - which might lack the normal complement of RNAs -directly into eggs. So far, there are no clear signs of problems among children conceived by ICSI, although long-term follow-up is needed to confirm the safety of the technique.

Finally, new research efforts in sperm biology are moving from RNA to proteins. Recent work reported the first proteomic study of male infertility. The aim of that study was not to produce a complete proteome for sperm, but evaluated differences in the protein profiles in the sperm of an infertile and a fertile man. The researchers found at least 20 proteins present in significantly different quantities (Pixton *et al.,* 2004), giving them a starting point to study cases of unexplained infertility and suggesting targets for new contraceptives.

1.5   Sperm chromatin packaging quality

The focus on the genomic integrity of the male gamete has been further intensified by the growing concern of transmission of genetic diseases through assisted reproductive techniques (ART), specifically intracytoplasmic sperm injection (ICSI). Accumulating evidence indicates that a negative correlation exists between disturbances in the organization of the genomic material in sperm nuclei and the fertility potential of spermatozoa, whether *in vivo* or *in vitro*. (Sun *et al.,* 1997; Spano *et al.,* 2000) This emphasizes that stable DNA, which is capable of decondensation at the appropriate time in the fertilization process, is one of the criteria needed to consider a spermatozoon fertile (Amann, 1989).

Conventional semen analysis *per se* cannot cover the diverse array of biological properties that the spermatozoon expresses as a highly specialized cell (Zini *et al.,* 2001, Evenson *et al.,* 2002). In addition, the results of semen analyses can be very subjective and prone to intra- and inter observer variability (Keel and Webster, 1990). At the present time, it is clear that a sperm chromatin structure of poor quality may be indicative of male

subfertility, regardless of the number, motility and morphology of spermatozoa. Sperm chromatin structure evaluation is an independent measure of sperm quality that provides good diagnostic and prognostic capabilities.

Therefore, it may be considered a reliable predictor of a couple's inability to become pregnant, (Evenson *et al.,* 1999) and may also have an impact on the offspring, resulting in infertility (Aitken, 1999). Many techniques have been described for evaluation of the chromatin status.

1.5.1   Human sperm chromatin structure

The nuclear status of sperm cells is determined by two major events that occur during spermiogenesis: acquisition of the final nuclear shape and the replacement of somatic-type histones by protamines (sperm-specific basic nuclear proteins) leading to highly packaged chromatin. Sperm DNA is organized in a specific manner to keep the chromatin in the nucleus compact and stable.

It is packed into a tight, almost crystalline status that is at least six times more condensed than mitotic chromosomes. It occupies nearly the entire nucleus volume, whereas somatic cell DNA only partly fills the nucleus (Fuentes-Mascorro *et al.,* 1999). This DNA organization not only permits the very tightly packaged genetic information to be transferred to the egg, but also ensures that the DNA is delivered in a physical and chemical form that allows the developing embryo to access the genetic information (Poccio, 1986).

Sperm nuclei do not have the volume required for the type of packaging present in somatic cells, because packing the DNA in a single, closely packed nucleosome requires $9.9\mu m^3$ (Agarwal and Damer 2004), which is more than twice the volume of an average sperm nucleus. Thus, a completely different type of DNA packaging must be present in mammalian sperm nuclei (Ward and Coffey 1991). Organization of chromatin for packaging in the spermatozoon takes place at four different levels: chromosomal anchoring, which refers to the attachment of the DNA to the nuclear annulus; formation of DNA loop domains as the DNA attaches to the newly added nuclear matrix; replacement of histones by protamines, which condense the DNA into compact doughnuts; and chromosomal positioning (Ward and Coffey, 1991).

The histones are first displaced by transition proteins (TNPs), which are removed from the condensing chromatin at later stages and replaced by protamines. It is of interest to note that the condensation of chromatin begins in the posterior pole and proceeds apically, which is a unique feature in humans that is not present in other mammalian species (Dadoune, 1998). Sperm epididymal maturation implies a final stage of chromatin organization involving protamine cross-linking by disulfide bond formation—a step that is supported by the fact that protamines contain a significant number of cysteine residues that participate in sperm chromatin compaction by forming multiple inter- and intra protamine disulfide cross-links. All these interactions make the mammalian DNA the most condensed eukaryotic DNA (Ward and Coffey, 1994).

1.5.2   Chromomycin A$_3$ Stain

Conventional semen parameters sometimes fail to predict fertilization outcome suggesting other hidden abnormalities, lying at sperm membrane level or at a chromatin

level. Chromomycin A_3 can detect these anomalies. It is a guanine-cytosine-specific fluorochrome that exposes chromatin that is poorly packaged in human spermatozoa via direct visualization of protamine deficient DNA (Bianchi *et al.*, 1996). The chromomycin A_3 and protamines compete for the same binding sites in the DNA which is also one limiting factor of CMA_3, because protamine levels vary according to spermatozoa maturity therefore limiting CMA_3 accessibility to the DNA (Bianchi *et al*, 1996).

A high CMA_3 fluorescence is a strong indicator of low protamination of the sperm DNA (Franken *et al*, 1999). A number of studies have shown that abnormal chromatin packaging relates to infertility in men (Evenson *et al*, 1980; Monaco & Rasch;, 1982; Foresta *et al*, 1992). The bright yellow spermatozoa (CMA_3-positive) are easily distinguished from the dull yellow spermatozoa (CMA_3-negative). Odds ratio analysis has shown (Esterhuizen *et al*, 2002) that cases where >60% CMA_3 staining were reported had a 15.6 fold increase in the risk of decondensation failure, relative to the CMA_3 staining <44% group. CMA_3 staining has a sensitivity of 73% and a specificity of 75% (Franken *et al*, 1999). Distinction between fertilization failure or success can therefore be made although it has been seen in some ICSI cases the CMA_3 positivity (very low protamination) does not indicate fertilization failure, but rather the failure of decondensation (although chromatin packaging is also responsible for the decondensation process) (Franken *et al*, 1999).

1.5.2.1 Advantages and limitations of the CMA_3 assay

The CMA_3 assay yields reliable results as it is strongly correlated with other assays used in the evaluation of sperm chromatin. In addition, the sensitivity and specificity of the CMA_3 stain are comparable to those of other DNA staining techniques; for example

acidic aniline blue stain (75% and 82%, 60% and 91%, respectively) if used in evaluation of the chromatin status in infertile men. However, it is important to note that all of these assays mentioned to this point are limited by observer subjectivity (Agarwal and Damer, 2004).

1.5.3 Chromosome abnormalities in human embryos.

After all the efforts that an in-vitro fertilization (IVF) clinic experience to achieve a successful, high implantation rate, the ultimate problem may well be that of excessive multiple pregnancies. Therefore, oocyte and embryo preference become of crucial importance. Furthermore, the numerical chromosome assessment also became one of the main tools for identifying selective criteria for human embryos (Munné and Cohen, 1998).

In order to analyze the numerical chromosome abnormalities properly, the following steps are important; first, individual chromosomes need to be assessed to determine specific aneuploidy rates. Second, all or most blastomeres from an embryo should be analysed to differentiate mosaicism from other abnormalities, and finally, developmentally arrested embryos should also be analysed.

1.6   Sperm morphology

Once sperm morphology assessments become consistent, reliable and repeatable, the concept of sperm morphology being the single most important semen parameter that correlates with the fertility potential during both in vitro (Kruger *et al*., 1986; Enginsu *et*

al., 1991; Ombelet *et al*., 1994; Coetzee *et al*., 1998; Franken *et al*., 2000a; Franken *et al.,* 2000b) and in vivo (Eggert-Kruse *et al*., 1995) results, may become a reality.

Controversy exists regarding the role of sperm morphology in the ICSI program (Nagy 1995, Nagy 1998, Tasdemir 1997; Miller *et al* 2001; Host *et al* l999; Host *et al.,* 2001; Parinaud *et al.*). Reports claim sperm cell morphology to be significantly correlated with blastocyst development (Miller *et al*., 2001), while others concluded that blastomere cleavage rate was also determined by sperm cell morphology (Salumets *et al.,* 2002). However, numerous studies showed that sperm cell morphology or any other sperm parameter is not correlated with the outcomes in ICSI (Nagy *et al*., 199; Nagy *et al.,*1998; Sallam *et al*., 1998; Kupker *et al*., 1998; Hammadeh *et al*., 1996).

On the other hand, it is known that strict morphology on the whole (raw) sample do not predict the ICSI outcome (Nagy *et al.,* 1995; Kupker *et al.,* 1998), but it seems as if individual sperm does have an effect. (Mansour *et al.,* 1995, de Vos *et al.,* 2003), De Vos et al., (2003) pointed out that the fertilization and pregnancy rate was affected by certain morphological abnormalities e.g. neck abnormalities, which definitely affected the outcome.

The approach of selecting the "best looking" sperm cells for injection is also based on findings by Bartoov *et al.,* (2003). The latter developed and described the so-called intracytoplasmic morphologically selected sperm injection (IMSI), during which high magnification techniques multiplies the image of individual sperm up to 6000 times through high power light microscopy (Bartoov *et al.,* 2003). This technique made it possible to discard sperm with abnormal shaped nuclei or contents. In short, for normally

shaped sperm, the head should be smooth, symmetric and with an oval configuration (any extrusion or invigilation of the nuclear mass is defined as malformations).

1.7   Acrosomal status

The acrosome reaction is a pre-requisite for fertilization in mammalian spermatozoa (Yanagimachi, 1994). In the mouse, one of the species best characterized so far, acrosomal exocytosis is physiologically induced by components of the zona pellucida (ZP), particularly the zona pellucida protein 3 (ZP3) (Bleil and Wassarman, 1980, 1983; Florman and Wassarman, 1985;). Binding of ZP3 to putative complementary receptor(s) on the sperm surface activates transmembrane signals that trigger cellular cascades resulting in the acrosome reaction (Wassarman, 1990a and b; Wassarman, 1999).
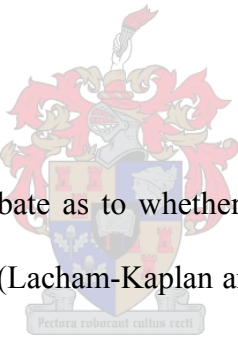
Several cellular pathways are involved in the stimulation of the acrosome reaction. It has been demonstrated that activation of pertussis toxin-sensitive heterotrimeric G proteins (G_i-class) is necessary for the ZP-induced acrosome reaction in the murine model (Kopf et al, 1986; Kopf, 1990). It has also been proposed that the ZP may alternatively activate a low voltage- activated T type calcium channel that is pertussis toxin-insensitive (Florman, et al, 1992; Florman, et al, 1998; O'Toole, et al, 2000).

Progesterone, present in high concentrations in the follicular fluid, is also a known stimulator of the acrosome reaction. It has been shown that progesterone exerts a priming effect on the ZP-stimulated acrosome reaction in the mouse (Roldan, *et al.,* 1994).

1.7.1   Acrosome reaction in *in vitro* fertilization

The induced-acrosome reaction assays i.e. zona pellucida induced acrosome reaction (Esterhuizen *et al.,* 2001) or human ZP-induced AR test (Liu and Baker, 1994) appear to be equally predictive of fertilization outcome and are simpler in their methodologies. Both these tests are used for diagnosing defects among sperm populations that revealed a poor or low acrosome response (<15% acrosome reacted) during the zona induced AR test. These patients were found to do better in the ICSI program compared to IVF (Esterhuizen *et al*., 2001; Liu and Baker, 1994).

1.7.2   Acrosome and ICSI

There is an ongoing debate as to whether the acrosome reaction is necessary for sperm incorporation after ICSI (Lacham-Kaplan and Trounson, 1995; Sathananthan *et al.,* 1997;. Takeuchi *et al.,* 2004). Earlier ultrastructural evidence showed that the acrosome reaction could occur in the ooplasm before sperm incorporation in mature human oocytes or the acrosome could be discarded intact before sperm incorporation in immature oocytes, matured in vitro. In that study, both germinal vesicle and growing follicular oocytes showed sperm chromatin decondensation, with discarded acrosomes close to the sites of incorporation, and were able to form male pronuclei (Sathananthan *et al.,* 1997). More recent work illustrated that aggressive immobilization of sperm prior to ICSI significantly improves fertilization rates. Immobilization of sperm for ICSI by compressing and rolling the sperm tails induces a variable disruption and sometimes loss of the acrosome. This could well be a reason for the higher success rates when ICSI is performed using immobilized sperm. (Takeuchi *et al.,* 2004).

It is well known that when the acrosome reaction occurs, it is preceded by acrosome swelling and is followed by vesiculation of surface membranes exposing the inner acrosome membrane, as observed on the surface of the zona during IVF or in the perivitelline space after subzonal sperm injection. These sperm are probably capacitated at the time of ICSI. Sathananthan *et al.*, (1997) provided evidence of leaching of the acrosomal matrix from intact discarded acrosomes and from partially depleted acrosomes attached to decondensing sperm heads.
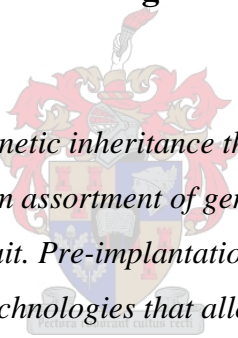
1.8   Acrosomal size

The clinical usefulness of strict criteria (Menkveld *et al.,* 1990) for sperm morphology evaluation has been demonstrated by Kruger *et al.* (1986) and thereafter been confirmed in many publications (Coetzee *et al.,* 1998). However, even in the so-called P-pattern or poor prognosis group ($\leq$ 4% morphological normal forms) of patients, fertilisation and pregnancies do still occur in some cases (Coetzee *et al.,* 1998).

