Ph.D. THESIS

SPERM SELECTION FOR HUMAN ASSISTED REPRODUCTION - EXAMINATION OF THE SAFETY OF DIFFERENT SPERM PREPARATION METHODS IN ELIMINATING SPERMATOZOA WITH NUMERICAL CHROMOSOME ABNORMALITIES AND CELLULAR IMMATURITY

Spermium szelekció humán asszisztált reprodukcióhoz – különböző spermium előkészítési eljárások hatásának vizsgálata a számbeli kromoszóma rendellenességgel és éretlenséggel sújtott ivarsejtek gyakoriságának alakulására

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Abbreviations

ABC – avidin-biotin complex

ASp – asthenozoospermy

BSA – bovine serum albumin

CI – Confidence Interval

CK- creatine phosphokinase

DAPI - 4’-6’ diamino-2-phenylin-dole

DNA – deoxyribonucleic acid

dUTP – deoxyuridil-triphosphate

FISH – fluorescence in-situ hybridization

FITC – fluorescein-isothiocianate

HA – hyaluronic acid, hyaluronan

HspA2 – heat-shock protein A2

ICSI – intracytoplasmic sperm injection

IUI – intrauterine insemination

IVF – in vitro fertilization

HTF – human tubal fluid

LIS – lithium salt

M1 – meiosis I.

M2 – meiosis II.

NS – not significant

NSp – normozoospermy

OASp – oligoasthenozoospermy

OAT - oligoasthenoteratozoospermy

OR – Odds Ratio

OSp – oligozoospermy

SD – standard deviation

SEM – strandard error mean

SSC – sodium chloride sodium citrate

WHO – World Health Organization

Introduction and Aims of the Thesis

With the advent of modern assisted reproduction techniques, especially with the intracytoplasmic sperm injection (ICSI) effective treatment has become available for men with severe male infertility. ICSI is efficiently used in clinical practice, but unfortunately, as a result of intensive research an increased risk of transmission of cytogenetic defects to the offspring has also been documented (Bonduelle *et al.*, 1998, 2002; Van Steirteghem *et al.*, 2002a, 2002b). These reports called our attention for the risks of bypassing the physiologic selection of abnormal spermatozoa with assisted reproductive techniques. In addition to this iatrogen risk, the incidence of sperm numerical chromosomal abnormalities (aneuploidy/polyploidy) among ICSI patients is known to be significantly higher (Martin, 1996; Van Dyk *et al*, 2000; Calogero *et al.*, 2001a, 2001b).

Since the introduction of the fluorescence in-situ hybridization (FISH) technique on decondensed sperm nuclei, which enables us to examine sufficient number of spermatozoa independently for numerical chromosomal abnormalities, large number of laboratories have already addressed the relationship between the numerical chromosomal abnormality of spermatozoa and male infertility. Despite of the extensive research, the relationship between the sperm morphology and functional parameters, such as motility or fertilization potential, and the frequency of numerical chromosomal abnormalities has not been established yet.

Also, insufficient data are available on the efficacy of the widely utilized sperm preparation techniques (the *gradient centrifugation separation* based on the sperm density and the *swim-up method* based on the sperm motility) in decreasing the frequency of chromosomal abnormalities in the sperm used for assisted fertilization.

There is close correlation between the proportion of immature sperm characterized with cytoplasmic retention and frequency of chromosomal aneuploidies, indicating that aneuploidies are primarily found in immature

spermatozoa (Kovanci *et al.*, 2001). Mature sperm without cytoplasmic retention selectively bind to the zona pellucida (Huszar *et al.*, 1994), but fertilization through ICSI may be successful using immature spermatozoa (Nagy *et al.*, 1994). ICSI as it is presently performed, is far from an ideal infertility solution, because sperm that are subjectively selected by the embryologist using morphology and motility criteria may have genetic impairments. The relationship between shape and genetic content in the same sperm is particularly important from the point of view of sperm selection for ICSI. The literature data are inconsistent regarding the relationship between the sperm morphology and aneuploidy.

During late spermiogenesis along with the extrusion of the cytoplasm the sperm plasma membrane also undergoes a maturation-related remodeling that promotes the formation of the zona-binding sites, as well as binding sites for the hialuronic acid (HA). Similarly to the zona pellucida, mature sperm are able to bind selectively to the HA coated surface (Huszar *et al.*, 2003). HA binding offers a new selection method to eliminate immature sperm.

Based on the safety concerns about the transmission of genetic impairment to the offspring with ICSI and the insufficiency of the scientific data regarding the efficacy of the presently used sperm selection methods, the key points of this thesis based on my research applying FISH on sperm were as follows:

1. What is the incidence of numerical chromosome abnormalities in spermatozoa in men with low sperm count who represent a population of potential candidates for assisted reproduction and ICSI and do the WHO definitions of spermiogram reflect the risk of genetic impairment? [*The Population Study: Determination of the incidence of the numerical chromosomal abnormalities (aneuploidy and diploidy) in infertile men with low sperm count and investigation of their relationship to the traditionally used WHO criteria of the spermiogram*]
2. Can one eliminate sperm with aneuploidy/diploidy using the present sperm preparation techniques? [*The Gradient Centrifugation and Swim-up Studies: Examination and comparison of the efficiency of the traditional sperm preparation techniques (the gradient centrifugation*

and the swim-up method) in eliminating aneuploid and diploid sperm, and sperm with diminished maturity.]

3. Can one visually select aneupolidy/diploidy-free sperm? [*The Morphology Study: Examination of the relationship of sperm morphology and numeric chromosomal abnormalities using objective computerized morphometry on the same sperm.*]
4. Can one select aneuploidy/diploidy-free individual sperm based on HA-binding? [*The HA-binding Study: Examination of the efficiency of selection of individual mature sperm with low frequencies of numerical chromosomal abnormalities by HA-binding.*]

Background

Safety Issues of Assisted Reproduction

Since the first report of pregnancies and births to result from the replacement of embryos generated by ICSI in 1992 (Palermo *et al.*, 1992), effective treatment has become available for those infertile couples with severe male factor (Van Steirteghem, 1994), for whom previously either the donor insemination or the adoption were the only choices to have a child. Despite its rapidly increasing use in assisted reproductive practice ICSI has been considered from the beginning as a risky procedure. ICSI is a more invasive procedure than routine in vitro fertilization (IVF), since one spermatozoon is injected through the oocyte membrane and since fertilization can ensue from a sperm which could never have been used previously in fertility treatment. There is a concern as to whether ICSI, the most invasive assisted reproduction technique, might lead to an increase in the proportion of children with de-novo chromosomal abnormalities or gene defects, or to inheritance of the father`s infertility problem. Although investigators of large cohorts failed to find any increased risk of major congenital malformations in ICSI children as compared with the general population, but an increased risk of de-novo chromosomal aberrations, mostly sex chromosomal aneuploidies was confirmed with major contribution of the Klinefelter syndrome (47, XXY) (Bonduelle *et al.*, 1995, 1996, 1998a, 1998b, 2002; Palermo *et al.*, 1996; Kurinczuk and Bower, 1997; In`t Veld *et al.*, 1997a, Liebaers *et al.*, 1995). An overwiew of seven studies on prenatal diagnosis after ICSI reported 73 abnormal karyotypes out of 2139 analysed (Van Steirteghem *et al.*, 2002), and forty of them were due to de-novo chromosomal aberrations. To the data of the most recent and relevant study (Bonduelle *et al.*, 2002b), there was a significant increase in the de-novo sex chromosome aneuploidy (0.6%) and structural autosomal abnormalities (0.4%) as compared with the general population (0.2% and 0.07% respectively).

Detection of Chromosomes and Estimation of Numerical Chromosome Abnormalities in Human Spermatozoa

Numerical chromosome abnormalities, aneuploidies or diploidies, occur when a sperm cell possesses more or less than a single copy of each autosomal or sex chromosome, or more than one copy of the entire genome. Mammalian spermatozoa are haploid cells (n=23) which contain 22 autosomes and one sex chromosome, either the X or Y. Aneuploidy is the condition in which a spermatozoon has an extra chromosome (disomy) or is missing a chromosome (nullisomy). Diploid spermatozoa have an entire extra chromosome set resulting in 44 autosomes and two sex chromosomes.

Earlier the capacity of the human spermatozoa to penetrate zona-free hamster oocytes was used to obtain the first preparations of human sperm chromosomes (Rudak *et al.*, 1978; Martin and Taylor, 1982; Martin *et al.*, 1982). This method yielded valuable data because it examines the entire chromosome complement of each spermatozoon and detects structural and numerical abnormalities. However, this technique was so complex and time consuming that it limited the number of spermatozoa can be examined, consequently its use was limited to a few laboratories, and was never applied in a clinical setting. Further, the results were potentially biased in that only those human spermatozoa which can fertilize oocyte were karyotyped.

In-situ hybridisation (ISH) involves hybridisation of a chromosome specific DNA probe to complementary sequences of a target chromosome followed by detection of the bound probe and hence the chromosome itself. The use of these chromosome specific DNA probes initially labelled with radioactive compounds (Joseph *et al.*, 1984) and later with fluorochromes (Guttenbach and Schmid, 1990), and especially the combination of several probes (Wyrobek *et al*., 1994) opened the way to indirectly study the chromosome constitution of large numbers of spermatozoa by multi-colour fluorescence in-situ hybridisation (FISH). Localisation of one chromosome using a single-probe FISH provides only limited detection of aneuploidy, so it is preferable to simultaneously localise several chromosomes to increase the sensitivity of

detection (multi-probe FISH). In practice, this is often limited to the detection of up to four chromosomes.

Mammalian spermatozoa are haploid interphase cells which have a unique packaging and arrangement of DNA, that differs significantly from somatic cells. (Ward and Coffey, 1991). The linear, side-by-side arrays of DNA, cross-linked by disulphide bonds between adjacent protamines, create a condensed, genomically-inert nucleus, which is inaccessible to DNA probes. It is now well recognized that to achieve efficient hybridisation, the sperm nucleus must be swollen and made accessible to probes by reducing disulphide bonds between protamine molecules (decondensation).

As aneuploidy for a given chromosome occurs at a very low frequency in human spermatozoa, it is recommended, that scoring 5000 spermatozoa per sample should be sufficient for comparisons if the results of donors are pooled, but at least 10 000 nuclei should be scored from each sample to provide an accurate estimate for each chromosome (Robbins *et al.*, 1993; Downie *et al.*, 1997). Assuming that a higher rate of non-disjunction exist for the sex chromosome (see below) but the autosomes all have a similar non-disjunction rate, the overall risk of aneuploidy in human spermatozoa can be roughly estimated.

Meiotic Disorders and Sperm Aneuploidy in Fertile and Infertile Men

Haploid spermatids (n=23) are results of the meotic cell divisions of the primary spermatocytes in the testis. Meiosis is a complex process that includes two successive cell divisions, without DNA replications between them. Any of the steps of meiosis may go wrong, giving rise to different types of chromosome abnormalities such as aneuploidy or diploidy. Meiotic disorders, i.e. chromosome abnormalities limited to spermatogenic cells and not detectable through the study of the somatic karyotype, are found ~6% in patients in whom meiotic studies are performed (Egozcue *et al.*, 1983) and this figure may grow to 17-18 % in patients with severe

oligoasthenozoospermia (OA) (Vendrell *et al.*, 1999). These data of the meiotic studies were later reflected in FISH studies investigating the chromosome constitution of spermatozoa: ~6% of spermatozoa of normospermic fertile men carries numeric chromosomal abnormalities and significant increase in the rates of aneuploidy and diploidy can be expected in patients with different degrees of OA (Downie *et al.*, 1997; Egozcue *et al.*, 1997; Shi and Martin, 2001). All chromosomes have now been investigated for the spontaneous incidence of disomy in human sperm. From these studies, the mean frequency of disomy is ~0.12% for the autosomes and 0.31% for the sex chromosomes in normospermic men (Egozcue *et al.*, 1997). Among the autosomes higher disomy frequency (>0.3% in average) of the chromosome 14 and the G-group chromosomes (21 and 22) were detected than in most other autosomes (<0.2% in average). Also, significantly higher frequencies of disomy for the sex chromosome were consistently found by most of the laboratory (Shi and Martin, 2000).

Meiotic disorders are frequent in infertile patients and are responsible for autosomal and sex-chromosome aneuploidies and diploidy in sperm (Egozcue *et al.*, 2000). The higher incidence of chromosomal aberrations after ICSI are explained by the higher frequency of chromosomal abnormalities of spermatozoa reported in olygospermic men with fertility problems, especially those of with oligoasthenoteratozoospermia (OAT) (Moosani *et al.*, 1995; Finkelstein *et al.*, 1998; Aran *et al.* 1999a; Pfeffer *et al.*, 1999; Colombero *et al.*, 1999; Pang *et al.*, 1999; Rives *et al.*, 1999; Nishikawa *et al.*, 2000; Ushijama *et al.*, 2000; Vegetti *et al.*, 2000; Ohashi *et al.*, 2001; Rubio *et al.*, 2001; Calogero *et al.*, 2001b). In contrast, if male infertility occurs with normal semen parameters, the risk of having increased frequency of aneuploidy in spermatozoa does not seem to be elevated (Miharu *et al.*, 1994; Lahdetie *et al.*, 1997; Bernardini *et al.*, 1997, 1998; Schultz *et al.*, 2000). Summarizing the data of studies scoring at least 1000 spermatozoa per donor, in a recent review Shi et Martin (2001) concluded, that infertile men with a normal karyotype and low semen concentration or certain types of morphologically abnormal spermatozoa have a significantly increased risk of producing aneuploid spermatozoa, particularly for sex chromosomes.

Most of these papers reported also higher rate of diploid spermatozoa in semen with low sperm count. Elevated diploid sperm frequency is also a common finding in infertile patients with meiotic disorders reaching a very significant proportion of sperm in some cases (In`t Veld *et a*l., 1997b, Benzacken *et al.*, 2001; Devillard *et al.*, 2002).

Clinical Significance of De-novo Chromosomal Aberrations

In general, most de-novo autosomal aneuploidies are non viable, and their products are eliminated during the pregnancy. For this reason, most of them are found in spontaneous abortion (Jacobs and Hassold, 1995). It is known, that fetal aneuploidy is responsible for the majority of the early pregnancy loss, most frequently the trisomy 16 (Eiben *et al.*, 1990). Also, it has been documented, that trisomies 21 and 22 are more frequently present than other trisomies in spontaneous abortions (Hassold *et al*., 1980), which supports the observation that the G-group chromosomes are more susceptible for aneuploidy, than other autosomes (Shi and Martin, 2000). In cases of recurrent miscarriage besides oligozoospermia the significantly elevated frequency of disomy 15, disomy 18 and sex chromosome disomy in sperm were confirmed with FISH studies (Rubio *et al.*, 2001). The only full numerical autosomal anomalies surviving to birth are trisomy 13 (probability of survival to birth of 2.8%), trisomy 18 (probability of 5.4%) and trisomy 21 (probability of 22.1%), but only trisomy 21 (Down syndrome) allow survival into puberty and adulthood (Egozcue *et al.*, 2000). Most of the autosomal trisomy are of maternal origin, the paternal contribution can be detected only in 10% of the cases (Egozcue *et al.*, 2000). These data are in good correlation with the epidemiological studies, according to which, unlike the sex chromosomal aneuploidies, there is no increase of the incidence of autosomal aneuploidies can be detected following the ICSI or IVF procedures.

Triploidy is also incompatible with human life, but also appears as a frequent etiology of the early pregnancy loss, as it can be found in 12-13% of spontaneous abortions (Eiben *et al.*, 1990). Chromosome studies following

recurrent miscarriages suggest, that most of the triploidies leading to abortion are of paternal origin (Aran *et al.*, 1999; Rubio *et al.*, 1999; Egozcue *et al.*, 2000; Zaragoza *et al.*, 2000, Egozcue *et al.*, 2002). ICSI patients with meiotic disorders, and with increased diploidy frequencies also had increased rates of abortion rates after the ICSI (Aran *et al.*, 1999b).

Sperm Shape and Aneuploidy

In the lights of the literature data above, in the present practice of ICSI, the relationship between sperm shape and genetic content in the same sperm is particularly important from the point of view of sperm selection for ICSI. The evaluation of sperm shape has been a difficult and inconsistent science because it is based on subjective parameters. Complete assessment of sperm shape includes analysis of the head, neck, midpiece, and tail. For a spermatozoon to be considered normal, the head should be oval and symmetrical. Insertion of the tail should be axial (in line with the long axis of the head). Abnormal sperm variants include those having heads that are large, small, round, asymmetrical, or amorphous, or those with tapered, bulging midpieces, or multiple heads or tails. In general, clinical laboratories use the sperm morphology parameters proposed by the World Health Organization (WHO), or the so-called “strict morphology,” developed by Dr. Kruger (WHO Laboratory Manual, 1999). There are earlier studies demonstrating an association between sperm shape properties and various chromosomal abnormalities. In men with oligoasthenoteratozoospermia (OAT) there was an increased frequency of numerical chromosomal aberrations suggesting that these patients produce a higher proportion of aneuploid gametes (Templado *et al.,* 2002; Rubio *et al.,* 2001; Calogero *et al.,* 2001; Colombero *et al.,* 1999). Infertile men with a normal karyotype and low sperm concentration or higher levels of morphologically abnormal have a significantly increased risk of producing aneuploid spermatozoa, particularly for the sex chromosomes (Shi and Martin, 2001). Semen samples with certain forms of morphological anomalies, such as megalo-pinhead spermatozoa were associated with increased rates of chromosomal aneuploidies (Yurov *et al.,*

1996; Yakin and Kahraman, 2001). There are further research data suggesting that the association between sperm morphology and incidence of sperm with aneuploidies and diploidies is not consistent. Lee *et al.* (1996) found that the incidence of structural chromosomal aberrations was about four times higher in semen samples with high incidence of sperm with amorphous, round or elongated heads than in those with morphologically normal sperm, however the incidence of aneuploidy was not significantly different between the two groups. No increase in chromosomal aberrations was found in human spermatozoa injected into mouse oocytes with abnormally large or small heads (Lee *et al.*, 1996). In another study by Rives *et al.* (1999), the disomy frequency in infertile males was directly correlated with the severity of oligospermia, however, no relationship was established between aneuploidy rate and morphology. Further, in a case study of men with increased levels of globozoospermia, shortened flagella syndrome or sperm with abnormal acrosomes, no association was found between sperm shape and chromosomal status (Viville *et al.*, 2000). Additionally, Ryu et al. (2001) studied approximately 100-150 morphologically normal sperm (according to Kruger's strict criteria) from both normal and infertile couples and found that normal morphology is not an absolute indicator for the selection of genetically normal sperm. In order to resolve these discrepancies, and to assess better the relationship between sperm shape and chromosomal aberrations, it is necessary to study the same spermatozoa for both parameters. However, so far the studies have focused upon the occurrence of sperm with abnormal morphology or aneuploidy in semen samples, but not on detection of both attributes in individual spermatozoa.

Sperm Maturity, Shape, Function and Aneuploidy

It is also of interest, that fertilization through ICSI may be successful using immature spermatozoa (Nagy *et al.*, 1994). One of these biochemical parameters is the concentration of the enzyme creatine phosphokinase (CK) in sperm, which reflects cytoplasmic retention, the hallmark of diminished maturity (Huszar *et al.,* 1988a, 1988b; Huszar and Vigue, 1990; Huszar and

Vigue, 1993). CK-immunocytochemistry studies of individual spermatozoa have also demonstrated a relationship between cytoplasmic retention and aspects of abnormal sperm morphology, including increased size of the sperm head, roundness of the sperm heads and the increased incidence of amorphous sperm. Efforts were also made to introduce objective computer assisted morphometry for the assessment of sperm shape, using sperm features that are related to the biochemical parameters of sperm maturation and function (Gergely *et al.*, 1999a). Further, in CK-immunostained sperm-hemizona complexes, sperm that bound to the zona were exclusively the clear-headed mature type, lacking cytoplasmic retention (Huszar *et al.,* 1994).

Another biochemical marker of sperm maturation is the testis specific HspA2 chaperone protein, a member of the highly conserved HSP70 chaperone family which were well studied in the mouse (Eddy, 1999; Son *et al.,* 1999). The developmental and clinical significance of the human homologue HspA2 in men has been established. (Huszar *et al.,* 2000). Using an HspA2 polyclonal antibody, two waves of HspA2 were indentified as the homologous hsc70 chaperon protein expression in spermatogenesis and spermiogenesis (Huszar et al., 2000). The chaperone protein first appears in the primary and secondary spermatocytes as a component of the synaptonemal complex, the structure formed between homologous chromosomes during meiosis. The second wave of HspA2 expression occurs during terminal spermiogenesis, simultaneously with cytoplasmic extrusion, plasma membrane remodeling and formation of the zona-binding site. It was suggested, that the common factor between sperm immaturity and chromosomal aneuploidies is the diminished HspA2 expression in immature sperm, that may cause both the higher incidence of aneuploidies and cytoplasmic retention. As was shown in the mouse, the HSP70-2, a homologue of the HspA2 chaperone protein, is a component of the synaptonemal complex, and also facilitates the intracellular movement of proteins (Dix *et al*., 1996; Eddy, 1999). This association may explain the relationship between meiotic errors, thus aneuploidies, and the presence of surplus cytoplasm in sperm of diminished maturity.

Accordingly, there was a significant correlation (r=0.7)) between the proportion of immature sperm with cytoplasmic retention and frequency of aneuploidies, indicating that aneuploidies are primarily found in immature spermatozoa with cytoplasmic retention and diminished HspA2 levels (Kovanci *et al*., 2001).

Since diminished expression of HspA2 is related to increased frequencies of disomies/diploidies, and because cytoplasmic retention and maturity are directly related to sperm shape, we hypothesized that there may be a relationship between sperm shape and disomies or diploidies within the same sperm. Such studies toward identification of abnormal morphology and aneuploidy in sperm have not been carried out previously because, (i) In order to perform fluorescence in-situ hybridization (FISH), the sperm DNA must be decondensed and denatured. (ii) These processes may alter sperm shape, thus the potential relationship between shape and aneuploidy cannot be assessed unless it has been established whether the shape attributes of sperm remain conserved prior to and after decondensation.