Acrosomal defects as seen with the light microscope can be classified as specific defects or as non-specific alternations. Specific acrosomal defects, which are mostly concerned with acrosome size, are genetically caused (Hofmann and Haider, 1985), for example globozoospermia (Bacetti *et al.,* 1991), and the mini-acrosome defect. These conditions are rare, but when occurring they are easy to detect by light microscopy. However, acrosomes can also be classified as too large, an abnormality which may in some cases be associated with a higher rate of spontaneous acrosomal reactions.

Staining defects included irregular acrosomes, multiple vacuoles, cysts and "empty" acrosomes (Jeulin *et al.,* 1986).   These staining defects may indicate damage of the acrosome membranes with subsequent leaking of (pro)acrosin from the acrosomes (Menkveld *et al.,* 1994).   Jeulin *et al.,* (1986) found low fertilization rates of semen samples containing predominant sperm with staining defects. They postulated that the low *in vitro* fertilization rates associated with increased abnormal acrosome morphology may not be due to the presence of the abnormal acrosomes *per se* but might be due to a relationship between acrosomal abnormalities and nuclear inmaturity of the spermatozoa, which may be caused by Reactive Oxygen Species (ROS) (Henkel *et al.,* 1997).

1.9   Pre-implantation genetic diagnosis (PGD)
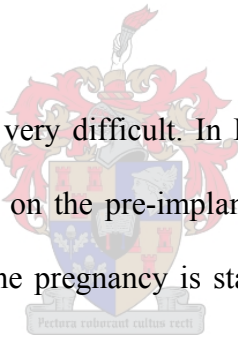
"Since Mendel elucidated genetic inheritance theories in the l860s, there has been an understanding that the random assortment of genes leads to a certain percentage of the offspring expressing a given trait. Pre-implantation genetic diagnosis (PGD) is the newest of a series of reproductive technologies that allows for the diagnosis of disease at the earliest possible juncture, even before an embryo is placed within the womb. This technique has the possibility of dramatically changing our conception of disease prediction, diagnosis, and prevention". Klipstein Fertil Steril 83; 2005

The idea of pre-implantation genetics first surfaced in the 1960s with work done by Edwards and Gardner on rabbits. By 1967 they succeeded in sexing rabbits at the blastocyst stage by diagnosing sex chromatin in cells excised from the trophectoderm of female embryos. They had predicted the use of similar technology in humans to avoid genetic disease. However, only until the revolution of molecular biology in the late 1980s was there a resurgence in interest in this work. Handyside *et al.,* (1989, 1990) worked on

sexing of day 3 embryos for x linked disorder by amplification of Y specific DNA, while Verlinsky *et al.,* (1990) tested the first polar body for autosomal recessive disorders.

Since the establishment of PGD as a diagnostic tool for human genetic disorders, more than 10 years ago, the major aim of the subsequent work was to offer an alternative to prenatal diagnosis for couples at risk of transmitting an inherited disease to their offspring (Handyside *et al.,* 1989; Harper and Delhanty, 2000.). It involves the genetic analysis of one or two blastomeres removed by micromanipulation from four to eight-cell stage embryos obtained by *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI). If prenatal diagnosis is performed and the fetus is found to be affected, the couple has to decide if they wish to continue with the pregnancy or undergo a termination.

These decisions can be very difficult. In PGD the couple undergoes IVF or ICSI and the diagnosis is performed on the pre-implantation embryo. Unaffected embryos are transferred to the woman, so the pregnancy is started knowing the fetus is unaffected at least for the chromosomes tested (Verlinsky *et al.,* 2004).

Therefore, the patients that request PGD are those who have had repeated terminations of pregnancy, with moral or religious objections to termination, those experiencing repeated miscarriages due to chromosomal abnormalities, infertile couples who are also at risk of transmitting an inherited disease and women of advanced maternal age. PGD involves three main stages: *in vitro* fertilisation (IVF), embryo biopsy and the genetic diagnosis. The IVF performed is the same as for routine IVF couples, except that for PGD at least 9 oocytes are required to give a good chance that some normal embryos will be available for transfer.

Three methods have been proposed for embryo biopsy;

- **polar body removal from the oocyte and zygote,**

Polar body biopsy involves the removal of the first and second polar body from an oocyte. It was initially used for the specific diagnosis of certain single gene defects but is now also being used for the detection of maternally derived aneuplodies in older-aged women undergoing IVF treatment (Harper, 1997:13). Polar bodies can be used to identify abnormal forms of segregation of chromosomes and some genes into the oocyte.

- **removing 1-2 blastomeres from the cleavage stage embryo**

Cleavage stage biopsy involves the removal of 1 or 2 cells from the cleavage stage embryo on day 3 of development in culture *in vitro*. Diagnosing 1 or 2 cells from a 6 −10 cell embryo may fail to detect mosaicism. The most commonly used method for obtaining genetic material for PGD is at the cleavage stage of embryo development.
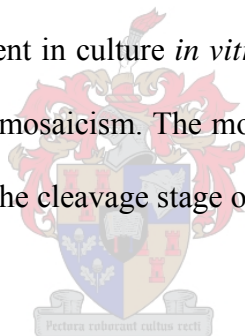
- **removing some trophectoderm cells from the blastocyst.**

Blastocyst biopsy is performed on day 5 of *in vitro* culture. It's advantageous because larger number of trophectoderm cells can be excised from the blastocyst. This would overcome the problem of identifying mosaicism which is problematic in the cleavage stage PGD because only 1 or 2 cells are available for diagnosis. This method is not commonly used because only about 30% of human embryos reach the blastocyst stage (Harper, 1997) and furthermore time required for the genetic tests would require extended culture of the blastocyst before a diagnosis is made. (Harper, 1997).

1.9.1   Growing application

The field of PGD is evolving very rapidly, mostly driven by the Human Genome Project and other technical developments. For example, several centres are now using probes designed for a specific family with a unique chromosome translocation. Such techniques have been made possible by increasingly comprehensive databases of DNA clones from which such probes can be obtained. Another field developing rapidly is the use of software to identify all 46 chromosomes by using FISH with the existing fluorochromes.
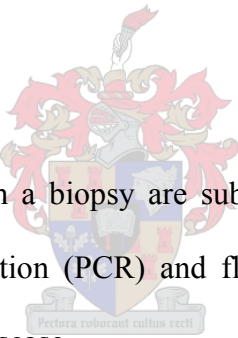
This has already been applied to the first polar bodies of freshly retrieved eggs. Furthermore, during refined investigations, the DNA of a single cell will probably be amplified and hybridized against a chip containing hundreds or thousands of markers to detect any chromosomal or sub-chromosomal imbalances. This will allow the detection of any numerical chromosomal abnormality, as well as chromosomal deletions, translocations and duplications. To date, PGD has been used successfully to screen for a number of genetic disorders in several centres throughout the world. These recent developments, together with recent advances in ICSI and IVF techniques, may make PGD an important clinica1 tool in a time when many couples, for whatever reason, are making increasing use of assisted reproduction techniques (Munne *et al.,* 1993, Munne and Weier, 1996, Munne and Cohen, 1998).

The data from the ESHRE PGD consortium contains detailed information on 1319 PGD cycles from more than 20 centres world wide (ESHRE Consortium, 2000). From this data, the PGD clinical pregnancy rate is 17% per cycle started. This is slightly lower than

routine IVF, probably because the embryo selection is on the genetic status rather than morphology and many good quality embryos are found to be genetically unsuitable for transfer.

The majorities of PGD clinics feel that PGD should only be offered for the diagnosis of inherited diseases, whether these are present at birth or are late onset. The late onset diseases include Huntington's and cancer predisposition. However, there have been many ethical debates on the use of PGD for non-medical reasons. PGD will continue to lead to many ethical discussions in the future (Harper and Delhanty, 2000).

1.9.2   Diagnostic methods for PGD

The cells obtained from a biopsy are subjected to one of two DNA techniques, namely polymerase chain reaction (PCR) and fluorescent in situ hybridization (FISH), depending on the category of disease.

These techniques allow diagnosis for three major groups of disease (Flinter, 2001).

(1)   Sex selection for sex (x) linked disorders, where the genetic defect is highly variable or the defect at the molecular level is unknown. The biopsy is tested by means of FISH to determine the sex of the embryo so that only a healthy female embryo is transferred to the mother.

(2)   Single gene disorders, where the abnormality at the molecular level is tested employing PCR.

(3)   Chromosomal disorders, involving a range of chromosomal rearrangements including translocations, inversions and chromosome aneuploidies are detected using FISH.

Once the blastomeres have been removed, the diagnosis is performed depending on the disease to be diagnosed; one of two procedures is used. The polymerase chain reaction (PCR) is used for the diagnosis of single gene defects, including recessive and dominant disorders, sexing and the triplet repeat diseases. Fluorescent *in situ* hybridisation (FISH) is used for the diagnosis of chromosome abnormalities, including translocations, age-related aneuploidy and sexing.

1.9.2.1   Polymerase chain reaction (PCR)

PCR is an in vitro technique for the amplification of specific DNA sequences. It is a cycling process in which the number of target DNA doubles in each cycle. The basic components of PCR include a DNA template, DNA polymerase, primers, dNTPs, buffer and MgCl2. PCR allows a gene to be copied a billion times in a matter of hours. It consists of 3 basic steps: denaturation, annealing and extension. Denaturation renders the double stranded DNA of the template into single strands. Annealing allows the binding/hybridisation of the primers to the template. Extension is the synthesis of the new DNA strand by DNA polymerase (Taq polymerase)(Wells and Sherlock, 1998).

Specific primers are used, which bind to the region of interest and copy the DNA. It is essential to know the specific sequence so that the primers can be designed. There are a number of techniques which can be used to identify PCR products, including heteroduplex
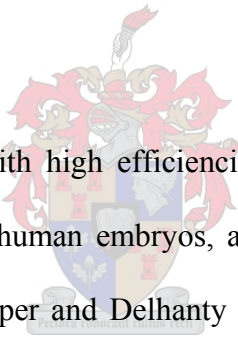
analysis, single strand conformational polymorphism, fluorescent PCR, restriction enzyme digestion, etc.

However, there are two problems with PCR. Because PCR is so sensitive, it is at high risk of contamination from external DNA. This can be from sperm embedded in the zona (so ICSI is used for all PCR diagnosis to ensure there is no sperm in the zona), cumulus cells (these must all be removed before the biopsy), cells from the PGD team, and DNA in the air. Therefore it is essential to perform PCR PGD under stringent working conditions to ensure that there is no contamination, as this could lead to a misdiagnosis. To overcome this problem, polymorphic markers which are different in the mother and the father (fully informative) are used in order to ensure that the DNA amplified is from the couple undergoing PGD. These markers are accordingly used in conjunction with the mutation analysis for the disease under investigation, usually in a multiplex PCR reaction.

The second problem with PCR diagnosis is a phenomenon termed allele dropout (ADO) or preferential amplification. This is where only one of the alleles is amplified, and is mainly a problem for the diagnosis of dominant diseases where the heterozygous state is affected with the disease. In this case, if the normal allele does not amplify, the embryo will be diagnosed as affected, which would not cause a problem. However, if the affected allele drops out of the PCR reaction, the embryo will be diagnosed as normal. Preliminary workup for dominant disorders needs to ensure that no misdiagnosis would occur.

1.9.2.2 Fluorescence *in situ* hybridization (FISH)

FISH is a procedure of specific annealing of fluorescein-labeled nucleic probes to complementary sequences of nucleic acids in a fixed specimen in which chromatin and/or chromosomes have been isolated. This is achieved by the denaturation of both the labeled nucleic acid probe and the specimen nucleic acids at melting temperatures of 68-73^{0}C. This is followed by incubation at 37^{0}C for reannealing which allows the binding of the probes to the target areas on the specimen. Unbound probe and non specific hybridization products are washed off at 73^{0}C using a salt solution and non-ionic detergent. This is followed by counterstaining and then visualization under fluorescent microscopy (Verlinsky & Kuliev, 2000).

FISH has been used with high efficiencies (85–95%) to study the chromosome constitution of cleavage-stage human embryos, arrested or not (Benkhalifa *et al*., 1993; Munné *et al*., 1993, 1995; Harper and Delhanty *et al*., 2000; Munné and Weier, 1996;). Using FISH with multiple probes can differentiate polyploidy from aneuploidy and also haploidy from monosomy, and when most or all cells of an embryo are analysed, mosaicism can be differentiated from FISH or fixation failure, as well from aneuploidy (Munné *et al*., 1993; Munné *et al*., 1995). However, FISH only supplies information on a limited number of chromosomes for which the probes are specific.

1.10 Ethics of PGD

With the progress of PGD over the past few years, the concerns raised are a continuing topic for discussion. The micromanipulation of embryos has initiated the

concern among scientists and others as to how far this technique may be applied without infringing on the beliefs of society in general. The ability of PGD to allow one to choose the sex of ones progeny, to transfer normal embryos so that the resulting offspring maybe a source of stem cells for transplantation to an already affected sibling, further raises questions about development of "designer babies" and more so asks "what is considered abnormal?"

1.10.1 Family balancing

Determining the sex of the embryo to avoid X-linked disorders remains a common indication for PGD, and the vast majority of such cases are carried out using fluorescence in-situ hybridization (FISH) with DNA probes derived from the X and Y chromosomes (Munné *et al*., 1994; ESHRE Pre-implantation Genetic Diagnosis Consortium, 1999). While the application of this technique to prevent sex-linked genetic disorders is now widespread, this method can also be used for pre-implantation sex selection for social reasons (family balancing).

Sex selection for family balancing continues to be highly controversial. The Ethics Committee of the American Society of Reproductive Medicine in 1999 indicated that couples not requiring IVF "should be discouraged" from requesting sex selection for non-medical reasons and that those undergoing IVF should "not be encouraged". A couple's wish for family balancing did not warrant the creation of embryos and the subsequent destruction of embryos of the undesired sex (Robertson, 1996, Robertson, 2002).