<u>Efficiency of Current Sperm Preparation Methods for Assisted Reproduction in the Elimination of Spermatozoa with Aneuploidy/Diploidy</u>

Sperm preparation techniques are frequently used in the methods of assisted reproduction. The *gradient centrifugation* separates sperm by their specific gravity. It takes advantage of the lower specific gravity of immature spermatozoa with cytoplasmic retention compared to that of mature spermatozoa. The other widely used sperm preparation, the *swim-up technique* is a method for the enrichment of the motile sperm and elimination of non-motile sperm. Enrichment of the motile sperm is beneficial in conventional assisted reproductive techniques, such as intrauterine insemination (IUI) and in vitro fertilization (IVF), but the question of genetic integrity is particularly important in light of sperm selection for ICSI. The relation of genetic integrity to sperm motility and density remained unclear.

Thus, the efficiency of the sperm preparation methods used by most of the assisted reproductive laboratories still also has to be determined.

Our interest in the efficacy of the sperm preparation methods in elimination of sperm with aneuploidies and diploidies stems from the previous study of our laboratory, in which the relationship between the proportions of sperm with diminished cellular maturity and the frequencies of sperm with numerical chromosomal aberrations was addressed (Kovanci *et al.,* 2001). In that study immature sperm were monitored by the presence of cytoplasmic retention and gradient centrifugation was used to enrich mature spermatozoa. In parallel with finding a strong relationship between the frequencies of chromosomal aneuploidies and diminished sperm maturity a decrease of aneuploidies in the pellet was observed. (Kovanci *et al.,* 2001).

Publications dealing with FISH and sperm preparation for assisted reproduction have primarily focused upon two questions, without reaching a consensus: (i) X and Y sex ratios in initial semen and prepared fractions, and (ii) the elimination of sperm with aneuploidies and diploidies in prepared fractions. There is substantial diversity in study findings which may, in part, have resulted from inconsistent patient selection and experimental design. However, the most significant confusing factor is the variation in number of sperm nuclei evaluated. For a reliable assessment of aneuploidy and diploidy rates, considering the mean frequency of 0.1% to 0.5% per chromosome, or 1 to 5 aberrant sperm nuclei per 1000, one should evaluate approximately 5,000-10,000 sperm in each sample. In the papers that dealt with sperm preparation methods as discussed below, the range of sperm per sample studied was 170-6000. In 5 of the papers cited, the number of sperm evaluated equaled 1000 or fewer.

Regarding the X and Y ratios between the initial semen and swim-up sperm fractions, some reports indicated no differences (Benet *et al.,* 1992; Calogero *et al.,* 2001a; Pfeffer *et al.,* 1999; Han *et al.,* 1993; Samura *et al.,* 1997). Other authors found an increase in the proportion of Y bearing sperm after swim-up (Li and Hoshai, 1998; Martinez-Pasarell *et al.,* 1997). With respect to elimination of aneuploid sperm, in two studies there were no differences

between the initial and swim-up fractions (Samura *et al.,* 1997; Van Dyk *et al.,* 2000), while others reported a decline of diploid sperm (Han *et al.,* 1993; Li and Hoshiai, 1998; Martinez-Pasarell *et al.,* 1997). In comparing sperm selection by three methods: swim-up, glass wool filtration, and two-phase discontinuous Percoll gradient centrifugation, Samura *et al.* 1997 found no difference between initial semen and treated sperm fractions with respect to frequencies of diploidy and disomy. The sex chromosome aneuploidy and diploidy rates after swim-up were found to be unchanged by Martinez-Pasarell *et al.* 1997, whereas other two studies indicated a decline of diploidy frequencies in swim-up fractions (Han *et al.,* 1993; Li and Hoshiai, 1998).

In a study of the author of present thesis we examined the efficiency of the swim-up technique in eliminating aneuploid or diploid sperm and compared it to the existing data of the gradient centrifugation. Since FISH analysis after both separation method requires a sufficient number of recovered sperm, this precluded the paralel investigation of them on the same semen samples, as well as the use of severely oligospermic and/or asthenospermic samples. To increase the validitation of the comparison we used semen samples with almost identical parameters in these studies. In addition to the insight based upon the role of HspA2, the experience with the gradient centrifugation study (Kovanci *et al.,* 2001) helped to an improved experimental design: (1) In general, in samples that are oligospermic or are in the 20-30 million/mL range, there are higher proportions of immature sperm (Huszar *et al*., 1988a, 1988b, 1990; Huszar and Vigue, 1993). For this reason, we have utilized samples primarily with <20 million sperm/mL (2). We monitored by CK-immunocytochemistry the proportions of sperm with diminished maturity in semen and in the swim-up fractions, as well as in the pellet after gradient centrifugation. (3) We studied motile sperm yield, or the recovery of motile sperm, in the swim-up fractions. (4) In order to further validate our study methods and results, we utilized five chromosome probes, and independently employed two-color and three-color FISH in evaluating at least 20,000 sperm in each patient.

Binding Properties of Spermatozoa to Zona Pellucida and Hyaluronic Acid

The relationship between zona pellucida binding competence and maturity has been identified earlier in our laboratory (Huszar *et al*., 1994), by focusing upon CK-immunostained sperm samples and their respective sperm-hemizona (halved unfertilized human oocytes) complexes. In the semen samples there were sperm with various degrees of cytoplasmic retention, but all sperm bound to the zona pellucida were mature as characterized with the absence of any cytoplasmic retention. Thus, immature sperm with retained cytoplasm, high CK content and low expression of HspA2 are apparently deficient in the zona-binding site (Huszar *et al*., 1994). It was found, that along with cytoplasmic extrusion and the initiation of HspA2 synthesis, a developmentally regulated plasma membrane remodeling also occurs, which facilitates the formation of the zona pellucida-binding site (Huszar *et al.*, 1997). The data provided evidence that the sperm plasma membrane undergoes a remodeling process during spermiogenetic maturation. These experimental data are supported by the clinical data, that low sperm HspA2 levels were also predictive for failure of pregnancies in two blinded studies of IVF couples (Huszar *et al*., 1992; Ergur *et al*., 2002), suggesting that immature sperm have low fertilization potential.

In another line of experiments, the effects of hyaluronic acid (HA) or hyaluronan was investigated, which is a linear repeating polymer of disaccharides, on human sperm function. HA in the medium increased the velocity and retention of motility and viability of freshly ejaculated, as well as in cryopreserved/thawed human spermatozoa (Huszar *et al.*, 1990; Sbracia *et al*., 1997), and it was concluded that the HA effects on sperm are receptor mediated. The presence of the HA-receptor in human sperm was established by two laboratories (Kornovski *et al*., 1994; Ranganathan *et al*., 1994).

In further studies it was also demonstrated (Huszar *et al.*, 2003), that mature sperm selectively attach and remain bound to solid state HA, similarly to the zona pellucida. The HA receptor, similar to the sperm zona-binding site, is developmentally regulated and is not present in immature sperm as identified by cytoplasmic retention and low HspA2 expression. Due to the exclusive

presence of HA receptors, it was possible to select mature sperm from a mixture of sperm of varying levels of maturity. Based on the strong correlation between the incidence of immature spermatozoa the frequency of the chromosomal aneuploidy, we hypothesized that HA binding would facilitate the selection of individual sperm with low levels of chromosomal aneuploidies.

Materials and Methods

Patient Population and Experimental Design

The study population was composed of 44 men who presented for semen analysis at Sperm Physiology and IVF Laboratories of the Department of Obstetrics and Gynecology, Yale University School of Medicine. Samples of ten moderately oligospermic patients were examined with CK immunocytochemistry and FISH before and after gradient centrifugation, another ten moderately oligospermic patients before and after swim-up preparation. Furthermore, samples of twelve oligospermic patients before and after HA binding selection and gradient treated samples of twelve normospermic patients before and after HA selection. The FISH studies of the swim-up and HA experiments (68 samples of 34 patients), cell scoring and data collection and analysis of the complete data-pool were performed by the author of this thesis in the Ward Laboratory of the Department of Genetics and in the Sperm Physiology Laboratory of the Department of Obstetrics and Gynecology, Yale University School of Medicine, while other parts of this research were performed by the co-authors. The sperm concentration and motility in the initial and prepared fractions were determined in a Makler chamber by computer assisted semen analysis (Hamilton-Thorne Scientific Co, Beverly, MA). All studies were approved by the Human Investigation Committee of Yale School of Medicine.

Preparation of Sperm Fractions by Gradient Centrifugation

Semen samples of ten individuals were utilized. For the study of the semen fractions, 7-10 μl of neat semen was used to prepare each sperm smear on a laboratory glass slide. In order to prepare the corresponding 80% Percoll sperm fraction, an aliquot of the same semen sample was centrifuged through 2 ml of an 80% single-phase Percoll gradient at 500 x g for 20 minutes at room temperature. The sperm pellet was re-suspended in 2 mL human tubal

fluid (HTF, Irvine Scientific, Santa Ana, CA) and centrifuged again at 600 x g for 10 minutes in order to eliminate the residual Percoll (**Figure 1.**). The pellet was re-suspended in HTF to a concentration of about 30-40 million sperm/ml and smears were prepared on glass slides. The smears of the re-suspended sperm (pellet fraction) and the initial semen fraction were fixed with methanol-acetic acid (3:1 ratio) for 10 minutes, air-dried, dehydrated in a series of 70%, 80%, and 100% ethanol, and stored at –70° C for the FISH experiments. Other sperm slides, prepared in an identical manner, were subjected to CK-immunocytochemistry to determine the proportion of sperm with cytoplasmic retention.

Preparation of Sperm Fractions by Swim-up

Semen samples of 10 individuals were studied. For the studies with FISH or with immunocytochemistry for detection of cytoplasmic retention in sperm of the initial semen 7-10 µl of neat semen were used to prepare sperm smears on laboratory glass slides. In order to prepare swim-up sperm fractions we have used a home-modified 15 mL Falcon tube to which 1 mL solid polymer is added, thus the conical sections is filled and the tube provides a flat centrifugation surface (Makler *et al.,* 1984). The following procedure is a standard component of our semen analysis, called the "migration test" (**Figure 2.**). Semen was diluted with HTF medium-0.5% bovine serum albumin in a 1:2 ratio (Irvine Scientific, Santa Ana, CA) and centrifuged at 400xg for 10 minutes. This sediments the motile and non-motile sperm and most cellular components of semen on the flat platform. The supernatant was then carefully discarded with the exception of about 1 mL fluid (this volume facilitates the calculation of the motile sperm yield), and the tube was placed into a 36°C incubator for 30 minutes. During the incubation, the motile sperm migrated into the supernatant, while the immotile and sluggish sperm and the particulate matter of semen remained at the bottom. After the incubation period, the top 0.5 mL of the supernatant, which is enriched in the motile sperm was withdrawn carefully and used for the FISH and CK-immunocytochemistry experiments.

Sperm Binding to HA: The Sperm Selection Process

During the course of the sperm HA-binding experiments we used HA of bacterial origin, which was permanently applied (Biocoat Inc. Fort Washington, PA.) to plastic Petri dishes as 100-300 μm dots. Sperm have indeed bound to HA, whether they were applied to the HA spot in seminal fluid, or if we used washed sperm (3-5 volume of HTF or other medium, 2000xg for 15 min, room temperature) re-suspended in medium.

Sperm selection from oligospermic men:

Sperm of twelve moderately oligospermic men washed and suspended in HTF were placed over HA spots bonded to Petri dishes (Biocoat Inc., Fort Washington, PA). The sperm progresses to the HA spots, and similarily to the zona binding pattern (**Figure 3.**), the mature sperm attaches to the HA-spot (**Figure 4.**). After incubation for 15 min (twice the period of maximum binding, determined previously), the HA-attached sperm were collected using an ICSI micropipette (**Figure 5.**) and placed onto a microscope slide. Aliquots of the initial sperm suspension and HA-bound sperm were examined after FISH, using centromeric probes for the X, Y and 17 chromosomes.

Sperm selection from normospermic men:

Gradient centrifugation (method described above) treated semen samples from twelve normospermic IVF patients were studied. It is well established that normospermic men have a lower frequency of chromosomal aneuploidy. In order to further enrich the semen samples in non-aneuploid and non-diploid mature sperm, we have passed through the semen an 80% Isolate gradient, according to the standard ICSI sperm preparation protocol at the IVF Laboratory. Thus, we created a “super sperm” fraction. The question was whether the HA selection of sperm would further improve this “super” sample with respect to the decline of sperm with aneuploidies and diploidies. As with the oligospermic samples, the gradient treated sperm washed and suspended

in HTF were placed over HA spots bonded to Petri dishes (Biocoat Inc., PA). After incubation for 15 min (twice the period of maximum binding, determined previously), the HA-attached sperm were collected using an ICSI micropipette and placed onto a microscope slide. Aliquots of the initial sperm suspension and HA-bound sperm, as with the oligospermic experiment above, were examined after FISH, using centromeric probes for the X, Y and 17 chromosomes.

CK Immunocytochemistry of Individual Spermatozoa

The washed sperm were allowed to settle onto polylysine-treated microscope slides overnight in a humidity box at 5°C. The overlying solution was carefully pipetted off and replaced by 1% formalin in phosphate buffer/sucrose (PB-suc) for 20 min at 37°C. After removal of the formalin, the slide was allowed to air dry. After three washings with PB-suc, the spermatozoa were exposed to 3% bovine serum albumin blocking solution in PB-suc at 37°C. After further washing with PB-suc, the sperm were exposed to a 1:1000 dilution of polyclonal anti-CK-B antiserum (Chemicon Co, Temecula, CA). After more PB-suc washes, the slide was processed with a biotinylated second antibody conjugated with horseradish peroxidase. The brown color representing the CK-content of spermatozoa was developed by the ABC method using the DAB reagent (Vector, Burlingame, CA and Sigma, St. Louis, MO). Spermatozoa were characterized as *mature* (no cytoplasmic retention) or *immature* (CK-staining in the head indicating cytoplasmic retention) (**Figure 6.**). For the determination of the proportion of immature spermatozoa, 3 x 100 spermatozoa were assayed in each of the initial, gradient centrifugated and swim-up samples (a total of 12 000 spermatozoa on 40 samples of 20 patients).

Fluorescence In-situ Hybridization on Spermatozoa

Preparation of sperm nuclei for FISH (decondensation)

For decondensation, the sperm slides were warmed to room temperature, and in order to render the sperm chromatin accessible to DNA probes were first treated with 10 mmol/l dithiothreitol (DTT, Sigma) in 0.1 mol/l Tris-HCl, pH 8.0 for 30 min, and then with 10 mmol/l lithium diiodosalicylate (LIS, Sigma) in Tris-HCl for 1-3 h.

DNA probes

The FISH studies were carried out using five probes: (1) a 20 Kb repeated family probe assigned to Xp11-Xp21 region of chromosome X (pXBR-1; Yang *et al.*, 1982); (2) microdissected probes for the Y chromosome (Guan *et al.,* 1996); (3) (3-5) alpha-satellite sequence specific centromeric probes for chromosome 17 (p17H8**;** Waye and Willard, 1986), for chromosome 10 (pα10RP8, Devilee *et al*., 1988), and for chromosome 11 (pLC11A; Waye *et al*., 1987). The DNA probes were labeled indirectly with a hapten conjugated nucleotide (biotin-11-dUTP for chromosome 10, 17 and X probes, or digoxigenin-11-dUTP for chromosome 11, 17 and Y probes) by nick translation (Rigby *et al*., 1977), and added to metaphase chromosome spreads to develop optimal conditions for hybridization.

Fluorescence In-situ Hybridization

In each individuals, the initial and post preparation fractions were examined using both two-color and multicolor FISH. In order to detect the frequency of autosomal disomy and diploidy using chromosome 10 and 11 probes, two-color FISH was utilized (10-11 assay). Since three probe is necessary to study the frequencies of disomy and diploidy in the sex chromosomes, multicolor FISH was performed when chromosome X, Y and 17 were hybridized together (X-Y-17 assay). In the triple-probe FISH experiments, the chromosome 17 was simultaneously detected with both biotin-labeled and digoxigenin-labeled probes, so that its fluorescence profile would be the

combination of two-colors (in our case red and green resulted in orange/yellow). A 12 µl sample of hybridization mixture (50% formamide, 10% dextran sulphate, 2xSSC) containing the probes was denatured at 75-80°C for 8 minutes and applied to the slide specimens previously denatured in 70% formamide, 2xSSC for 8 minutes at 70°C. The hybridization was carried out at 37°C in a moist chamber for 12-14 h. Post-hybridization washes were performed with 50% formamide-2xSSC three times at 42°C and another three times with 0.1xSSC at 60°C in order to remove the excess probe reagents. After a blocking step in 4xSSC/3% bovine serum albumin/0.1% Tween-20 for 30 minutes at 37°C, the sperm nuclei were incubated for 30 min at 37 ^{o}C with avidin-FITC (fluorescence green, Roche Biochemicals, Indianapolis, IN) for biotin-labeled probes, and anti-digoxigenin-rhodamine (fluorescence red) for digoxigenin-labeled probes. The slides were then washed with 4xSSC/0.1%Tween-20 at 42°C three times, and after staining with 4'-6' diamino-2-phenylin-dole (DAPI; Sigma), they were mounted with an antifade solution Vectashield (Vector Laboratories, Burlingame, CA).

Scoring criteria and data collection

For each patient in the gradient centrifugation group one slide (triple-probe FISH) of both the initial and the mature sperm fractions, in the swim-up group two slides (double-probe FISH and triple-probe FISH) of both the initial and the swim-up sperm fractions, in the HA binding group one slide (triple-probe FISH) of both the initial and the selected fractions were scored by, totaling > 4-7000 spermatozoa on each slides (with the exception of the HA-bound fraction, where the number of the available spermatozoa were limited). The overall hybridization efficiency in these experiments was >98%. Scoring was performed on an Olympus AX70 epifluorescence microscope primarily with the triple pass filter for DAPI, FITC and rhodamine (Chroma Technologies Co., Brattleboro, VT), with monochrome filters for DAPI, FITC and rhodamine for improved signal resolution and distinction (**Figure 7.**). Sperm nuclei were scored according to published criteria (Martin and Rademaker, 1995). Since it is hard to interpret, that an absence of a signal indicates nullisomy or failure of hybridization, nullisomies were disregarded, as it is generally accepted

(Egozcue *et al.*, 1997). Nuclei were eliminated from the scoring if they overlapped, or if they displayed no signal due to hybridization failure. In the case of aneuploidy, the presence the sperm tail was confirmed. A spermatozoon was considered disomic when it showed two fluorescent domains of the same color, comparable in size and brightness in the approximately same focal plane, and clearly positioned inside the edge of the sperm head and at least one domain apart (**Figure 8.**). Diploidy was recognized by the presence of two double fluorescence domains with the above criteria (**Figure 8.**). If extra intracellular chromosome signal was observed with the triple bandpass filter, it was always examined with the monochrome filters for DAPI (blue-only), FITC (green-only) and rhodamine (red-only) to confirm the decision of extra chromosome. Aneuploid or diploid spermatozoa were always examined also with a phase-contrast objective in order to verify the presence of the tail and to exclude apparent diploidy in two spermatozoa in close proximity.

For the assessment of aneuploidy frequencies, ~4-10 000 spermatozoa were evaluated in each sample (472 345 sperm nuclei in the 88 fractions of 44 subjects, 330 277 by the author of this thesis).

Estimation of the cumulative incidence of numerical chromosome abnormalities in a sample

Assuming that a higher rate of non-disjunction exist for the sex chromosomes but the autosomes all have a similar non-disjunction rate, the overall risk of aneuploidy can be rougly estimated. Using the mean autosomal and sex chromosome disomy rates for the conservative estimation for the aneuploidy frequencies, the overall disomy frequency in spermatozoa can be calculated. If the incidence of nullisomy is assumed to occur in equivalence with the disomy, then by doubling the overall estimated disomy rate the overall aneuploidy rate can be reached. Adding the diploidy frequency to the estimated aneuploidy frequency will result in the estimated frequency of numeric chromosome abnormalities in the spermatozoa (Downie *et al.*, 1997, Egozcue *et al.*,1997).

Morphologic Analysis and Objective Computerized Morphometry of Individual Spermatozoa

These studies were carried out in two phases: (A) In the first set of experiments, we investiged whether sperm maintain their shape after decondensation and denaturation. (B) In subsequent experiments directed to both FISH and morphometry analysis, we investigated whether sperm shape and numerical chromosomal aberrations are related.

Preparation of slides

Aliquots of liquefied semen (100-200 µl) from eight patients (sperm concentration: 16.9 ± 8.8 million sperm/ml, motility 43.2 ± 7.6%) were diluted with physiologic saline containing 0.3% BSA and 30 mM imidazole (SAIM) up to a final volume of 5-8 ml. We selected low sperm concentration samples as a model for the shape-decondensation studies, because our earlier work demonstrated that these ejaculates are likely to contain a higher proportion of immature sperm with cytoplasmic retention, abnormal morphology and chromosomal aneuploidies (Huszar *et al.*, 1988; Kovanci *et al.*, 2001). The semen samples were centrifuged at 400 x g for 18 minutes at room temperature. After the supernatant was discarded, the pelleted sperm were resuspended in the SAIM solution to a concentration of 10-25 million sperm/ml. Slides were prepared by applying 10 µl of sperm suspension as a smear on a clean glass slides and allowed to air-dry. Slides were fixed in a 3:1 methanol-acetic acid (MAA) solution for 15 minutes and then air-dried for 20 minutes at room temperature. Further, the sperm smears were dehydrated in 70%, 85%, and 100% ethanol for five minutes at each step.

Decondensation and Pre- and Post- Decondensation Imaging of Sperm

Our basic experimental design, including preparation of slides, pre- and post-decondensation imaging of sperm, and morphometric analysis is consisted of the next steps: (a) The fixed slides were stained with one drop of antifade

mounting medium (Vectashield™, Vector) and were observed with a black and white digital camera under a 40x phase-contrast using an Olympus BX51 microscope. Although the antifade-mounting medium is not a dye, via the digital camera it facilitated the recording of consistently dark sperm contours, which are necessary to establish a threshold level for the separation of the sperm from the background for the Metamorph™ assessments. (b) The individual sperm in the fields were digitized using a Sanyo VCB-3524 B/W CCD camera with the Metamorph™ program, and saved. The X, Y coordinates of each digitized microscopic field were noted. (c) Spermatozoa then underwent DNA decondensation. The cover slips that were placed for the initial microscopic observation were carefully removed by rinsing with distilled water. Slides were placed in a humidity chamber and decondensed by flooding with a 10 mM solution of dithiothreitol (DTT, Sigma, St. Louis, MO) in 0.1 M Tris-HCl (pH 8.0), for 25 minutes at room temperature. Subsequently, the DTT was replaced with a 10mM solution of lithium 3,5-diiodosalicylicacid (LIS, Sigma, St. Louis, MO) in 0.1 M Tris-HCl (pH 8.0), and slides were incubated in the dark for 2.5 hours at room temperature. The LIS solution was discarded and the slides were rinsed gently in distilled water.