Others believe that if people can choose when and how many children to have, and also to terminate unwanted pregnancies, why not permit them to choose the sex of their child if they so desire. (Malpani*., 2002).

1.11.2 HLA matching

Pre-implantation HLA matching has recently emerged as a tool for couples desiring to conceive a potential donor progeny for transplantation in a sibling with a life-threatening disorder. This procedure involves a mini-sequencing-based genotyping of HLA regions A, B, C and DRB combined with mutation analysis of the gene regions. (Fiorentino *et al.,* 2004). .

Couples who have an already affected child with congenital or acquired bone marrow disease or cancer may choose to undergo IVF and PGD to have a non-affected embryo transferred and the cord blood collected at delivery can be used for stem cell transplantation for the older child (Robertson, 2004). Critics of this practice believe that to choose an embryo that may produce a child who would provide stem cells for an older sibling will not be valued in their own right. Furthermore there is the objection to the destruction of healthy embryos that may be produced but are non-compatible tissue donors (Boyle & Savulescu, 2001).

CHAPTER 2

2. MATERIAL & METHODS

2.1 Patients and study design

Thirty nine patients were recruited for the study from a private infertility clinic, namely the Cape Fertility Clinic, located in Cape Town. The clinic is under supervision of Dr K Wiswedel a former member of the UCT department of Obstetrics & Gynaecology. All cases in this study were included after informed consent was provided by both male and females partners allocated to the study. The studies received the approval of the Institutional Review Board at the University of Stellenbosch.
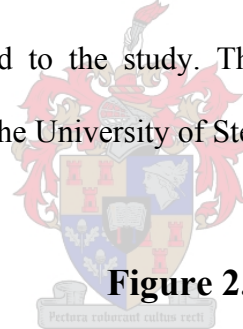
Figure 2.1

Experimental design

Semen samples of 39 men adhering to inclusive criteria set for ICSI therapy

PART I

SPERM IMAGE CAPTURING OF INJECTED SPERM
Semen samples from 12 randomly selected men were used for individual sperm image capturing.

EMBRYO BIOPSIES & FISH
PGD (FISH) was performed on single blastomeres retrieved from embryos that originated after ICSI using sperm from this group. These embryos showed no further developmental potential after 3 days in culture.

PART II

SPERM FUNCTIONAL TESTS
Semen from 27 patients of the study group was used to perform (i) morphology, morphometrical analysis (acrosomal size), chromatin packaging, and spontaneous acrosome reaction.

PART I

Sperm image capturing, embryo biopsy and FISH

In the first part of this study, we randomly selected 12 patients undergoing assisted reproductive treatment with or without egg donation. For this study, only couples with male factor infertility were selected. Oocytes were obtained from female partners with a mean (±SD) age of 31.1 ±2.1 years and egg donors from the Cape Fertility Egg Donation Program with a mean age of 28.7 ± 2.3 years. Patient sperm selected for ICSI were captured on tape and quadruplet smears of their raw specimen were made for analysis in the second part of the study. The resulting poorly developed embryos from these patients were subjected to embryo biopsy and FISH analysis.
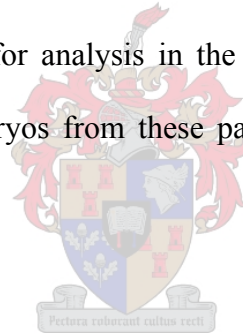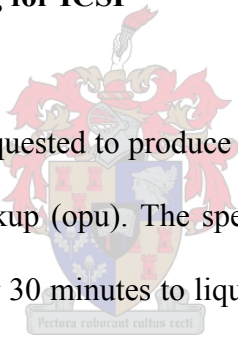
2.1.1   Ovulation stimulation

The standard protocol used was the flare up short protocol. Leuprolide acetate (Lucrin, Abbott Laboratories, Johannesburg, South Africa) at a concentration of 5mg/ml (0.2ml/day) was administered subcutaneously from day 2 of the stimulation cycle. On day 3, HMG (Menogon, Ferring Kiel, Germany) was taken at a daily dose of 225IU for 3 days and then decreased to 150IU per day. Ultrasound of the pelvis was carried out on day 8, 10 and 12 of the cycle. When the leading follicles reached 18 – 20 mm, the medication was stopped and ovulation was triggered by 10 000IU of HCG (Profasi, Serono, Rome, Italy). Thirty six hours later the oocytes were retrieved under transvaginal ultrasound guidance.

2.2 Sperm Characteristics

All patients in the study presented with semen analysis that adhered to the criteria set for ICSI therapy at the Cape Fertility Clinic namely, (i) couples with repeated poor or failed fertilization in the absence of known female factors (ii) cases with <9% normal sperm morphology (teratozoospermic) (iii) patients on the day of treatment presented with poor sperm counts or motility and (iv) poor sperm recovery rate i.e. failed sperm processing.

2.2.1 Sperm handling for ICSI

Patients were requested to produce their specimen in sterile collection jars 1 hour prior to ovum pickup (opu). The specimen were placed in a $37^{o}C$ incubator and allowed to stand for 30 minutes to liquefy. Density gradient centrifugation was used as a method for sperm preparation. The gradients consisted of two solutions: 40% and 80% (sperm gradient kit, Sidney IVF, K-SISG plus Sidney IVF sperm buffer).

Two ml of the 80% solution was placed in a conical tube and overlaid by 2ml of the 40% gradient. Up to 2 ml of the ejaculate was overlaid on the 40% gradient. This was centrifuged for 20 minutes at 2800 rpm. The resulting sperm pellet produced at the bottom of the 80% gradient layer was removed, resuspended in 10ml of sperm buffer and centrifuged for 10 minutes at 2800rpm. The supernatant was removed

and the pellet resuspended in 0.5ml of fertilization medium. This was placed in 37°C incubator until ICSI.

2.2.2 Videographic sperm imaging

Prior to the injection of a single sperm cell, video clips were recorded on a dual TV and Video Cassette Recorder. A Panasonic colour cctv camera was attached to an inverted microscope (Nikon) and connected to the TV/VCR-system. The sperm that were used during ICSI were selected and then recorded on videotape at x200 magnification. Sperm selection was based on the concept of "best-looking" sperm i.e. sperm lacking gross and obvious malformations such as broken necks, cytoplasmic droplets, amorphous or elongated head.
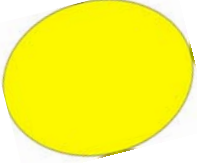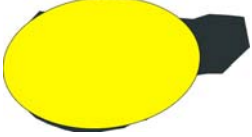
2.2.3 Graphic sperm imaging
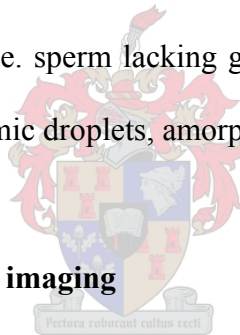
High quality photomicrographs of each sperm cell were produced from the video footage. The photographic material were used to determine the basic shape and the actual length:width ratio of the injected sperm heads (Figure 2.8).

Figure 2.8

Sequence of steps during image capturing using a graphic computer programme

Figure 2.8.1	Using a graphic computer programme (CorelDraw version 8 and CorelDraw PhotoPaint), the peripheral outline and shape of each sperm cell is captured. This was achieved by using the Bezier tool of the CorelDraw software to carefully trace the outline of each cell. Tracings were converted to a silhouette and stored as jpg-files. These jpg-files were used to provide the basic peripheral or outline of the sperm image.
Figure 2.8.2	A silhouette of the outline was then used to establish the basic morphologic image of the injected sperm cells i.e. oval, elongated, round or amorphous.
Figure 2.8.3	Using the tool role-out menus of CorelDraw 8 a yellow overlay was created that best fitted the silhouette. Graphical information i.e. length and width measurements were recorded and was subsequently used to calculate the length: width ratio of each injected sperm head (Figure 2.3.4 to 2.3.6)

Figure 2.8.4 Length:width ratio: 1.1	Figure 2.8.5 Length:width ratio 1.67	Figure 2.8.6 Length:width ratio 1.90

2.3 Intracytoplasmic sperm injection (ICSI)

Two hours post ovum pick up (opu), oocytes were evaluated for their maturity. ICSI was performed on metaphase II oocytes. Media used was Cook, Sidney IVF.

A four well Nunc dish (Nunc Plastics) was equilibrated overnight with 1.0ml hyaluronidase in one well and 1.0ml fertilization medium in each of the three remaining wells. 10% Hepes (Sigma) is added to the three wells containing fertilization medium prior to denuding on the day of ICSI.

Four to six oocytes are transferred to the hyaluronidase in the first well. The oocytes are repeatedly pipetted in the hyaluronidase for 30 seconds to remove the cumulus cells surrounding the oocytes. They are transferred to the second well and narrower pulled pipettes are used to further denude the oocytes of any adhering corona cells. The oocytes are move to the third and fourth wells for further rinsing before they are placed in holding dishes containing fertilization medium without Hepes until ICSI.

2.3.1 Micromanipulation

ICSI was performed on a Nikon inverted microscope with Narishige micromanipulators. Micropipettes and media used were Cook, Sidney IVF.

The inverted microscope and micromanipulators are set prior to the commencement of ICSI. The holding pipette (30° angle) is inserted into the pipette holder and lowered to the heated microscope stage. It is brought into focus using

the fine controls of the manipulator. The pipette is positioned so that it is perpendicular to the microscope stage. The injection pipette (30° angle) is prepared in a similar way.

The microinjection dish (lid of Falcon product 3001, Lasec Pty., Ltd, Cape Town) is prepared by adding a 50ul of PVP (polyvinylpyrrolidone) to the center of the dish. Droplets of 20 ul Hepes (Sigma) buffered fertilization medium are added around the PVP droplet. The droplets are overlaid with mineral oil. Patient sperm is added to the PVP. Immediately after the dish preparation, single oocytes are transferred to each of the medium droplets.

The dish is placed on the heated stage of the microscope. The injection pipette is lowered into the PVP using the coarse controls and lowered further and brought into focus using the fine controls. PVP is aspirated into the pipette so as to prime the inside of the pipette. The pipette is moved toward a population of sperm. Sperm that appear morphologically normal and which move progressively forward are selected. The tip of the pipette is lowered onto the tail just below the midpiece at a right angle. The sperm is immobilized by further lowering the needle onto the tail and pulling the needle over the tail. The immobilized sperm is slowly aspirated tail first into the injection pipette.

The pipette is moved over to the droplet containing the first oocyte. The holding pipette is slowly lowered down and with gentle suction, the oocyte is held into place by the holding pipette. The oocyte is carefully rotated so that the polar

body is either in the 6 o' clock or 12 o' clock position. The oocyte is further lowered so as to touch the bottom of the dish to stabilize the oocyte. The injection pipette is brought into focus. It is lowered to the 3 o' clock position. The sperm is brought closer to the tip of the injection pipette. The pipette is then firmly pushed through the zona pellucida traversing the oolemma into the ooplasma of the oocyte. By gentle suction a small amount of cytoplasm is aspirated into the injection pipette and then expelled together with the sperm into the oocyte. This ensures that the plasma membrane has been broken and that the oocyte has been activated.

The injection pipette is slowly removed from the oocyte and the oocyte is carefully released from the holding pipette. This procedure is repeated for the remaining oocytes in the micro-injection dish. Once all oocytes have been injected, they are removed from the injection dish, transferred to wash droplets and then moved to a culture dish containing 50ul droplets of cleavage medium for continued culture in an incubator at $37^{o}C$, 6% CO_2. The oocytes were observed for fertilization 16 – 18 hours post injection. Only normally fertilized oocytes were selected for culture until day 3.

2.4.1 Embryo biopsy

Embryos were evaluated on day 3 for development and embryo transfer. The embryos used for this study were late day 3 slow developing (4 cells or less) embryos, embryos with a high percentage of fragmentation (greater than 30%), embryos with granular, dark or pitted blastomeres.

2.4.1 Micromanipulation

The day before the embryo biopsy a micromanipulation dish is prepared. Media and micropipettes used were Cook, Sidney IVF.

Pipette 5 x 10 ul equilibrated biopsy medium droplets and a 1 x 30ul droplet of acid tyrodes (pH2.4, Sigma) onto a dish and overlay with mineral oil.

The biopsies were carried out on a Nikon inverted microscope fitted with Narisheghe micromanipulators with a double pipette holder for the assisted hatching pipette and biopsy pipette. The holding pipette (30° angle) is inserted into the pipette holder and set as described for ICSI. The assisted hatching pipette (30° angle) and the biopsy pipette (30° angle) are inserted into the double pipette holder. The holder is slowly lowered to bring the pipettes just above the microscope stage. Using their corresponding fine controls, both pipettes are simultaneously brought into focus.

The embryo to be biopsied is loaded into the first of the biopsy medium droplets on the micromanipulation dish. The dish is placed on the heated microscope stage of the microscope and the double pipette holder is lowered just over the surface of the mineral oil. Using the fine controls of the assisted hatching pipette, the pipette is gently lowered into the acid tyrodes droplet and the acid is aspirated into the pipette.

The pipette is moved over to the biopsy droplet holding the embryo. The holding pipette is lowered into the biopsy medium and by gentle suction the embryo is held into place. The embryo is orientated so that the cell to be biopsied lies directly opposite the holding pipette and is easily accessible to the biopsy pipette. A blastomere with a nucleus is selected for the biopsy. Once the embryo is immobilized by the holding pipette, the assisted hatching pipette is brought to the area of the zona to be drilled. A hole is drilled into the zona by a steady stream of acid tyrodes. Once this is achieved, the assisted hatching pipette is removed by use of it fine controls.