(d) In control experiments (after we established that decondensation does not alter sperm head size or shape further), the decondensed sperm were treated with denaturation solution [28 ml of 70% formamide and 4 ml of 20xSSC (1x SSC is 0.15 M NaCl plus 0.015 M sodium citrate) in 8 ml of ddwater] and heated to 75°C for 8 to 10 minutes. The denatured slides were immediately cooled to -20°C in 70% EtOH for 2 minutes and then in 100% EtOH for 2 minutes at the same temperature. Slides were allowed to air-dry.

e) The decondensed sperm were stained, as initially, with Vectashield™ antifade medium. Slides were viewed and the same fields that were initially studied were recaptured, and the now decondensed sperm were digitized, saved, and the further morphometric comparisons were carried out with Metamorph™. The imaging step and shape determination were carried out under phase contrast microscopy, because the fluorescence illumination does not allow the visualisation of the sperm contour and the sperm tail (**Figure 9.**).

Morphological classification of the sperm head

In order to evaluate better sperm head shape changes due to decondensation, or to detect differences between normal and abnormal sperm forms, which also reflect cytoplasmic retention and sperm maturity, we classified the sperm cells, prior to Metamorph™ assessment into four groups as normal, intermediate, abnormal, and amorphous (**Figure 9.**).

Computerized morphometry measurements

Calibration was performed by viewing an objective micrometer scale (OB-M 1/100) at 40x magnification and digitizing the image with the Metamorph™ program. The automated, computerized conversion of pixels to µm was 0.29 µm/pixel.

After digitizing the images, Metamorph™ overlay tools were used to delineate the head versus tail regions of individual spermatozoa in order to measure head and tail parameters separately. In the assessment of *head parameters* Metamorph™ recognize the following elements (**Figure 10.**): Area (area of entire object); Perimeter (distance around edge of object, measuring from midpoints of each pixel that defines its border); Long head axis (length of longest diameter through the object); Short head axis (width measured perpendicular to the longest diameter); Shape Factor (a value from 0-1 representing how closely object represents a circle, with 1 being a perfect circle)**.** For the *sperm tail measurements* Metamorph™ distinguishes the Fiber length (the length of an object, assuming that it is a fiber). In addition, in our laboratory, we have developed two sperm parameters that reflect well sperm cellular maturity: Roundness Ratio (short head axis/long head axis) and Tail length / Long head axis that are not standard to the Metamorph™ program, and thus were calculated using Microsoft Excel (Gergely *et al.*, 1999).

Integration of the morphometry and FISH studies

In the second phase, the FISH studies focusing upon the relationship between sperm morphology and aneuploidy were performed by the author of this thesis, using the methods described in details above. Briefly, semen samples from another 15 men with borderline oligospermia (sperm concentration: 21.9 ± 2.8 million sperm/ml, motility 45.6 ± 1.9%) were fixed on slides and decondensed using DTT and LIS solutions, as described above. FISH was performed using centromeric probes for the X, Y, 10, 11, and 17 chromosomes. Non-aneuploid, aneuploid, and diploid sperm were classified, and images were captured and further analyzed with computerized morphometry.

Statistical Analysis

All the values were expressed as mean ± SEM. Differences between the frequency of chromosomal abnormalities in different WHO spermatogram groups were characterized using Odds Ratio (OR) with the confidence interval of 95% (SAS System for Windows v8, SAS Inst. Inc.). Other statistical analyses were performed using SigmaStat 2.0 (Jandel Corporation, San Rafael, CA). Differences in disomy and diploidy frequencies, as well as immature sperm rates were analyzed using the χ^2 analysis of contingency tables. Mann-Whitney Rank Sum Test were used to analyze the motility differences between the fractions. Correlations between the motility, the proportion of immature spermatozoa and aneuploidy frequencies were examined with Pearson correlation test. At the morhometry analysis paired t-tests were performed for sperm parameters before and after decondensation within each group. Percent changes in means of each morphometric parameter before and after decondensation were calculated and tabulated. ANOVA tests were performed in order to test the similarities and differences between the sperm shape categories both before and after decondensation. Level of significance was selected as $p<0.05$.

Photography and Imaging Methods

Photographic images of various sperm preparations were captured via an Olympus Camedia Zoom C-4040 digital camera employing a bracketing technique and uploaded via Olympus Camedia Master 2.5 software (Olympus Optical Company, LTD, Tokyo, Japan). Underexposure was used for fluorescent signals. The images were sized, and processed for printing using the Adobe Photoshop 5.0 program (Adobe Systems Inc., San Jose, CA)

Results

The Population Study: Frequency of Numeric Chromosomal Abnormalities in the Study Patients

Sperm concentration and motility of the study population and the groups of WHO criteria

Neat semen of 32 patients with low sperm concentrations were examined throughout these studies described above. Mean sperm concentration was $18.2 \pm 1.5 \times 10^6$ sperm/mL semen (range 8.0-45.5, all data Mean±SEM, range), the mean motility 49.4±1.65% (30.0-69.2). A total of 200,696 sperm in the 32 initial semen were scored with a mean of 6272 (range 5030-7340) in each man. The samples were grouped according to the WHO definitions of the spermatogram: normozoospermic group (NSp, N=5), asthenozoospermic group (ASp, N=6), oligozoospermic group (OSp, N=12), oligoasthenozoospermic group (OASp, N=9). Characteristics of these groups are summarized in **Table 1**.

X/Y ratio

In all but two of the study subjects the X/Y ratio was above 1.0 with a mean of 1.07±0.01 (0.97-1.32), which suggest a slight, but consistent excess of the X-bearing sperm in the ejaculates. Considerable shift on favour of the X-bearing sperm was observed in one man only, whereas the X/Y ratio was 1.32. There were no differences in X/Y ratio among the spermatogram groups.

X, Y and 17-disomy frequency

In the 32 men, the mean frequency of the sex chromosome disomies (XX, YY and XY) was 0.36%, while that of the disomy 17 was considerably less (0.16%). The distribution of the different sex chromosome disomies is also not uniform (0.1% for YY disomy, 0.12% for XX disomy and 0.14% for XY disomy, $p<0.05$ in each relation). Further, the disomy frequencies show considerable

inter-individual variations, as we observed a range of 0.12-0.83% for the sex-chromosome disomies and 0-0.49% for the disomy 17.

There were no differences in any examined disomy frequencies among the spermatogram groups (**Table 1., Figure 11.**). However, considering only the sperm concentration disregard to the motility, the frequency of sex chromosome disomy was significantly higher in the oligozoospermic men than in normozospermic men with sperm concentration above $20x10^6$ sperm/mL. (0.37% vs. 0.32% OR=1.18, 95% CI= 1.01-1.39, P<0.001) (**Figure 12.**). More importantly, there is a change in the distribution of the sex chromosome disomy frequencies with the low sperm count. In the oligozoospermic subjects the excess of the XY disomy in all sex-chromosome disomies became more prominent, the increase of its frequency was more than two-fold (0.17% vs. 0.08%, OR=1.99, 95% CI=1.48-2.67, P<0.001), i.e. the increase of the sex-chromosome disomies in the oligozoospermic men is caused by the elevation of the incidence of the XY disomy (**Figure 12.**).

Diploidy frequency

Diploidy frequencies showed an even more considerable interindividual variation with a mean of 0.59% (range 0.03%-1.94%), higher than it was observed in a large scale analysis of normozoospermic population (0.24-0,32%, Downie, 1997, Egozcue, 1997). The highest diploidy frequency occurred in the OASp group (0.96%, range 0.13-1.94%), the increase was statistically highly significant when compared to the average of the other groups (OR=2.39, 95% CI=2.13-2.69, P<0.001) (**Figure 13.**). If the diploidy frequencies are analyzed according to the sex chromosomes components in the oligozoospermic (N=21) and normozoospermic (N=11) patients, the increased frequency of the XX and YY diploids with the origin of meiosis II. can be observed in the oligozoospermic men (0.35% vs. 0.26%, OR=1.39, 95% CI=1.17-1.65, P<0.001), but there is no increase in the frequency of diploids with the origin of meiosis I. (0.24% vs. 0.24%, NS) (**Figure 13.**).

Unusually high diploidy frequencies exceeding the rate of 0.5% were found in 10 men, and 8 of them were oligozoospermic. In all but one oligozoospermic men with elevated frequencies of diploidy the high incidence of spermatozoa with extra chromosome set occurred due to the defect of meiosis II. (XX and YY diploids). In one the two normospermic subject with high frequency of diploidy the excess of M1 diploids, while in the other the excess of the M2 diploids were observed.

It is also of interest, that the higher the total frequency of diploid sperm the greater the difference between the proportion of M1 and M2 diploids was. Among the ten men with >0.5% diploidy rate there were 7 (70%) with more than a 3-fold difference between M1 and M2 diploids (M1>M2 in 2 cases, M1<M2 in 5), while there was only one such patient (M1>M2) among the 22 men (4.7%) with <0.5% diploidy rate. Using Pearson correlation analysis we confirmed a very high correlation ($r=0.596$; $P<0.0005$) between the total diploidy rate and the ratios of M1/M2 (or M2/M1, if M1/M2<1) diploids.

Estimated frequency of numerical chromosome abnormalities

The mean of the estimated frequency of numeric chromosome aberrations of our study population was 8.3% (0.4-22.7%), with considerable inter-individual differences (**Figure 14.**), but without differences among the spermatogram groups (**Figure 15.**).

Relationship between sperm concentration, motility and the results of FISH examinations

The mean cumulative 17 and sex chromosome disomy frequency was not elevated in oligospermic men (N=21) than in those of with sperm count higher then 20×10^6 sperm/mL (N=11) (0.53 vs. 0.49, NS), in contrast with the increased frequency of diploidy in the oligozoospermic subjects, as described above. Nevertheless, correlation was not found between the diploidy frequency and the sperm count. Similarly, in the cases of decreased motility (motility<50%, N=15) there was no difference in the mean cumulative 17 and sex chromosome disomy frequency as compared to the group of normal

motility (N=17) (0.54% vs. 0.48%, NS), but the diploidy frequency increased significantly (0.77% vs. 0.38%, OR=2.03, 95% CI=1.80-2.30, $P<0.001$), although correlation was also not found between the motility and diploidy frequency.

The Gradiens Centrifugation Study: Frequency of Immature Sperm and Numerical Chromosomal Aberrations in the Initial Semen and the Pellet

Sperm concentration, motility and cellular immaturity in the semen and 80% Percoll pellet sperm fractions

We have studied ten oligospermic men (conc: 13.3±1.4 x 10^6 sperm/mL, motility: 50.3±3.4%, SEM, **Table 2.**). The selection of this patient population was based on previous work in which we established a relationship among cytoplasmic retention, lack of HspA2 expression and sperm immaturity. In oligospermic vs. normospermic semen samples the proportion of immature sperm was higher.