The biopsy pipette is lowered into the droplet and brought to the hole in the zona. It is slowly inserted into the hole and by gentle suction the selected blastomere is aspirated into the pipette. The pipette is removed and the blastomere is expelled into the droplet.

This procedure was performed on all subsequent embryos. In this study where possible all blastomeres were biopsied from the embryos so as to serve as backup material if cells were lost during biopsy or fixation for FISH. Individual biopsied cells were placed on prepared slides (ethanol/ether) and transported to Unistel for fixation and FISH.

Figure 2.3

Embryo biopsy procedure
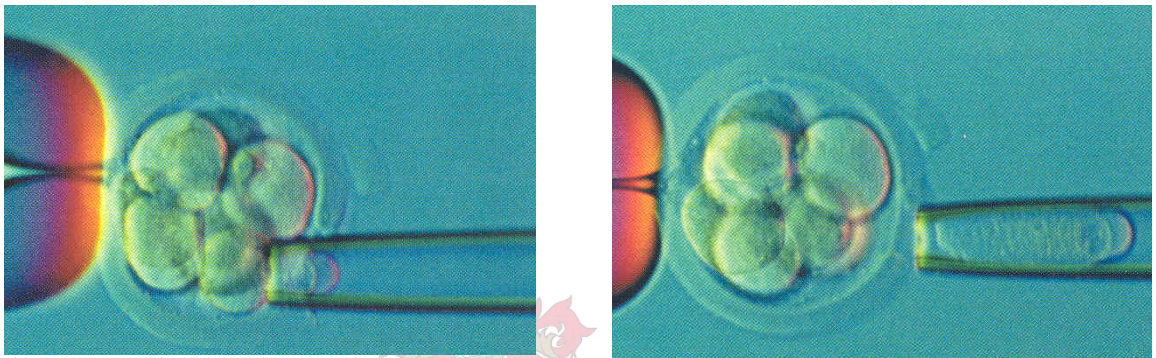
Micromanipulation pipette is used to penetrate zona and aspirate a single blastomere

Single blastomere is removed for FISH analysis

2.5   Fluorescent *in situ* Hybridization (FISH)

Blastomeres were fixed and used to determine presence of X or Y sex chromosomes (FISH technique). FISH of the biopsied cells were carried out at the Genetics Laboratory of Dr. Munroe Marx (Unistel). Within 45 minutes of the biopsy procedure, the slides containing the cells were transported to Unistel.

2.5.1   Preparation of slides

Slides for holding the biopsied cells were prepared 48 hours prior to the embryo biopsies.

Slides are kept overnight in 50% Ethanol/50% Ether. They are removed and thoroughly wiped with cotton swabs. A small circle is made in the middle of the slide with a diamond pen. The slides are washed again in 50% ethanol/50% ether and wiped dry and ready for use.

2.5.2   Fixation Procedure

Once the blastomere is biopsied it is removed from the biopsy medium, washed in KCL (2,79g/500ml) and transferred to the circle made on a prepared slide. The slide containing the cell was sent to Unistel Genetics Department where the preparation and isolation of the nuclei was carried out. On receiving the slide, cold fixative is added to the cell and then placed in a freezer at -20°C for 40 minutes. Thereafter it is dried in front of a fan. The slide is ready for the FISH procedure.
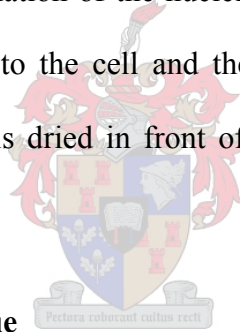
2.5.3   FISH Technique

The slides are subjected to pretreatment steps prior to the application of the fluorescent probes so as to remove any residual cytoplasm and also to maintain chromatin morphology.

2.5.3.1 Rnase treatment

Add 100ul of 0.1ug/ul Rnase with 2 x SSC to the blastomere slide. The slide is placed for 49 minutes at 37°C in a humidification chamber (beaker with

several layers of wet tissue). Slowly rinse in 2 X SSC three times for 5 minutes each and dehydrate in 70%, 90%, 100% ethanol series.

2.5.3.2 Pepsin treatment

After Rnase treatment, the slide is placed in a solution of 18ul pepsin in 100ml PBS with 100ul HCL in an incubator at 37˚C for 15 minutes followed by 4 steps of rinsing in three different solutions for 5 minutes each at room temperature. Solution 1) 1 X PBS, solution 2) 95ml PBS + 5ml MgCl2 (1M), solution 3) 50ml PFA (8%) + 45ml PBS (1 X PBS) + 5ml MgCl2 (1M) and final rinse in 1 X PBS. The slide is then dehydrated in 70%,90%,100% ethanol series and dried in front of a fan. 10ul of probe mixture (CEP) for X and Y is added to the target DNA area on the slide and covered with a cover slip avoiding air bubbles. The edges are sealed with rubber cement.
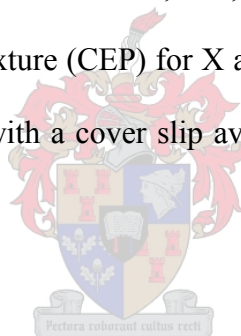
2.5.3.3 Denaturation of slides

The probe and target DNA are simultaneously denatured for 5 minutes by placing the slide on a hotplate set at 75˚C.

2.5.3.4 Hybridisation

Following denaturation, the probes are allowed to anneal to complementary sequences on the target DNA by placing the slide in a humidification chamber in a 37˚C incubator for 6 – 8 hours. On completion of hybridization the cover slip and rubber cement is removed and the slide is washed immediately in 3 washing

solutions .The first solution, 0.4 X SSC/0.3% Tween is heated to 68˚C and the slide is placed in it for 3 minutes. The second solution, 2 X SSC/0.1% Tween is at room temperature and the slide is soaked for 3 minutes. The final wash is in PBS for 1 minute. The slide is allowed to dry completely in a dark area. DAPI with antifade is added to the slide and covered with a cover slip and sealed with clear nail polish. Once the seal is dry, the slide can be analysed under a fluorescent microscope.

2.6   Fluorescence Microscopy

Signals were viewed under x1000 magnification on a Zeiss fluorescent microscope fitted with dual red and green filters using fluorescence immersion oil. The centromeric enumeration probes (cep) hybridize to a greater number of alpha repeat sequences on the X and Y chromosomes. The X chromosome fluoresces green and Y fluoresces red. Microscope is fitted with a CCD camera and computer with imaging software.
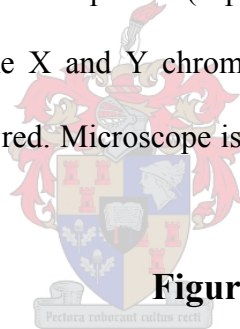
Figure 2.4

Photomicrographs of X chromosome (green fluorescing) and Y chromosomes (red fluorescing) as detected the FISH technique

| XX bearing blastomere with 2 green fluorescing chromosomes | XY bearing blastomere with green (X) and red (Y) fluorescing chromosomes. |
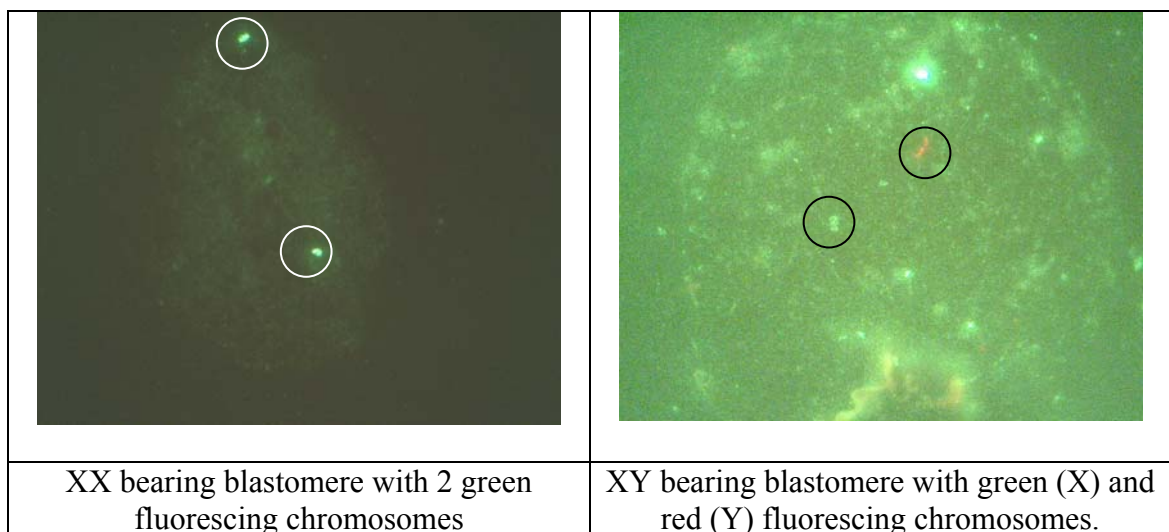
PART II

SPERM FUNCTIONAL TESTS

A total of 39 patients were selected for this part of the study. This included the 2 patients from the first part of this study. The aim is to compare morphology, acrosomal size, chromatin packaging and spontaneous acrosome reaction.

2.7   Sperm handling and slide preparation

After complete liquefaction at room temperature, a basic semen analysis was performed according to the WHO manual for semen examination (WHO 1999). Hence, recordings were made of the semen volume, sperm concentration, motility and forward progression.
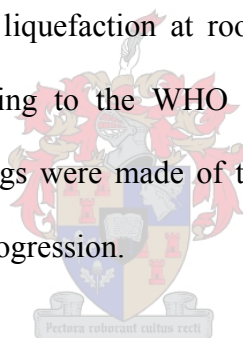
Sperm concentration and motility were evaluated according to World Health Organization criteria [WHO], and sperm morphology was assessed by strict Tygerberg criteria after Hemacolor staining (Merck Chemicals Darmstadt, Germany) (Kruger *et al.,* 1986).

A sperm cell was considered morphologically normal if the head was normal (shape, size having an acrosome, and lacking mid-piece and tail defects). Assessment was made using the Tygerberg strict criteria guidelines to the extent possible given the limited magnification and lack of proper differential staining. Before the onset of the study, morphological reading skills of the observer were

standardized. All readings were within expectable margins of error, namely within 0.2 SD of the mean of the reference (training and QC slides) slides.

The main defect recorded in morphologically abnormal cells were elongated or tapered heads (head showing dramatic tapering so that head length:width ratio >1.75) , amorphous heads (any deviation form normal head morphology) , broken necks (central head axis deviates $>30^0$ from midpiece axis caused by a breakpoint between head and midpiece) and presence of cytoplasmic droplets (any size droplet was regarded as abnormal).

Separate quadruplet slides were prepared to evaluate;

(i)   the percentage normal cells using light microscopy, (manual method, WHO 1999)

(ii)   the percentage normal cells using the Metrix system (Metrix Hamilton Thorne Research, Beverly MA, USA)

(iii)   acrosomal size (Metrix, Hamilton Thorne Research, Beverly, MA, USA)

(iv)   the quality chromatin packaging and

(v)   acrosome reaction

Making a Smear

The smears were prepared on cleaned frosted slides. The droplet size for a smear was dependant on the sperm concentration. For specimen with concentrations less then 20 x 10^6/ml, a 10ul droplet was used and for concentrations more than 20 x 10^6/ml the droplet volume was 5ul. The droplet was dispensed in the middle of a slide toward the frosted end (of the slide). A second slide was

placed on the drop at an angle of 25˚ - 30˚ and pulled evenly over the first slide and pushed back toward the frosted end of the first slide. Four slides per patient were prepared. The slides were allowed to air dry.

2.8   Functional Tests

2.8.1   Human Automated Sperm Morphology Analysis

Manually prepared semen slides were stained with the Hemacolor staining technique and evaluated for percentage normal sperm cells using strict criteria (Kruger *et al.,* 1986, Menkveld *et al.,* 1990, Coetzee and Kruger 1998).

2.8.1.1   The Metrix Operating System

Hemacolor stain
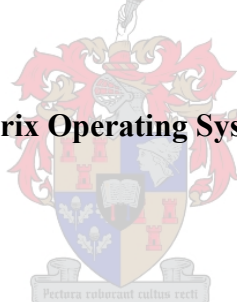
Metrix is compatible with the Hemacolor diffrential stain. Hemacolor is a dip stain and were dispensed in a Coplin staining jars.

A general mounting medium i.e. DPX was used to mount the stained slides

Hemacolor Staining Procedure for Human Sperm

1)   Time = 10 seconds: Fix sperm by immersing slide in Hemacolor fixative solution 1. Blot edges of slide.

2)   Time = 20 seconds: Stain spermby immersing in Hemacolor solution 2. Blot edges of slide.

3)   Time = 20 seconds ;Counterstain sperm by immersing in Hemacolor

        solution 3. Blot edges of slide.

4)   Rinse slide gently in distilled water.

5)   Blot and allow to air dry.

Mounting

1)   Place 3 -4 drops of mounting medium along the center of the slide.

2)   Carefully place a clean coverslip on the slide.

3)   Press coverslip gently to disturb mounting medium.

4)   Allow mounting medium to dry sufficiently before analyzing.

2.8.2   Measurement of sperm acrosomal size

        Hemacolor (Merck Germany) pre-stained slides were used to record the
following measurements of the sperm head: Length, width, area, acrosomal size of
each sperm population (METRIX system, IVOS-HTR).

2.8.3   Automated sperm morphology analysis (ASMA)

        Automated analysis of human sperm is performed using 2 separate systems
namely, computer assisted sperm analysis (CASA) and automated sperm
morphology analysis (ASMA). CASA systems are used to evaluate motility
parameters, while ASMA systems are utilized to determine the morphometry of
spermatozoa.

Spermatozoon morphometry refer to the actual dimensions of spermatozoon structures such as head area, head width and length acrosomal area. The ASMA system comprises of hardware that consists of a microscope, a video camera, a computer, a frame grabber and the morphometrical software used to evaluate the images captured.

Figure 2.5

A typical on screen display of individual sperm cells evaluated with the ASMA software
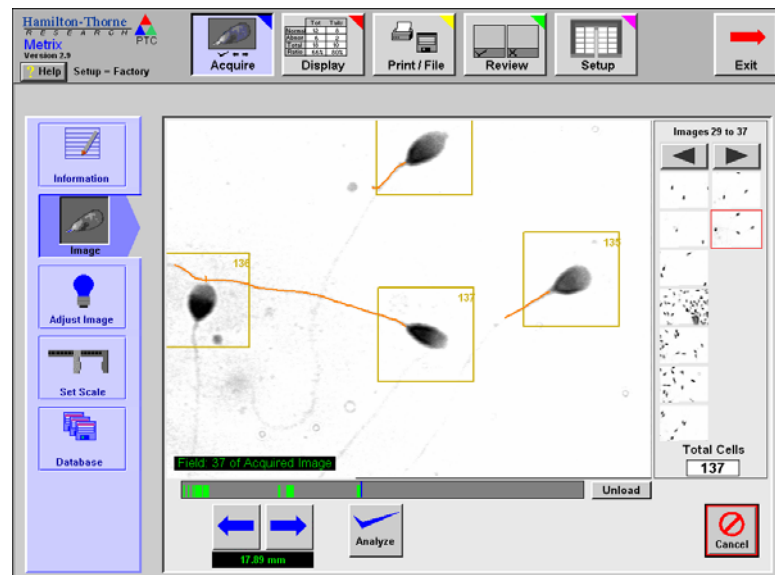
Spermatozoa containing a normal tail are boxed and classified as normal or abnormal.

Figure 2.6

A Zoomed image showing the morphological appearance of a normal sperm cells as recorded by ASMA software
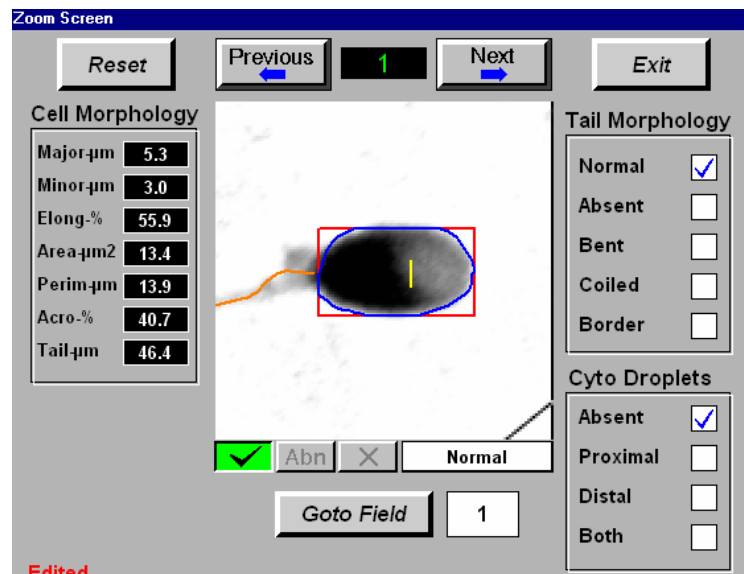
Individual spermatozoa morphometric parameters are analysed with an ASMA system. The total area of the sperm head is indicated with a blue line, while the posterior position of the acrosome is marked with a yellow line. Red square: Corresponds to the values for the minor (width) and major (length) axes; Yellow line: Corresponds to the end of the acrosome; Blue line: corresponds to the perimeter.

Figure 2.7

Typical example of an abnormal sperm cell as evaluated by ASMA software
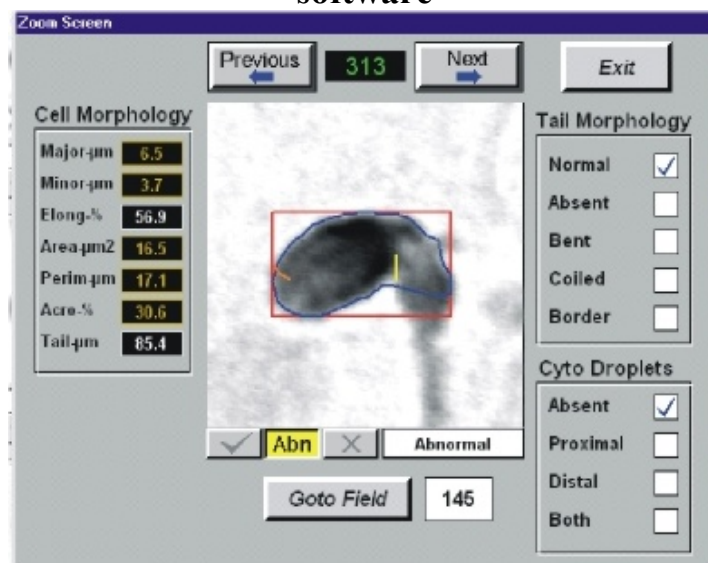
2.8.4 Sperm chromatin packaging quality

2.8.4.1 Chromomycin A3 staining

Staining and evaluation procedures

The quality chromatin packaging was determined using CMA_3 staining (Sigma Chemicals, St Louis, MO USA Cat 2659), techniques (Sakkas *et al.,* 1995, Manicardi *et al.,* 1995).

- Semen smears were prepared and fixed in methanol/glacial acetic acid 3:1 at 4^0C, for 20 minutes. Each slide was air dried and treated in a dark chamber for 20 minutes with 100 µL CMA_3 solution (0.25mg/mL in McIlvane's buffer, pH 7.0 containing 10mM $MgCl_2$). (Sakkas *et al.,* Franken *et al.,* 1999). The slides will be kept at 4^0C overnight.

- Slides were then washed in McIlvaine buffer (Sakkas *et al.,* 1996) and mounted using Dabco (Aldrich Chemicals Co, Milwaukee, USA cat No. 29-073-4).

- Two hundred spermatozoa were evaluated under an Olympus B40 fluorescent microscope (Wirsam Scientific Cape Town, South Africa Filter Fx 465-495). The lens allowed the use of both phase and fluorescence. (Sakkas *et al.,* 1996, Franken *et al.,* .1999).

- Slides will be rinsed in buffer and mounted with Dabco. The evaluation of the CMA_3 staining is easily done with the bright yellow spermatozoa (CMA_3 positive) being distinguished from the dull yellow spermatozoa (CMA_3 negative). CMA3-positive cells are associated with DNA damage.

Figure 2.8

CMA3 –Fluorescence of human spermatozoa.
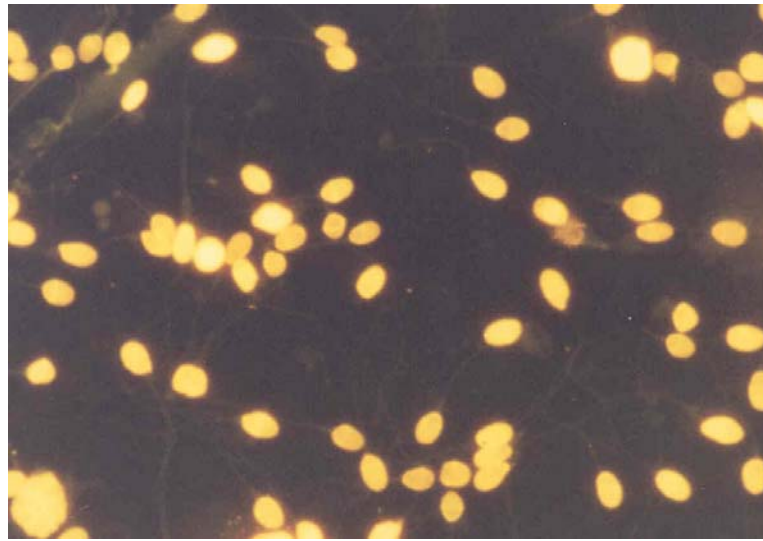
Bright yellow fluorescence is regarded as CMA3-positive, while dull yellow staining in CMA3-negative.

2.8.5 Spontaneous acrosome reaction

Fluorescein isothiocyanate labeled with pea (*Pisum sativum*) agglutinin (FITC-PSA; Sigma) was used to evaluate the acrosomal status of the sperm. Pisum Sativum agglutinin (PSA) has a specificity for alpha–methylmannose residues and labels the acrosomal content (Cross et al., 1986).

2.8.5.1 Preparation and Staining

Semen smears were prepared on the day of ICSI and fixed in 95% ethanol at $4^{o}C$ for 24 hours.

Thereafter the spermatozoa were stained for 2 hours at room temperature with 30µg/ml PSA (Pisum sativuma agglutinin) labelled with fluorescein-isothiocyanate FITC (L-0770, Sigma Aldrich, Vorna Valley, Republic of South Africa). Finally, slides were washed in Dulbecco's Phosphate Buffered saline (DPBS) and mounted with DPBS. A minimum of 100 spermatozoa were counted under a Olumpus B40 fluorescent microscope (Wirsam Scientific, Cape Town), Filter Ex 465-495 0 with 400X magnification.

2.8.5.2. Evaluation of Staining Patterns

The acrosomal status of spermatozoa was classified according to the lectin staining into: (i) intact acrosome, complete staining of acrosome; (ii) reacting acrosome, partial or patchy staining of acrosome; and (iii) reacted acrosome, complete staining of the equatorial segment only or no staining of the whole sperm head. The proportions of intact, reacting and reacted acrosome were expressed as percentages of the respective patterns in the total number of spermatozoa counted (Perry *et al*, 1995).

2.9   Statistics

Statistical calculations were done using Medcalc for Windows version 8.0.1.0 (Mariakerke, Belgium). Comparisons between the different patient's percentage normal sperm cells, acrosomal morphometry as well as the chromatin packaging quality (% CMA$_3$) were done using paired student's t-test and multiple

regression was used to calculate the correlation coefficients for morphology versus chromatin packaging and acrosome reaction. Receiver Operator Characteristic analyses (ROC) curves were used to determine the sensitivity and specificity for chromatin packaging quality vs. sperm morphology and spontaneous acrosome reaction vs. sperm morphology.
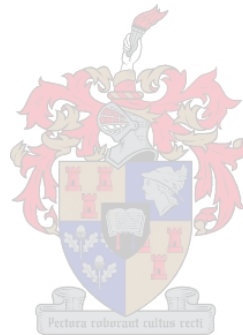
CHAPTER 3

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3. RESULTS

3.1 Semen parameters

The mean (±SD) values for the semen parameters of the 39 men of the study group are depicted in Table 3.1.

Table 3.1

Mean (±SD) results of semen parameters of 39 men undergoing ICSI treatment

Sperm concentration 10^6 cells/ml	Motility % live cells	Morphology % normal cells	Progressive motility %	Spontaneous acrosome reaction % reacted spermatozoa	CMA3 staining % positive
18.0±2.1	22.3±2.6%	7.9±2.8%	20%	12.0±5.1%	48.6±11.4%

3.2 Sperm morphometry

Sperm morphometrical recordings were done with an IVOS Metrix morphology program. The mean (±SD) values for sperm head length, head width, head area and percentage of the head area that constitutes the acrosome size are represented in Table 3.2.

Table 3.2

Sperm morphometric results of 39 men attending the ICSI program

MORPHOMETRY	NORMAL CELLS		ABNORMAL CELLS	
	Mean	**±SD**	**Mean**	**±SD**
Head Length (μm)	5.06a	0.36	5.9b	0.71
Head Width (μm)	3.28c	0.25	3.48d	0.26
Area occupied by sperm head (μm^2)	13.9e	1.93	14.0f	1.27
Acrosomal size (% of area of sperm head)	37.7g	9.94	28.5h	6.99

Student's paired-test: a vs b: p= 0.00001;

 c vs d: = 0.01; e vs f: p= 0.97; g vs h: p= 0.0004

Length width ratio: Normal cells: 1.54; Abnormal cells: 1.70

3.3   Oocytes

All oocytes that were used during the present study adhered to the criteria described for Metaphase II oocytes (Figure 3.1). Strict guidelines were followed during the oocyte classification and metaphase II oocytes were identified according to their nuclear maturity status (Veeck 1988).

Figure 3.1

Photomicrograph example of a typical metaphase II oocyte used in the study
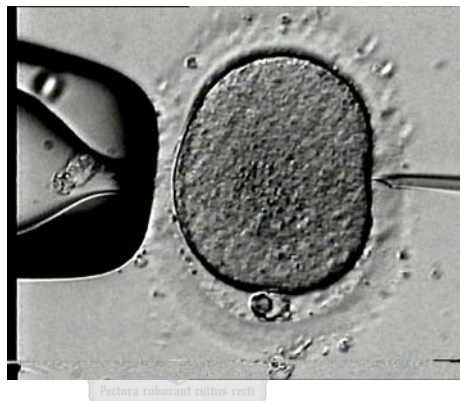
3.4   Intracytoplasmic sperm injection (ICSI)

The clinical results i.e. number of oocytes fertilized, fertilization rates, morphology values and chromatin packaging quality are represented in Table 3.3. A mean (±SD) number of 7.46(±4.2) metaphase II oocytes among 39 patients were aspirated. We used only metaphase II oocytes and all metaphase I oocytes were discarded and not include in the ICSI program. A total of 276 metaphase II oocytes were used for sperm injection. Two hundred and forty six oocytes of the 276 injected (89.1%) fertilized. Pregnancies were reported among 20 of the 39 couples (51%). In all reported pregnancies, these cases were defined as ongoing pregnancies with a positive fetal heart.