The proportion of mature and immature sperm in the study samples was determined by immunocytochemistry which identifies immature spermatozoa with cytoplasmic retention (**Figure 6.**). Accordingly, the proportion of immature sperm in the initial semen and pellet fractions was 45.4±3.4% vs. 26.6%±2.2 (medians: 48.2% vs. 25%, $P<0.001$, N=10 sample pairs, **Table 2.**).

X/Y ratios

The X/Y ratios were somewhat higher in the pellet fractions vs. semen, but the differences did not reach significance (1.08 vs. 1.05, ranges: 1.0-1.13 vs. 0.98-1.08, medians: 1.05 vs. 1.08, respectively, **Table 3.**).

Disomy frequencies in the semen and 80% Percoll pellet fractions

For the assessment of aneuploidy frequencies we evaluated about 7000 sperm in each semen and 80% Percoll pellet sperm fractions (142,068 sperm nuclei in the 20 fractions).

There were substantial differences in aneuploidy frequencies between the sperm nuclei arising from semen and those arising from pellet fractions. Each individual had significant differences (using the stricter level of $P \leq 0.02$) between the semen and the pellet fractions in the detected disomy categories (**Table 3.**). Among the 33 significantly different comparisons within the ten individuals, 14 comparisons were at the level of $P<0.001$, all of these differences occurred among the disomy comparisons. In all subjects there were significantly different comparisons between the semen and pellet sperm fractions in the aggregate disomy frequencies. With respect to wide variations in disomy frequencies within the semen among the subjects, there was only one of note: XY disomies (0.03-0.68%, subjects #8 and #1).

The cumulative data of the ten subjects (**Table 3.**) indicate that the disomy frequencies are significantly lower in the 80% Percoll pellet fractions compared to the semen sperm fractions ($P<0.001$, N= 71,385 and 70,683 sperm) in all disomy categories. Analysis of the aggregate disomy and diploidy categories illustrates well the differences in numeric chromosome aberration frequencies in sperm from semen vs. the 80% Percoll pellet (**Table 3.**). There are two major findings: First, there is a significant decline in aggregate disomy (0.54 vs. 0.17%), diploidy: (0.26 vs. 014%) ($P<0.001$ in both comparisons). Second, the man-to-man variations in disomy frequencies are also diminished in the 80% Percoll pellet fractions. The distribution of the values is closer and the distribution of immature sperm in the semen vs. Percoll pellet fractions also follows the same pattern. These findings are in line with the enhancement of mature sperm in the 80% Percoll pellet fraction from which the immature sperm with various degrees of cytoplasmic retention are eliminated. The 80% Percoll pellet fraction is more homogeneous from the

point of view of maturity, and the aneuploidy frequencies are similar to those reported in normal men.

The decline in the ten sample pairs was more distinct in the comparison of disomies (3.2-fold, range: 2.5-5.1) than of diploidies (1.9-fold, range: 1.5 – 3.0). This indicates that disomies are more related to the elimination of immature sperm from the semen than are diploidies, and gradient density centrifugation eliminates primarily sperm with aneuploidy.

Diploidy frequencies in the semen and 80% Percoll pellet fractions

Relatively narrow range of 0.2-0.3% in the initial samples of diploidy frequencies was observed with the exception of two subjects (0.01-0.62%, subjects #10 and 5). Although after gradient treatment there was a significant decline in the mean diploidy frequency of the ten men, the diploidy frequencies in the pellet fractions were different only in subjects #6 and 7 (**Table 3.**).

The proportion of immature sperm and sperm concentrations in the samples

We have shown previously that the biochemical parameters of sperm maturity (CK activity, chaperone ratio and proportion of mature/immature sperm) are independent form the sperm concentrations in the samples. The data of the men in this study, although the group is small, well supports this observation.

If the ten semen samples are divided according to the five lower and five higher sperm concentrations, the group with the lower sperm concentrations (subjects #1,2,3,7 and 10, **Table 2.**) have an average sperm concentration of 10±0.6 million sperm/ml, whereas in the other 5 men (subjects 4,5,6,8 and 9, **Table 2.**) the average is 16.5±1.2 million sperm/ml/, closer to the 20 million sperm/ml normospermic range. However, the proportion of immature sperm is higher in the group with 16.5 versus 10 million sperm/ml (49.8±4.5% vs. 41.4±5.2%, **Table 2.**). This inverse relationship is also evident in the two cases of the men with the lowest and highest sperm concentrations (subjects #2 vs. #5, sperm concentrations of 8 and 19 million sperm/ml), in whom the

proportions of immature sperm are 37% and 59%, respectively. Finally, the lack of a consistent relationship between sperm maturity and sperm concentrations is best demonstrated by the 3 men with sperm concentrations of 10 million sperm/ml (subjects #1,7 and 10). The proportions of immature sperm in these 3 semen samples are 42%, 24% and 55%, respectively, bridging across the entire range of the ten men.

<u>The Swim-up Study: Frequency of Immature Sperm and Numerical Chromosomal Aberrations in the Initial Semen and the Swim-up fraction</u>

Sperm concentration, motility and cellular maturity in semen and swim-up fractions

We studied ten men with mean sperm concentrations of $20.3 \pm 3.8 \times 10^6$ sperm/mL semen (range 8.9-45.5, **Table 4.**, all data Mean±SEM). We used 3 samples each from the <10 and the 10-20 million sperm/mL concentration ranges**,** two samples from the 20-30, and two samples from the > 30 million sperm/mL ranges. The selection of these groups reflects our intent to include patients with differing proportions of mature and diminished maturity sperm, and thus different levels of chromosomal aneuploidies. *Sperm motility* (motile sperm are defined as curvilinear velocity >7.0 µm/sec) in the swim-up fractions vs. the initial semen was significantly higher (74.2±6.3% vs. 45.2±2.4%, P=0.005, N=10 pairs). The motile sperm concentration in the ten initial samples was $8.8 \pm 1.2 \times 10^6$ motile sperm/mL. The efficiency of the swim-up procedure was measured by the *motile sperm yield* (proportion of motile sperm from the initial semen recovered in the swim-up fraction). Motile sperm yield in this group was 44% (**Table 4.**). Along with increases in the percentage of motile sperm, the proportion of sperm with diminished maturity (reflected by CK-immunostaining, which highlights cytoplasmic retention) declined in the swim-up fractions, as compared to initial semen (29.6±3.7% vs. 44.4±4.3%, P<0.001) with a reduction rate of 1.5.

X/Y ratios

In the assessment of two independent aliquots from each sperm fraction, we used probes for the X, Y and 17 chromosomes, and for the 10 and 11 chromosomes, scoring approximately 105,000 and 103,000 sperm nuclei, respectively (**Table 5.**). Although the X/Y ratios were close to 1:1, the swim-up fractions showed ratios consistently lower than those of initial semen fractions, with the exception of samples from patient #1. Regarding the increase in the proportion of Y-bearing sperm, significance was reached only in the samples of patients #9 and 10. However, after totaling counts, the group of ten patients showed a difference at the $P<0.001$ level (means: 1.09 versus 1.04).

Disomy frequencies in the semen and swim-up sperm fractions

A variation in disomy frequencies was observed among the patients, as well as among the 5 chromosomes evaluated (**Table 5.**). The mean aggregate disomy frequencies for X, Y and 17 chromosomes in initial semen and swim-up fractions were 0.32% and 0.21%, a 1.5x reduction ($P<0.001$). The mean aggregate disomy frequencies with probes for autosomes 10 and 11 were very similar to those for X, Y, and 17 at 0.29% and 0.21%, respectively, approximately a 1.4-fold reduction ($P<0.01$). The mean frequencies of disomies were different within patients. For instance, the ranges of X, Y and 17 disomies were 0.12%-0.64% in the initial semen and 0.08%-0.33% in the swim-up fractions. It is of interest that proportions of the different sex chromosomal disomies were not evenly distributed among the men. The prevailing disomy, in the initial semen, is XY in men #1,3 and 5, while X disomy is predominant in men #4, 8, and 10, and Y disomy is most prevalent in patient #9.

There was no relationship between sperm concentrations and frequencies of aneuploidies. For instance, the 5 men in the upper sperm concentration range (#2,3,5,6,and 10) and the 5 men in the lower sperm concentration range (#1,4,7,8 and 9), had disomy frequencies of 0.33% (range: 0.12-0.64) and 0.31% (range: 0.25-0.39). This finding is in agreement with the previous and

the gradient centrifugation studies, which demonstrated that both the proportions of immature sperm and the frequencies of aneuploidies were independent from sperm concentrations in semen samples (Huszar *et al.,* 1988b, 1990c, 1992). However, in line with the association between diminished sperm maturity and the frequencies of aneuploidies, there was a moderate correlation between the incidences of sperm with cytoplasmic retention and disomies in the initial semen and in the swim-up fractions (r=0.46, P<0.05, N=20).

Although disomy frequencies showed a declining trend in the swim-up fractions, these changes did not reach statistically significant levels for any of the five chromosomes investigated, except in one patient (#6). In this patient, the total frequency of 17, X and Y chromosome disomies was significantly higher in semen and lower in swim-up fraction (0.64% vs. 0.34%, P<0.05). Considering the data from all five probes following the swim-up procedure, the overall disomy frequencies decreased from 0.61% (range 0.34-1.13%) to 0.42% (range 0.21-0.59), with a reduction rate of 1.5x. In spite of variations in aneuploidy frequencies among the chromosomes studied, the declines of the various disomies were proportional, as indicated by the correlations between aneuploidy frequencies in the swim-up vs. initial semen fractions for Y disomy (r=0.75, P<0.01), 11 disomy (r=0.76, P=0.01), 17 disomy (r=0.9, P<0.001) and all 5 disomies (r=0.75, P=0.01). Conversely, the motile sperm yield was related to the clearance factor of eliminating disomies from the swim-up fractions (r=0.65, P<0.05, N=10).

Diploidy frequencies in the semen and swim-up fractions

The diploidy frequencies and reductions in the swim-up fractions showed outcomes quite different from those of the disomies (**Table 6.**). With respect to the three-color FISH approach, there was a significant reduction in six of the ten patients, with an approximately three-fold decline in diploidy rates for the group of ten patients. Similarly, with probes 10 and 11, there was a significant decline in six of the ten patients. Thus, the decline in diploidy frequencies was statistically significant in eight of the ten samples at the level of P<0.01. Five of these men showed declines with both the two and three-color FISH probes,

whereas diploidy frequencies with the X, Y and 17 probes were reduced in patient 2, and with the two-color FISH in patient 1. There was also considerable inter-individual variations, with a mean 0.65% (range 0.13-1.76%) in the initial semen, which decreased significantly to 0.24% (0.03-0.79%) in the swim-up fractions (P<0.001). This decline represents a 2.7-fold reduction rate for diploidies.