Table 3.3

Results of ICSI outcome, sperm morphology, chromatin packaging quality, spontaneous acrosome reaction recorded for 39 couples

	Number oocytes	Number oocytes Fertilized	% Oocytes Fertilized	Diff-Quik moprhologygy (% normal cells	Metrix morphology	%CMA3 positive cells	Spontaneous AR % acrosome reacted cells
Mean	7.5	6.65	90.4%	7.9%	10.2%	48.7%	12.0%
±SD	4.2	3.8	11%	2.8%	2.3%	11.3%	5.1%

Based on the ICSI outcome we divided the couples into pregnant and non-pregnant. Results of fertilization rates, sperm morphology, chromatin packaging quality and sperm morphometric values of 20 pregnant and 19 non-pregnant couples are represented in Table 3.4 and Table 3.5. Individual data for each patient is presented in the Appendices 2, 3 & 4.

Table 3.4

Mean (±SD) results for fertilization rates, sperm morphology, chromatin packaging quality and sperm morphometric values of 20 pregnant and 19 non-pregnant couples

	Oocytes fertilizaed	No oocytes	% Ferttilized	DQ-MOR	Metrix MOR	% CMA3 positive cells	% acrosome reacted cells	MORPHOMETRY Normal forms Length (µm)	Width (µm)	Area (µm²)	Acro size (% of head)	MORPHOMETRY Abnormal forms Length (µm)	Width (µm)	Area (µm²)	Acrossize (% of head)
PREGNANT GROUP (n=20)															
Mean	**7.4**	**8.2**	**92.0**	**8.3**	**11**	**48**	**11**	**5.1**	**3.3**	**14**	**36a**	**6**	**3.5**	**14**	**30c**
SD	4.0	4.3	13	2.7	2.2	13	4.9	0.4	0.2	1.8	7.5	0.7	0.2	1.6	6.8
NON-PREGNANT GROUP (n=19)															
Mean	**5.7**	**6.6**	**88.8**	**7.6**	**9.8**	**49**	**12.8**	**5.0**	**3.3**	**13**	**39b**	**5.8**	**3.5**	**14**	**26.9d**
SD	5.1	4.1	10	2.8	2.4	9	5.3	0.3	0.3	2	11	0.7	0.2	0.7	6.8

Student's paired t-test results showed no significant differences between all the parameters tested except for acrosomal size as recorded for the normal spermatozoa compared to abnormal sperm (p=<0.001) in both pregnant and non-pregnant groups. The pregnant and non-pregnant group's results revealed no statistical difference among the parameters tested.

Student's two-tailed t-test for independent samples showed no statistical differences between the values recorded for the pregnant and non-pregnant groups. The p-values for each parameter tested are provided in the appendix.
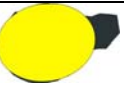
3.5   Sperm morphology and embryo biopsies
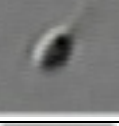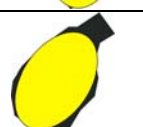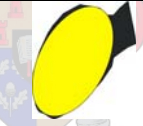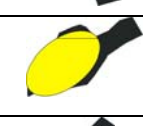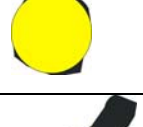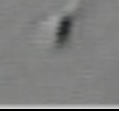
The results of embryo biopsies and sperm morphologic appearance are represented in Table 3.5.

Eleven of the sixteen (69%) injected sperm that resulted in embryos that had no developmental potential and of which a single blastomere were removed for FISH were shown to be male.

Table 3.5

Comparison of embryo biopsy results and sperm morphologic appearance presented as graphic images

Patient	Photomicrograph of injected sperm cell	Sperm number	Diagram of injected sperm cell	Length/width values	Length:width ratio	FISH results	PREGNANCY	Seminal Morphology
4		1			1.50 normal	Male	**P**	8
		2			1.40 abnormal	Female		
16		3	No image	No image	No image	Male	**NP**	

		4			1.60 normal	Female		
		5			1.6 normal	Female		
		6			1.3 abnormal	Male		
22		7			1.58 normal	Male	**NP**	5
23		8			1.5 normal	Male	**P**	7
		9			1:50 normal	Female		
24		10			1.55 normal	Male	**NP**	11
26		11			1.5 normal	Male	**P**	2
27		12			1.7 normal	Male	**NP**	11
31		13			1.8 abnormal	Male	**NP**	3
32		14			1.5 normal	Male	**NP**	
34		15			1.1 abnormal	Male	**NP**	7
37		16			1.5 normal	Female	**P**	4

3.6 Sperm morphology: ASMA vs. manual method

The mean (±SD) percent normal forms as recorded by the automated sperm morphology program (ASMA) was 7.95±2.8% compared to 10.2±2.3% recorded by the manual method.

3.7 Sperm morphology and Chromatin packaging quality

Using the mean CMA_3-positive value for the patients in the study namely, 48.6±11.4% (Figure 3.2) as a cut off value for chromatin packaging defects, the Receiver Operator Characteristic (ROC) curve analysis showed an area under the curve of 0.95, which implies that a randomly selected individual from the CMA_3-positive group has a test value smaller (i.e. <48% CMA_3 positive) than that of a randomly chosen individual from the negative group in 95% of the time. With 48% as cut off value, the ROC curve analysis revealed a sensitivity of 87% and specificity of 95% (Table 3.2).

Figure 3.2

Receiver Operator Characteristic curve analysis between sperm morphology and CMA$_3$ staining results.
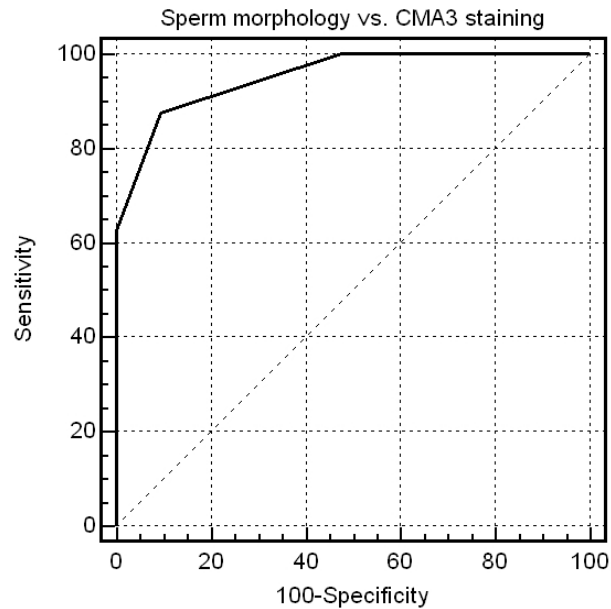
3.8   Sperm morphology and spontaneous acrosome reaction

Using the mean percent spontaneous acrosome reacted sperm, namely 12% as a cut off value, the Receiver Operator Characteristic (ROC) curve analysis showed an area under the curve of 0.73, which implies that a randomly selected individual from the <4% morphology group has a test value smaller (i.e. <12% acrosome reacted sperm) than that of a randomly chosen individual from the negative group in 73% of the time. With 12% as cut off value, the ROC curve analysis revealed a sensitivity of 60%, whiles a specificity of 91% (Figure 3.3).

Figure 3.3

Receiver Operator Characteristic curve analysis between sperm morphology and percent sperm with spontaneous acrosome reaction.
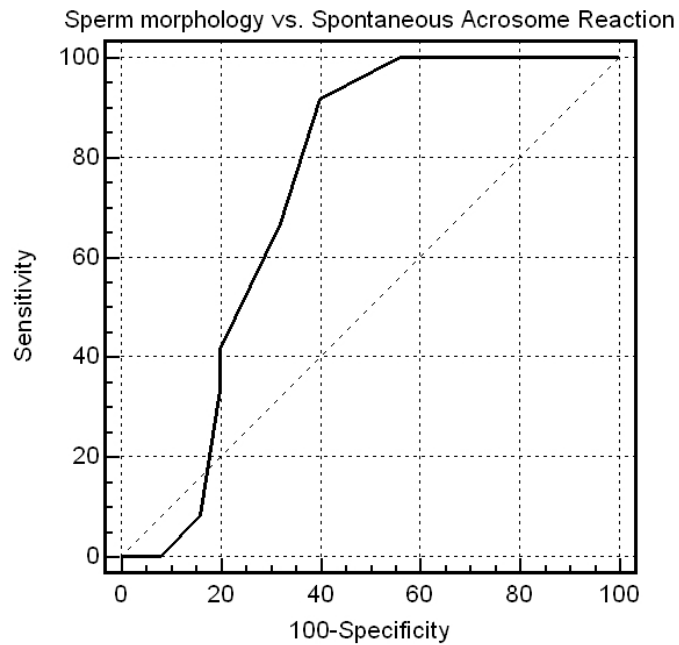
Multiple regression analyses between the percentage normal spermatozoa, chromatin packaging quality and spontaneous acrosome reaction correlation coefficients of r=-0. 77 for CMA_3 (p=<0.0001) and r=-0.18 (p=0.86) for acrosome reaction.

CHAPTER 4

DISCUSSION

4.1   Paternal influence on fertilization failure

We removed single blastomeres for X/Y chromosome analysis from 16 embryos obtained from 12 couples (see Figure 2.2 material and methods for technique). From the results obtained with the FISH-technique, 11 (69%) embryos were found to be Y chromosome bearing embryos.

The number of Y-bearing embryos from the group of embryos that underwent biopsies underlines the importance of the paternal role played during fertilization. Abnormal sperm is known to fertilize oocytes, but some of the consequences on fertilization (Figure 4.1) include embryo fragmentation, delayed embryo development and lower fertilization rates recorded during ICSI treatment (de Vos et al 2003). The paternal influence of sperm may originate at various levels; nuclear, cytoskeletal and at an organelle level. Justifiably, one of the most important factors influencing embryo development and subsequent reproductive outcome is an abnormal nucleus in the spermatozoa.

Moreover, it is well documented that the fast cleaving embryos lead to the best pregnancy rates after transfer i.e. day 2 or 3 (Menezo and Janny, 1997) `If we consider a

scenario in IVF where poor quality sperm are used, delays in fertilization process are often encountered. Furthermore delays associated with the epigenetic problems together with the fertilization delays might have a cumulative effect that lead to prolonged cell cycles and late divisions (Ron-El, 1991).

These factors lead to developmental arrests around genomic activation in relation to the depletion of the mRNA maternal store. ICSI allows saving time at least for the fertilization process, and this saving possibly helps in overcoming part of the epigenetic defects. This fits with the observations of Oehninger *et al.,* (1996) namely, ICSI embryos in case of severe teratozoospermia have higher morphological scores and developmental potential that their corresponding IVF embryos.

Figure 4.1

Consequences of fertilization with abnormal sperm
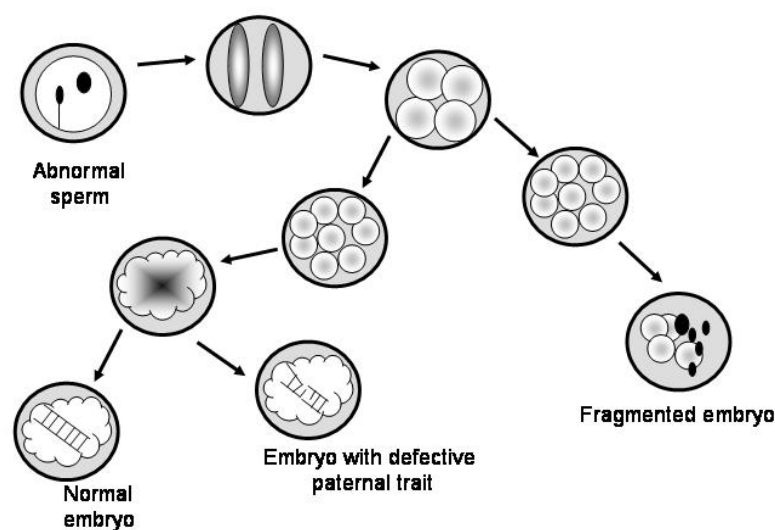
Twenty pregnancies (52%) were reported (elevated beta-HCG on day 21) among the 39 couples treated in the study. However, among the 11 couples that underwent embryo biopsies 5 full term pregnancies (41%) occurred. Of these 5 pregnancies, 2 were singleton females, 1 pair of twin males and 2 singleton males. Although we did not perform FISH-techniques on all the embryos with fertilization arrest in the study, we regard the prevalence of 69% Y-bearing embryos among the group as a tendency as far as the influence of the paternal effect on pre-implantation embryos are concerned. Further investigations might detect an apoptotic marker in the Y-chromosome bearing sperm that is responsible for the defect that occurred in those embryos that did not develop.

In conclusion, if we assume that the defective embryos were a direct result of the paternal trait manifested in the Y-chromosome bearing sperm population of a specific individual, then future research might be directed to evaluate methods to the remove Y-chromosome bearing spermatozoa from the ejaculates of the men diagnosed with severe male factor infertility. Elimination can be accomplished with techniques such as flow cytometry (Johnson 1995). Recent work demonstrated that significantly more Y-bearing spermatozoa were capable of zona binding than X-bearing spermatozoa at 48h at 37 °C incubation (van Dyk *et al.*, 2001).

4.2 Individual selected sperm and ICSI

The use of ICSI to treat severe cases of male-factor infertility results in injection of more morphologically abnormal sperm cells or immature spermatozoa. Concerns about the safety should be considered mainly due to the following reasons; (i) fertilization and

subsequent embryos are obtained and transferred to the patient (ii) a clear correlation has been shown between morphology and chromatin packaging quality (Bianchi *et al.,* 1996; Evenson *et al.,* 2002; Franken *et al.,* 1999) and chromosomal constitution of human sperm cells (Lee *et al.,* 1996).

Earlier studies have concluded that sperm morphology in ejaculates as recorded with strict criteria is not correlated to the outcome of ICSI (Küpker *et al.,* 1995; Mansour *et al.,* 1995; Nagy *et al.,* 1995). In these studies the fertilization rates obtained with semen samples having severe teratozoospermia (<4% normal forms) did not differ from those with better overall sperm morphology. A possible explanation for this is the fact that the embryologist selects a motile, normal looking sperm cell for injection. As long as morphologically well-shaped live spermatozoa are used for injection, fertilization rates and pregnancy rates after ICSI is not affected (Mansour *et al.,* 1995; Berkovitz *et al.,* 1999; de Vos *et al.,* 2003).
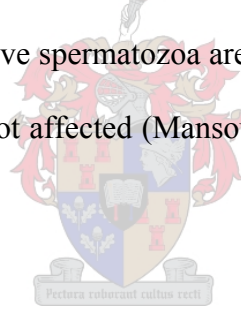
We studied the effect of morphology of individual sperm cells used during ICSI. From the sperm images depicted in Table 3.5, it is clear that we used sperm that presented as "normal looking" under the inverted microscope. In general all sperm except sperm number 13 had an oval shape appearance with length: width ratio within the limits set for normal sperm i.e. between 1.5-1.7. Computer graphic techniques showed that the majority sperm (i.e. 11 out of 15 images 73%) length:width ratio was within the limits set for normal spermatozoa.

The approach of using an intracytoplasmic morphologically selected sperm injection technique, originally described by Bartoov *et al.,* (2003), is of clinical importance since we consistently obtained fertilization rates of 90% and more with pregnancy rates of >52%. The pregnancy rate of 52% after a single intracytoplasmic morphologically selected sperm injection is in agreement with earlier reports by Bartoov *et al.,* 2001) who reported a pregnancy rate of 67% among couples with a history of failed ICSI cycles. In that study the authors claimed the significant higher pregnancy rate (66% vs. 30%) achieved could be attributed to the intracytoplasmic morphologically selected sperm injection technique (Bartoov *et al.,* 2001, 2003).

Compared to the high magnification technique that multiplies the image (thus providing a clear impression of the sperm nucleus) of individual sperm up to 6000 times through high power light microscopy used by Bartoov and colleagues, the low magnification (X400) and subsequent low resolution of the sperm morphology assessment can be regarded as a limitation of the study.

Using the "best-looking" sperm selection technique made it difficult to provide detailed deviations from the well-described normal form as defined by the Tygerberg strict criteria approach. The selection technique used in the present report is a combination of a variety of parameters namely, (i) strict criteria guidelines for normality i.e. sperm lacking major defects such as broken neck, an elongated or amorphous head or the presence of cytoplasmic droplets (ii) length:width ratio for normal spermatozoa as defined by the WHO (1999).

Evaluation of length : width ratio combined with the morphology guidelines for normal forms, high magnifications were not necessary in the present study. The distinction between normal and abnormal forms was clear, since we did not use any sperm that had the slightest deviation from normal as could be detected with the low magnification and poor resolution.

Despite the magnification restriction, the sperm selection technique of choosing the "best-looking" in the current study provided satisfying results as far as fertilization (90%) and pregnancy rates (52%) are concerned. There was no difference found among any of the evaluated parameters i.e. morphology, chromatin packaging, acrosomal status and size and fertilization rates when comparing the pregnant (n=19) with the non-pregnant group (n=19).

Furthermore, according to de Vos *et al.,* (2003), number of morphologically defective sperm different between surgically retrieved and fresh ejaculated spermatozoa differs significantly. Fresh ejaculated sperm show less morphologic defective sperm cells compared to surgically retrieved samples. Results from previous studies indicates a possible trend in lower fertilization and pregnancy rates obtained with morphologically abnormal cells compared to that reported for normal sperm cells (de Vos *et al.,* 2003). The relatively high percentage normal cells reported in the present work namely, 7.9±2.9% is in agreement with results reported by de Vos *et al.* (2003).

Finally, from the present results and data published by others (de Vos et a., 2003, Bartoov *et al.,* 2001, Bartoov *et al.,* 2003), one may assume that during IVF/ICSI

procedure, the survival of the embryo in the uterus is associated with the fine morphological state of the sperm nucleus. Therefore based on the exclusive selection of spermatozoa, free of any subtle nuclear morphological malformation, leads to a higher pregnancy rate.

4.3   Chromatin packaging

Presently it is accepted that, human sperm quality differs remarkably not only between males but also within a single ejaculate. These differences are not only evident when examining the classical parameters of sperm concentration, motility and normal morphology, but also in the manner that the sperm chromatin is packaged. Semen quality is traditionally determined according to sperm concentration, motility and morphology features in a given ejaculate (World Health Organization, 1999).
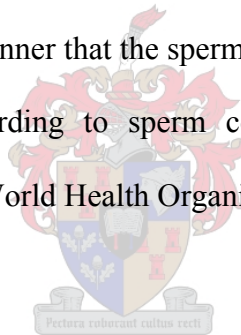
The mean ±SD for chromatin packaging quality of the ejaculated sperm was 54.0±15.1% (CMA_3 positive cells) in this study. This value is slightly higher compared to those values reported in other studies, namely 44% (Franken et al., 1999, Esterhuizen et al., 2001a). This slight difference can be attributed to the fact that the present study included patients with a longstanding history of poor or failed fertilization during IVF and ICSI therapy. In close agreement with previous published reports the present results (Franken et al, 1999, Sakkas et al., 1996, Esterhuisen *et al.,* 2001a., Esterhuisen *et al.,* 2001b, Bianchi *et al.,* 1996) illustrated a positive highly significant relation between sperm morphology and CMA_3 - positive cells. Furthermore, it has been shown (Bianchi *et al*., 1996) that

lower packaging quality is highly prevalent in morphologically normal spermatozoa in male factor patients.

Sperm chromatin integrity assessed by the sperm chromatin structure assay exhibits no correlation with the fertilization rate after ICSI (Larson *et al.,* 2000). However, chromatin abnormalities appear to influence later embryonic development and can possibly be the major cause for the existence of the so-called ICSI-resistant couples.

4.4   Acrosomal status and size

The mean ±SD percentage recorded for spontaneous acrosome reaction was 12.0±5.1%. It is well known that spontaneous acrosome reaction has little or no relevance with fertility capacity of spermatozoa. (Planchot *et al*., 1984, Cummins *et al.,* 1991). However, acrosomal morphology is known to be related to impaired sperm function (Jeulin *et al*., 1986, Menkveld *et al*., 1996, Bastiaan *et al*., 2003) and extensive studies on acrosomal biochemistry (Lee *et al*., 1992) and acrosome reactions (Liu and Baker 1994, Esterhuizen *et al*., 2001a, Esterhuizen *et al*., 2001b) have defined associations between structure and function.

During the morphometric analysis of the ejaculated spermatozoa used in the study, the acrosomal size of the cells was expressed as a percentage of the head area. The abnormal sperm cells (Table 3.2) had significantly smaller acrosomes (28.5% of head area) compared to the normal sperm cells 37.7% of head area, p=0.004). Earlier findings by Menkveld *et al.* (2003) concluded that the acrosomal size and thus morphological

appearance, reflects the physiological potential of sperm cells. It indicates that spermatozoa with small acrosomes are more susceptible to apoptosis and non-physiological acrosomal loss. However, the molecular reasons for this functional disability are still unknown (Menkveld *et al.,* 2003).

In the present study we found no correlation between the percent of spermatozoa that have undergone the acrosome reaction i.e. the spontaneous acrosome reacted population in the sample and sperm morphology (Figure 3.2 r=0.18). The specificity and sensitivity was 91% and 60%, respectively. These results imply that the spontaneous acrosome reaction cannot be used as a screening test for sperm morphology defects.

Although the acrosome *per sé* does not seem to influence ICSI results, it's reaction can be used as an indicator of sperm fertilizing potential. The ability of the acrosome to react in the presence of the physiological inducer of the acrosome reaction, namely the zona pellucida, provides valuable information regarding the dysfunctional spermatozoon and can be used as an additional diagnostic test in an assisted reproduction program (Liu and Baker 1994, Bastiaan *et al.,* 2003, Esterhuizen *et al.,* 2000).

4.5   Sperm morphology

The mean ±SD percentage normal sperm cells recorded was 7.9±2.8% for manual readings and 10.2±2.3% as evaluated by the Metrix system (p=0.0001). Results are in

agreement with results reported by (Kruger *et al.,* 1995). The difference in the results of the two methods namely, the manual versus IVOS can be caused by several factors, namely, (i) too many cells per microscopic field, thus causing a discrepancy between manual and IVOS readings and (ii) poor slide quality; ICSI cases often presents ejaculates that contain a high number of debris and cellular components.

Poor slide quality will cause overlapping of sperm cells and debris interference with the image analyzing process of the computer, thus leading to poor agreement between manual and computerized analysis. This will increase the chance of a computer error, as overlapping cells or clumps of cells cannot be judged individually. This is not only true for computer assisted analyses but also manual analysis (Menkveld *et al.,* 1990).
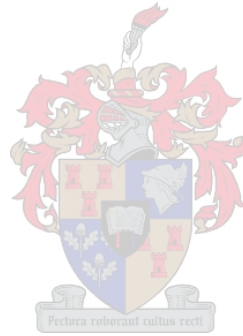
4.6   Conclusions and future directions

Based on the results of our study we conclude that;

1.   The quality of the sperm sample used during ICSI seems to have a regulatory role in the chromosomal composition of the embryo. Seventy percent of the embryos that failed to meet the developmental timetable were Y-chromosome bearing cells. In this regard it is important to underline the effect of sperm DNA packaging on

early embryonic development. Dissimilarities observed in chromatin packaging in sperm samples might well result in defective DNA decondensation, pronuclear formation and delayed cell division events. Impaired sperm morphology is closely associated with DNA damage and chromatin packaging.

2.   If we accept that the defective and dysfunctional Y-chromosome bearing embryos are in part a consequence of a DNA-damaged sperm that was injected during ICSI, the present results leaves the impression that the injected sperm is a Y-chromosome bearing cell. On the other hand, oocyte abnormalities in ICSI and IVF include; aneuploidy (38.4%), hyper-and hypohaploidy) (Martini *et al.*, 1997), disomy and hypoploidy (22.2%) (Martini *et al.*, 1997, Vatev & Toncheva, 1998, ambiguous chromatin complement (34.4%), hypohaploidy and euploidy (Frydman *et al.*, 1998), chromosome breakage (45%), fragmented and broken chromatids and single oocyte chromatids (Martini et al., 1997). A previous report even concluded that 58.7% of unfertilized oocytes in cases of normozoospermic spouses revealed chromosome abnormalities (Almeida and Bolton 1994). Future research might address the possibility to perform a X/Y sperm separation technique in order to inject only X-chromosome bearing sperm in these severe male factor cases.

3.   Sperm selection for ICSI based on the approach of choosing the "best looking" spermatozoon in the ejaculate surprising enough seems to provide cells that can be classified as normal based on the length width ratio set by the WHO for normal cells. Even in cases where the initial morphologic assessment of the whole semen sample showed that ICSI was indicated due to the teratozoospermic (<9% normal forms) nature of the sample, we encountered high numbers of "normal looking"

sperm in the sample. Normal looking sperm typically lack major defects such as broken neck, an elongated or amorphous head or the presence of cytoplasmic droplets.

4. The evaluation of the infertile man in the clinical setting includes a thorough history and physical examination followed by repeated semen analyses. An endocrine, urological and/or imaging workup should be performed as appropriate. Genetic testing (peripheral karyotype and/or mutations screening by PCR and aneuploidy by FISH) should be performed based on defined indications. The analysis of gene expression in mature spermatozoa by novel microarray technology appears to be a promising technique to be introduced among the battery of genetic test.

5. Although ICSI bypasses certain sperm functional aspects i.e. sperm-zona binding and acrosome reaction, the assessment of sperm DNA quality, the identification of the yet putative egg activating factor and the molecular basis of events leading to sperm head decondensation and pronuclear formation, and the impact of the timing and dysfunctions of the activated male genome, will help us understand the role of the male gamete during fertilization. Subsequent acquired knowledge will underline the paternal contributions to embryogenesis in normal and abnormal situations.

6. As the human genome project and the area of proteomics advance, the results and those of studies performed in combination with more classis reproductive biology-

endocrinology techniques will unveil the basis of sperm oocytes interaction dysfunctions to alleviate male infertility.

7. The aim of this study was to hone the skill of embryo biopsy by the worker, to further develop the aptitude of the worker for the sperm functional tests carried out and to determine whether results obtained are supported by current thinking. The results presented provide evidence that the set goals of the project were achieved.
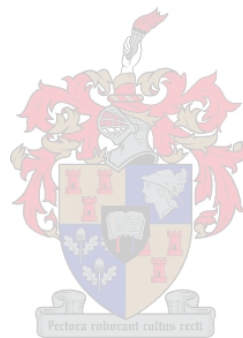
CHAPTER XX

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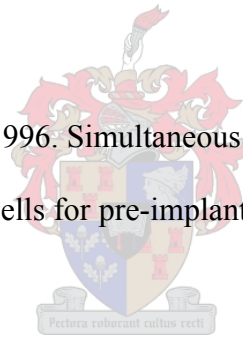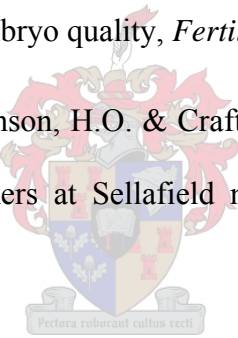
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APPENDIX 1

Fertilization rates, morphology value, chromatin packaging quality spontaneous acrosome reaction and morphometric recordings of 39 couples that underwent ICSI therapy.