Diploidy frequencies with three-color and two-color FISH, which represent independent measures in the various samples, showed a correlation of r=0.99 (P<0.001, N=10) for the initial semen fractions. In the swim-up fractions, the correlation was a comparable r=0.89 (P<0.001, N=10). The inter-assay correlation of the diploidy frequencies between the three-color and two-color FISH was also a comparable r=0.99 (P<0.001, N=20). These relationships were valid for diploidies detected by all chromosome probes and in all men, as indicated by the close correlation between the reductions of chromosomal aberrations in the semen and swim-up fractions (r= 0.93, P<0.001). These data suggest a general clearance factor specific for all diploid sperm.

Association between diploidy and the number of tails in the semen and swim-up fractions

During the phase contrast microscope examination of the diploid spermatozoa we observed a difference of the distribution of diploid sperm with one tail and with two tails (**Figures 16. and 17.**). Although there was a decline in diploidies in swim-up fractions as compared with semen, whether sperm were double or single tailed; the reduction rates were 7.1:1 and 1.7:1 respectively (P<0.001, N=10). Considering individual men, the double-tailed diploid sperm declined after swim-up in nine of the ten men, whereas the one tailed diploid decreased in only one men (**Table 6.**).

The Morphology Study: Maintenance of Sperm Shape After the Decondensation and Relationship between Sperm Morphometry and Chromosome Content

Conservation of sperm shape before and after decondensation

(1) Sperm shape classification

Initially, prior to the Metamorph™ studies, we performed sperm classification based on microscopic observation of the sperm shape. The visual determination of the sperm shape was relevant from the point of view of sperm selection for ICSI. We distinguished the following sperm types (**Figure 9.**).

(i) Sperm that most resembled "normal-shaped" sperm, those with oval, symmetrical heads, symmetrical tail insertion and long tails, were classified as *normal* (N=115); (ii) *Intermediate* sperm did not satisfy the descriptions of normal or abnormal sperm (often elongated or slightly rounded heads, N=115); (iii) Sperm with large, round, or asymmetrical heads and evidence of cytoplasmic retention were classified as *abnormal* (N=115) (iv) Sperm with grossly asymmetric heads were considered *amorphus* sperm (N=50).

In evaluating the effect of decondensation on sperm shape, we digitized 307 total slide fields that represented the four categories of spermatozoa in the 8 men studied. We were able to re-localize 277 fields (90%) after the decondensation step.

(2) Conservation of morphological attributes before and after decondensation

In order to examine whether sperm with normal or abnormal shape would be altered differently by the decondensation/denaturation treatments, sperm images within each of the normal, intermediate, abnormal and amorphus categories were examined for similarities and differences between the images both before and after decondensation. We detected no shape changes in any of the sperm shape categories before and after decondensation (**Figure 9.**). The overall shape of the sperm head and the degree of increase after

decondensation for total area, perimeter, long axis, and short axis were all maintained across the board. An exception was the larger increase in the head area of the amorphus category (**Table 7.**).

Our initial classification of the spermatozoa into four sperm categories was confirmed with the comparison of sperm dimensions before and after decondensation. After performing ANOVA and post hoc Dunn's tests, significant differences were seen in nearly all pairwise comparisons of mean initial vs. decondensed values of the normal, intermediate, abnormal, and amorphus sperm groups (**Table 8**, P<0.001).

FISH and morphometry: relationship between sperm shape and chromosome content

Once we had demonstrated that sperm shape is preserved after decondensation and denaturation, we could then examine the relationship between sperm shape and aneuploidy, and evaluate the role of sperm shape in sperm selection for ICSI. In this second phase of the study, we evaluated 1073 individual sperm from 15 men: 900 non-aneuploid, 110 aneuploid and 63 diploid sperm, using centromeric FISH probes for the X, Y, 10, 11, and 17 chromosomes.

In attempting to evaluate whether sperm shape may be used for selection of non-aneuploid sperm, we *first* sorted and divided the 900 non-aneuploid sperm into three groups according to morphometrical attributes of the sperm head, and classified them into "small head", " intermediate head", and "large head" groups. *Second,* we sorted the 110 aneuploid and 63 diploid sperm according to the size categories established in the non-aneuploid sperm groups, and determined the frequencies of aneuploid and diploid sperm within the three head size groups (**Table 9.**).

Aneuploidies and diploidies were present within all three groups. The frequency of chromosomal aberrations positively correlated with sperm head size, as size reflects cytoplasmic retention and immaturity. The frequencies of chromosomal aneuploidies were also related to the other sperm head

parameters, indicating that study of any sperm head parameter is relevant to the relationship between sperm shape and disomies or diploidies (**Table 9.**). When we asked the question, “How many of the disomic or diploidic sperm will fall into the most normal sperm category, within the lowest tertile of the 900 non-aneuploid sperm”, we found that sperm of any head size or shape may have chromosomal abberations. Furthermore, also about 18% of sperm with disomy and 2% with diploidy of the 173 sperm selected for this analysis fell into the category of the normal 300 sperm within the lowest tertile, whether we considered one or more of the four basic morphometrical parameters.

In another approach (**Table 10.**), we sorted and then classified the same 1073 sperm according to their shape characteristics of normal (N=326), intermediate (N=337), abnormal (N=357), and amorphus (N=53). Again, aneuploid and diploid sperm were present in all four groups with an increasing frequency of 7%, 12%, 21% and 70%, in line with the severity of the sperm shape abnormality.

Using both approaches, our results support a relationship between abnormal sperm shape and disomies/diploidies, as the combined rates of diploidy and disomy increased within each morphological category from normal to amorphus, reflecting the direction of diminished sperm maturation. According to our results, diploidy frequency showed a distinct increase from normal to amorphus sperm shape categories, being highest in the amorphus class. Disomy rates also increased relative to sperm shape aberrance. Moreover, with the exception of the amorphus class, normal, intermediate and even the abnormal sperm shape categories showed similar disomy frequencies. These data are similar to those in **Table 9.**, in which the sperm with normal head dimensions, considering any of the parameters, exhibited an about 20% aneuploidy and diploidy frequency. The data thus further confirmed that shape assessment is an unreliable method for selection of non-aneuploid sperm.

The HA-binding Study: Frequency of Numerical Chromosomal Aberrations in the Initial and and in the HA-bound Sperm

Washed sperm of twelve moderately oligospermic men (sperm conc.±SEM: 20.6±1.7x10^6/ml, motility: 54.1±2.5%) and gradient treated sperm of twelve normospermic men (sperm conc.: 121.3±21.4x10^6/ml, motility: 59.5±4.9%) were studied. With respect to the initial samples, in each man we analyzed a mean 4,500 sperm, or 110,000 sperm in the 24 men. In the HA-bound fractions we were limited with the sperm numbers because the selection was carried out with the ICSI pipette. In the 12 oligospermic men, we evaluated a mean of 753 sperm (range: 224-1142, total 9036), and in the 12 normospermic men a mean of 972 sperm (range: 373-1955 sperm, total 11,666) were scored.

X/Y ratio

Although a slight decrease of the X/Y ratio was observed in both study groups, this decline reached statistically difference only in the cumulative data of the normospermic group (1.13 in the initial versus 1.05 in the HA-bound sperm, **Tables 11. and 12.**), which sperm was pretreated with gradient centrifugation. This finding may be explained by the observation from the Gradient Centrifugation Study above, that the gradient centrifugation enrich slightly X-bearing spermatozoa. Thus, initial sperm in this group may not reflect physiologic distribution of X and Y spermatozoa. Nevertheless, these differences are not clinically significant, and we suggest, that HA-binding capacity is not affected by the sex-chromosome component of the spermatozoon.

Disomy frequencies in the initial and HA-bound sperm

Regarding the obvious technical limitation of the study design to score sufficient number of spermatozoa to reach statistically significant differences in each individuals, the cumulative data of both groups were analysed only. In the oligospermic group (**Table 11.**), the cumulative disomy and diploidy frequencies declined in the HA-bound fractions with a factor of 5-fold for the

17 disomy, 4-fold for the sex chromosome disomy. In the normospermic 80% Isolate “super” sperm group, as expected, the frequency of disomies and diploidies were lower compared to the oligospermic group (**Table 12.**). However, after HA selection there still was a two-fold decline in 17 disomy (NS), 4-fold decline in sex chromosome disomies (P<0.001). Whether the HA-bound sperm were selected from the oligospermic or the normospermic groups, the frequency of aneuploidies were in the very low range of < 0.10%. Thus, HA selection eliminated sperm with disomy efficiently reaching a low level of frequency which is even less than that of characteristic for the normal men. Interestingly, the approximately 4-4.5-fold decline of sex chromosome disomies is consistent with the increase of such chromosomal aberrations in ICSI children.

Diploidy frequencies in the initial and HA-bound sperm

Diploidy frequencies also declined in the HA fractions of both groups (**Tables 11. and 12.**), from 0.81% to 0.13% in the oligospermic group and from 0.58% to 0.10% in the normospermic group, representing a consistent 6.1-fold reduction (P<0.001) for diploid sperm frequency regardless to the initial incidence.

Discussion

In the 32 men involved in these studies the frequencies of sperm with 17 and sex chromosome disomies in the neat semen (0.16% and 0.36%) correlate with the literature data in their magnitude, although our rates are in the upper range of the reported disomy frequencies of normospermic men (Downie *et al.*, 1997; Egozcue *et al.*, 1997; Shi and Matrin, 2000). With respect to the fact, that the validity of our methodology was confirmed by a very high correlation between the detected diploidy frequencies using two- and three-color FISH in ten men, the underlying cause of the slight increase should be the sampling difference, namely that we examined men with low sperm count. Based on the detected disomy and diploidy frequencies, the estimated mean frequency of numerical chromosome abnormalities in these men was 8.3%, which characterizes the risk of choosing a single spermatozoon for ICSI from a non-selected sperm population of an infertile patient with low sperm count. In some cases, due to the considerable inter-individual variations the aneuploidy or diploidy frequencies can be significantly higher (**Figure 14.**). It is also clearly shown by our data that the WHO definitions, such as the olygozoospermy below the sperm count of $20x10^6$ sperm/mL do not characterize the risk of elevated aneuploidy frequency. Although the sex chromosome disomy frequency alone was found to be elevated in the oligospermic men, there was not any correlation between the sperm concentration and the frequency of any numerical chromosome abnormalities in the examined sperm count range. In contrast, there is a strong relationship between the aneuploidy frequency and the rate of immature sperm (Kovanci *et al.*, 2001), and there is an inverse relation between the sperm count and immaturity. The latter was confirmed also in a Hungarian population (Gergely *et al.*, 1999b). In agreement with the finding that in 25-30% of normospermic men, especially in those with moderately decreased sperm concentration (20-$30x10^6$ sperm/mL) the increased rate of immature spermatozoa can be detected (Ergur *et al.*, 2002; Huang *et al.*, 1999; Huszar *et al.*, 1988a), slightly elevated risk of aneuploidy appears in our material characterized with the sperm concentration less than $50x10^6$ sperm/mL.