									Normal forms				Abnormal forms			
									MORPHOMETRY							
	PREGANCY	No fertilized	No oocytes	% Ferttilized	DQ morphology	Metrix morphology	CMA3 positive (5)	%Spontaneous acrosome reacted(5)	Length (μm)	Width (μm)	Area (μm²)	Acro size (% of head)	Length (μm)	Width (μm)	Area (μm²)	Acro size (% of haed)
1	P	19	20	95	10	14	35	11					5.6	4	16.2	21
2	NP	6	8	75	5	9	56	17	5.5	3.3	13.3	41	5.6	3.1	12.6	44.6
3	NP	2	2	100	4	7	54	21	5.1	3.5	14.7	33.6	5.3	3.6	14	24.2
4	P	7	9	77.8	8	12	45	9	5.4	3.2	15.8	43	6	3.4	12.2	39
5	P	4	4	100	12	13	33	12	5.1	3.1	17.7	37.9	5.8	3.7	15.9	27.5
6	NP	7	7	100	11	14	39	6	5.4	3.6	15.2	33.5	5.6	3.4	13.8	30.4
7	P	12	13	92.3	12	14	43	11								
8	P	13	14	92.9	9	10	47	7								
9	NP	15	18	83.3	8	12	43	13	5	3.1	16.1	21.7	6.5	3.5	14.6	22.2
10	P	3	3	100	8	11	34	6								
11	P	9	9	100	9	11	54	11								
12	NP	3	3	100	5	12	60	23					7.1	3.4	15.2	20.5
13	P	3	3	100	4	7	67	9	5.2	3.5	14.1	31.5	5.7	3.5	14.4	33.4
14	NP	9	11	81.8	10	13	47	10	4.5	3.3	12	32	5.3	3.9	15.2	23.8
15	P	3	3	100	7	11	44	12					5.8	3.2	13.5	35.5
16																
17	NP	3	3	100	9	9	47	11	5	3.3	14.1	38.5	5.9	3.4	14.2	25.2

Appendix 1 continue

	PREGANCY	No fertilized	No oocytes	% Ferttilized	DQ morphology	Metrix morphology	CMA3 positive (5)	%Spontaneous acrosome reacted(5)	Normal forms				Abnormal forms			
									MORPHOMETRY							
									Length (µm)	Width (µm)	Area (µm^2)	Acro size (% of head)	Length (µm)	Width (µm)	Area (µm^2)	Acro size (% of haed)
18	P	6	7	85.7	11	11	34	9								
19	NP	2	2	100	5	9	56	17					4.7	4.2	14.6	25
20	P	7	7	100	9	12	44	12	5.3	3.3	12.8	31.8	5.5	3.4	13.1	32
21	P	3	3	100	8	11	67	21	5.5	3	15.9	31.7	6	3.7	16.3	26
22	NP	6	7	85.7	5	10	54	8								
23	P	7	7	100	7	9	78	12	4.8	3.1	11.8	52.1	7	3.2	11.6	38.4
24	NP	7	8	87.5	11	12	38	12	5.5	3.5	14.6	49.3	5.8	3.4	14.3	19
25	P	8	8	100	8	9	44	22								
26	P	12	12	100	2	6	67	10	5.3	3.4	13.8	36.8	5.5	3.2	12.8	16.9
27	NP	4	5	80	11	7	34	8	4.9	3.2	11.9	52.9	5.1	3.3	12.8	34.5
28	P	6	7	85.7	7	11	49	19	5	3.6	15.3	38.8	5.6	3.7	15	29.3
29	NP	10	11	90.9	6	9	54	16								
30	NP	5	5	100	9	11	48	14					5.7	3.6	14.2	19.9
31	NP	3	4	75	3	5	70	3	4.6	2.6	9	62.7	7.2	3.2	13.3	30.5
32																
33	NP	3	4	75	13	12	33	9								
34	NP	3	4	75	7	7	45	10								
35	P	8	8	100	12	11	32	10	4	3.3	12.3	24	5.8	3.5	15	23.1
36	NP	10	10	100	7	9	55	19	5	3.7	15.6	27.2	5.6	3.4	14.2	30

Appendix 1 continue

	PREGANCY	No fertilized	No oocytes	% Ferttilized	DQ morphology	Metrix morphology	CMA3 positive (5)	%Spontaneous acrosome reacted(5)	Normal forms MORPHOMETRY				Abnormal forms MORPHOMETRY			
									Length (µm)	Width (µm)	Area (µm²)	Acro size (% of head)	Length (µm)	Width (µm)	Area (µm²)	Acro size (% of haed)
37	P	6	10	60	4	7	55	2					4.9	3.2	11.2	33
38	P	6	10	60	7	8	51	16	5	3	12.2	29	7.6	3.4	13.6	24.1
39	P	6	7	85.7	11	13	45	6	5.2	3.3	13.2	43.3	7	3.4	13.3	39.5
		246	276													
	20/37	6.65	7.46	90.4	7.95	10.2	48.7	12	5.062	3.28	13.9	37.7	5.9	3.48	14	28.5
		3.87	4.26	11.8	2.78	2.34	11.3	5.12	0.361	0.25	1.93	9.94	0.71	0.26	1.27	6.99
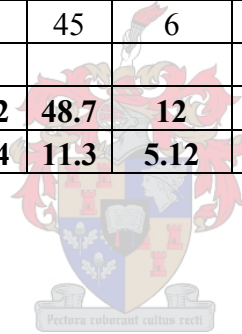
APPENDIX 2

Results of fertilization rates, sperm morphology, chromatin packaging quality and sperm morphometric values of 20 couples where a <u>pregnancy</u> after ICSI therapy was reported

| | | | | | | | | MORPHOMETRY | | | | | | | |
| | | | | | | | | Normal forms | | | | Abnormal forms | | | |
Pat number	NO fertilizaed	Nooocytes	% Ferttilized	DQ MOR	Metrix MOR	CMA3	%Spon-AR	Length	Width	Area	Acro size	Length	Width	Area	Acro size
1	19	20	95	10	14	35	11					5.6	4	16	21
4	7	9	78	8	12	45	9	5.4	3.2	16	43	6	3.4	12	39
5	4	4	100	12	13	33	12	5.1	3.1	18	38	5.8	3.7	16	28
7	12	13	92	12	14	43	11								
8	13	14	93	9	10	47	7								
10	3	3	100	8	11	34	6								
11	9	9	100	9	11	54	11								
13	3	3	100	4	7	67	9	5.2	3.5	14	32	5.7	3.5	14	33
15	3	3	100	7	11	44	12					5.8	3.2	14	36
18	6	7	86	11	11	34	9								
20	7	7	100	9	12	44	12	5.3	3.3	13	32	5.5	3.4	13	32

Appendix 2 continue

Pat number	NO fertilizaed	Nooocytes	% Ferttilized	DQ MOR	Metrix MOR	CMA3	%Spon-AR	MORPHOMETRY							
								Normal forms				Abnormal forms			
								Length	Width	Area	Acro size	Length	Width	Area	Acro size
21	3	3	100	8	11	67	21	5.5	3	16	32	6	3.7	16	26
23	7	7	100	7	9	78	12	4.8	3.1	12	52	7	3.2	12	38
25	8	8	100	8	9	44	22								
26	12	12	100	2	6	67	10	5.3	3.4	14	37	5.5	3.2	13	17
28	6	7	86	7	11	49	19	5	3.6	15	39	5.6	3.7	15	29
35	8	8	100	12	11	32	10	4	3.3	12	24	5.8	3.5	15	23
37	6	10	60	4	7	55	2					4.9	3.2	11	33
38	6	10	60	7	8	51	16	5	3	12	29	7.6	3.4	14	24
39	6	7	86	11	13	45	6	5.2	3.3	13	43	7	3.4	13	40
Mean	7.4	8.2	92	8.3	11	48	11	5.1	3.3	14	36	6	3.5	14	30
SD	4.0	4.3	13	2.7	2.2	13	4.9	0.4	0.2	1.8	7.5	0.7	0.2	1.6	6.8

APPENDIX 3

Results of fertilization rates, sperm morphology, chromatin packaging quality and sperm morphometric values of 19 <u>non-pregnant</u> couples

	Pat number	No oocytes	No Fertil	No used	% Ferttilized	DQ MOR	Metrix MOR	CMA3	%Spon-AR	Normal forms				Abnormal forms			
										MORPHOMETRY							
										Length	Width	Area	Acro size	Length	Width	Area	Acro size
	2	10	6	8	75	5	9	56	17	5.5	3.3	13.3	41	5.6	3.1	12.6	44.6
	3	2	2	2	100	4	7	54	21	5.1	3.5	14.7	33.6	5.3	3.6	14	24.2
	6	7	7	7	100	11	14	39	6	5.4	3.6	15.2	33.5	5.6	3.4	13.8	30.4
	9	22	15	18	83.3	8	12	43	13	5	3.1	16.1	21.7	6.5	3.5	14.6	22.2
	12	3	3	3	100	5	12	60	23					7.1	3.4	15.2	20.5
	14	11	9	11	81.8	10	13	47	10	4.5	3.3	12	32	5.3	3.9	15.2	23.8
	17	4	3	3	100	9	9	47	11	5	3.3	14.1	38.5	5.9	3.4	14.2	25.2
	19	3	2	2	100	5	9	56	17					4.7	4.2	14.6	25
	22	11	6	7	85.7	5	10	54	8								
	24	12	7	8	87.5	11	12	38	12	5.5	3.5	14.6	49.3	5.8	3.4	14.3	19

Appendix 3 continue

|	Pat number	No oocytes	No Fertil	No used	% Ferttilized	DQ MOR	Metrix MOR	CMA3	%Spon–AR	Normal forms MORPHOMETRY				Abnormal forms			
										Length	Width	Area	Acro size	Length	Width	Area	Acro size
	27	5	4	5	80	11	7	34	8	4.9	3.2	11.9	52.9	5.1	3.3	12.8	34.5
	29	14	10	11	90.9	6	9	54	16								
	30	5	5	5	100	9	11	48	14					5.7	3.6	14.2	19.9
	31	5	3	4	75	3	5	70	3	4.6	2.6	9	62.7	7.2	3.2	13.3	30.5
	33	5	3	4	75	13	12	33	9								
	34	4	3	4	75	7	7	45	10								
	36	10	10	10	100	7	9	55	19	5	3.7	15.6	27.2	5.6	3.4	14.2	30
Total		133	98	112													
Average	20/37	7.82	5.76	6.59	88.8	7.59	9.82	49	12.8	5.05	3.31	13.7	39.2	5.8	3.49	14.1	26.9
SD		5.17	3.58	4.17	10.6	2.85	2.41	9.5	5.31	0.326	0.29	2.05	11.9	0.71	0.28	0.77	6.81

Appendix 4
Results of ICSI outcome, sperm morphology, chromatin packaging quality spontaneous acrosome reaction recorded for 39 couples

No	Pregnant/ non-Pregnant	No oocytes	No Fertilized	% Fertilized	Diff-Quik	Metrix	CMA3	Spontaneous AR
1	P	20	19	95	10	14	35	11
2	NP	8	6	75	5	9	56	17
3	NP	2	2	100	4	7	54	21
4	P	9	7	78	8	12	45	9
5	P	4	4	100	12	13	33	12
6	NP	7	7	100	11	14	39	6
7	P	13	12	92	12	14		
8	P	14	13	93	9	10		
9	NP	18	15	83	8	12	43	13
10	P	3	3	100	8	11	34	6
11	P	9	9	100	9	11	54	11
12	NP	3	3	100	5	12	60	23
13	P	4	3	75	4	7	67	9
14	NP	11	9	82	10	13	47	10
15	P	3	3	100	7	11	44	12
16								
17	NP	3	3	100	9	9	47	11
18	P	7	6	86	11	11	34	9
19	NP	2	2	100	5	9	56	17
20	P	7	7	100	9	12	44	12
22	NP	7	6	86	5	10	54	8
23	P	7	7	100	7	9	78	12
24	NP	8	7	88	11	12	38	12
25	P	8	8	100	8	9	44	9
26	P	12	12	100	2	6	67	9
27	NP	5	4	80	11	7	34	7
28	P	7	6	86	7	11	49	19
29	NP	11	10	91	6	9	54	16
30	NP	5	5	100	9	11	48	14
31	NP	4	3	75	3	5	70	3
32								
33	NP	4	3	75	13	12	33	9
34	NP	4	3	75	7	7	45	10
35	P	8	8	100	12	11	32	10
36	NP	10	10	100	7	9	55	19
37	P	10	6	60	4	7	55	2
38	P	10	6	60	7	8	51	16
39	P	7	6	86	11	13	45	6
Total	20/37	276	246					
Mean		**7.5**	**6.65**	**90.4**	**7.95**	**10.2**	**48.7**	**12.0**
SD		**4.2**	**3.8**	**11**	**2.78**	**2.34**	**11.3**	**5.1**

APPENDIX 5

Student's paired t-test results of ICSI results and sperm functional assays of pregnant vs. non-pregnant

	No fertilizaed	No oocytes	% Oocytes Ferttilized	DQ morphology	Metrix morphology	% CMA3 positive cells	%acrosome reac Spontaneous ted cells
p-values	0.1	0.3	0.9	0.48	0.36	0.87	0.41
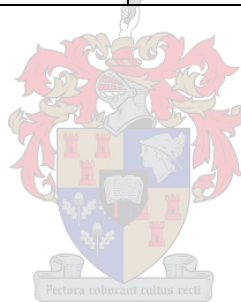
APPENDIX 6

Student's paired t-test results of sperm morphometric results and sperm functional assays of pregnant vs. non-pregnant

	MORPHOMETRY							
	Normal forms				Abnormal forms			
	Length (μm)	Width (μm)	Area (μm²)	Acro size (% of head)	Length (μm)	Width (μm)	Area (μm²)	Acro size (% of head)
p-values	0.89	0.62	0.53	0.53	0.51	0.78	0.67	0.2