For any given chromosome, the frequency of disomy observed in sperm nuclei using FISH is always higher than the incidence of trisomic individuals in the populations, estimated from epidemiologic studies (Egozcue *et al.*, 1997). However, in the case of XXY Klinefelter`s syndrome and XYY males, the differences are not as considerable, probably because most of these cases are viable. For XXY patients, the possibility of survival to birth is relatively low (55.3%), while for XYY males it is close to 100%. XXYs are of maternal and paternal origin in similar proportions, but XYY males are obviously of paternal origin (Jacobs and Hassold, 1995). The concern about the increased incidence of viable sex chromosome aberrations in relation to the ICSI procedure has been raised earlier (Int`Veld *et al.*, 1995; Int`Veld *et al.*, 1997). Later, the increased risk of the transmission of de-novo chromosomal abnormalities, mostly sex chromosomal aneuploidies was confirmed on large ICSI populations (Bonduelle *et al.*, 1998, 2002). In our FISH studies on large number spermatozoa patients the most frequent disomy was the disomy XY, and a more than two-fold increase of the disomy XY was detected in oligozoospermic men (0.17% vs. 0.08%). This finding is consistent with the observation that there is an increase in sex chromosome aberrations in ICSI children with a major contribution of Klinefelter`s syndrome (XXY).

Considering the clinical aspects of male infertility, an association between oligospermy and synaptic anomalies was already suggested (Vendrell *et al.*,1999; Aran *et al.*, 1999). Indeed, it has been shown that many 46,XY males are affected by synaptic anomalies during meiosis, resulting in various degrees of meiotic arrest and, consequently, aneuploid and diploid sperm (Egozcue *et al.* 1983; Martin *et al.*, 1996). It is also known that trisomies were found in about 5% of clinically evidenced pregnancies. The trisomies arise from disomic gametes originating in non-disjunction during meiosis. Trisomies of paternal origin are rare in newborns and in abortion materials, but the low rate may just reflect a more strict selection process against zygotes with paternally derived trisomies. The variability of the rate of non-disjunction among men is well demonstrated by the wide range of fluctuations in XY disomy frequencies (0%-0.66%) among the 32 subjects of our studies. Another issue is the apparent fertilization by sperm with aneuploidies in the

paradigms of conventional intercourse or in vitro fertilization. It appears that there is a low incidence of sperm with “intermediate” maturity in which the synchrony among HspA2 expression, cytoplasmic retention and membrane remodeling are lost, but completion of some of these cellular events is advanced enough to retain the ability of binding and penetration of the zona pellucida by the incompletely mature sperm which carry aneuploid chromosomes (Ergur *et al.*, 2002).

In the range of sperm concentration examined by us (<50x10^6 sperm/mL) there was not any correlation observed between the disomy 17 frequency and the sperm count. However, the incidence of the disomy 17 was in the upper range of the frequencies reported by other laboratories, which also suggests that aneuploidy frequencies might be elevated already in case of moderately decreased sperm count and the application of WHO criteria in spermatogram does not give information for the estimation of the risk. Nevertheless, the autosomal aneuploidy frequencies in our study subjects do not mean clinically significant increase risk, which supports the fact that no increase of the autosomal trisomies was observed in ICSI children (Bonduelle *et al.*, 1998, 2002b).

Fetal triploidy is a frequent cause of early pregnancy loss, it occurs in 12-13% of spontaneous abortions (Eiben, 1990), mostly of paternal origin (Aran *et al.*, 1999; Egozcue *et al.*, 2000; Rubio *et al.*, 1999; Zaragoza *et al.*, 2000). Diploid spermatozoa is the most common anomaly to all chromosome aberrations related to human male infertility. The frequency of triploids in humans is unusually high, the incidence of it was estimated at 1% of clinically recognized pregnancies, which corresponds to a frequency close to 9% for all conceptions (Jacobs, 1992). The frequency of triploids of 4.5% in binucleated embryos (to exlude dispermy) obtained by ICSI was found (Munne and Cohen, 1998).

Although the frequency of diploidy in sperm exceeds the individual frequencies of disomies, but in general the diploids are responsible only for a minor proportion of the cumulative frequency of numeric chromosomal aberrations. Increased frequencies of diploid spermatozoa up to 10% are

common findings in patients with meiotic disorders (Bernardini *et al.*, 1997; Aran *et al.*, 1999; Pang *et al.*, 1999), in some cases may reach impressive proportions (Pieters *et al.*, 1998; Int`Veld *et al.*, 1997; Devillard *et al.*, 2002) or even 100% (Aviram-Goldring *et al.*, 1997; Bergere *et al.*, 1998). We observed significant increase in diploidy frequency in samples with reduced motility, especially with oligoasthenozoospermy. This finding supports the relationship between the diploids and meiotic defects, and it also suggests a reduced motility of sperm with extra chromosome set.

We made some novel observation with regard to the diploid spermatozoa. Defective chromosome separation of either meiosis I. or meiosis II. can be responsible for the production of diploid spermatozoa. In sperm with higher than average diploidy rates, the dominance of either the M1 diploids or M2 diploids can always be observed. This suggests, that in the majority of the cases with moderately elevated frequencies of diploid spermatozoa, chromosomal non-separation during meiosis I. and defective nuclear cleavage during meiosis II occur separately. However, in most cases of decreased sperm concentration and increased frequencies of diploids the failure of the meiosis II. is responsible for the formation of sperm with extra chromosome set, since frequency of diploids of M2 origin are elevated. Consequently, the failure of the nuclear cleavage during meiosis II. will result not only in increased number of diploid spermatozoa, but also in decreased sperm count.

We studied the frequencies of numerical chromosomal aberrations and incidences of sperm with diminished maturity in both initial semen and sperm fractions prepared with conventional methods widely used in assisted reproduction. We were also interested in comparing the efficacy of gradient centrifugation and swim-up approaches in the elimination of sperm with diminished maturity and chromosome abnormalities.

We optimized the experimental design of the gradient centrifugation and swim-up studies based on experience with previous experiments: (i) In general, men having oligospermy or low normal sperm concentrations have a higher proportion of sperm with diminished maturity. Thus, we primarily used semen samples with sperm concentrations around the 20 million sperm/mL

range. However, since swim-up separation required a sufficient number of motile sperm, this precluded the use of severely oligospermic and/or asthenospermic samples. (ii) In the swim-up study we monitored "motile sperm yield," a parameter which reflects recovery of motile sperm in the swim-up fraction. The motile sperm yield is related to the clearance rate of disomic sperm ($r=0.65$, $P< 0.001$, $N=10$). (iii) In order to increase the internal control of the methodology, in the swim-up study we used 5 different chromosomal probes on two independent slides: X, Y and 17 as three-color FISH, and 10 and 11 as two-color FISH (10,000 sperm in each fraction and 20,000 sperm in each man evaluated). This way, our results were further validated by the correlation of $r>0.9$ between the comparable data with three-color and two-color FISH. (iv) Finally, in addition to monitoring chromosomal aberrations, we also assessed the proportions of immature sperm both in initial semen and in the prepared fractions. Sperm with diminished maturity were identified by the presence of retained cytoplasm as highlighted by CK-immunocytochemistry. As it was reported earlier, there is a relationship between the rate of immature sperm and the incidence of aneuploidy (Kovanci *et al.*, 2001). In the swim-up study the proportions of sperm with diminished maturity and with chromosomal aberrations also showed a correlation ($r=0.46$, $P<0.05$, $N=20$). This correlation was not as close as that for the gradient centrifugation study ($r=0.7$, $P<0.001$, $N=20$, Kovanci *et al.*, 2001). This difference indicates that sperm motility is less discriminatory than sperm density and buoyancy, which are the bases of the gradient fractionation. This supports our further finding, that sperm motility is less related than the cellular maturity to the incidence of aneuploidy.

In comparing the efficiency of the swim-up and gradient methods in eliminating aneuploid and diploid sperm, one has to consider whether, in spite of moderate differences in actual sperm concentrations and motilities (20.3 ± 3.8 vs. 13.3 ± 1.4 million sperm/mL, 45.2 ± 2.4 vs. 50.3 ± 3.4%, respectively), the two study populations are similar. We suggest that this is the case, and this notion is supported by three factors: 1) *The motile sperm concentrations*, which are the important determinants regarding the efficiency of sperm separation by either swim-up or gradient centrifugation, are similar in

the two groups (8.8±1.6 vs. 6.7±0.8 million sperm/mL, P>0.2; medians: 6.0 and 6.6, respectively). 2) *The proportions of diminished maturity sperm*, which are related to the frequencies of aneuploidies, are virtually identical in the initial semen samples (45.5±3.6% vs. 44.4±4.3%). 3) The third argument, that *sperm concentrations are not a reliable measure of sperm quality and maturity,* is based on the various biochemical marker experiments, and is evident in the both study (See inconsistencies between sperm concentrations and % diminished maturity sperm, **Tables 2. and 4.**).

The relationships between diminished sperm maturity and chromosomal aneuploidies may be explained by the roles of the HspA2 chaperone in both supporting meiosis as a component of the synaptonemal complex, and in facilitating cellular movement of proteins, a function which, we believe, may involve cytoplasmic extrusion (Eddy, 1999; Huszar *et al.,* 2000). Thus, sperm with diminished maturity and low HspA2 expression level may show both increased frequencies of meiotic errors, causing numerical chromosomal aberrations and cytoplasmic retention, which in turn affect shape and density of sperm (due to the fact that the excess cytoplasm is lighter than DNA and the nuclear components). Relationships between synaptic anomalies during meiosis, chromosomal abnormalities, and male infertility were recognized earlier (Egozcue *et al*., 1983; Vendrell *et al*., 1999).

Our data indicate that both the gradient centrifugation and the swim-up step eliminates sperm with disomies, diploidies, and sperm with diminished maturity with an overall significant reduction at the level of P<0.001. However, the results were not consistent. In the gradient centrifugation study all the ten patients showed a decline in disomy frequencies, and only two in diploidy frequencies in the Percoll pellet vs. semen sperm fractions (Kovanci *et al*., 2001). In the ten patients in the swim-up study there were seven that reached significant declines in proportions of immature sperm, and only one in the proportion of disomic sperm (**Tables 4. and 5.**). Regarding diploidies, six of the ten men reached a significant reduction in the swim-up fraction, and this pattern was similar whether we considered data from three-color or two-color FISH. (**Table 6.**). Also, the difference between the gradient centrifugation and

swim-up study approach was evident considering the clearance rates for disomic sperm of 3.2-fold and 1.5-1.4-fold (the three- and two-color FISH), respectively. It is of further interest that diploid sperm show much higher rates of clearance with swim-up (2.7 fold) as compared to those for disomic sperm (1.5-1.4 fold). In line with our swim-up results, in a recent study, utilizing swim-up fractionation of sperm, a reduction in frequencies of disomies and diploidies with a clearance rate of approximately 1.5-fold, was reported (Ong *et al.*, 2002).

The higher efficiency of gradient centrifugation vs. swim-up for the aneuploidies is due to the fact that, in the Percoll gradient, sperm with cytoplasmic retention do not reach the pellet, whereas, in swim-up fractionation, the differing swimming efficiency of the mature vs. diminished maturity sperm (particularly diploid sperm), by virtue of sperm head shape and swimming pattern, is a likely contributory factor. The >4-fold lower rate of reduction of single-tailed compared with double tailed diploid sperm in the swim-up fractions indicates that diploidy does not hinder swim-up efficiency. Conversely, swim-up favours single tails sperm because they are more efficient swimmers compared with two tailed sperm, in which flagellar movements are not coordinated.

Differences in sperm velocity between sperm with normal and abnormal morphology have been recognized previously (Katz *et al.*, 1985). We have found earlier that cytoplasmic retention, as evidenced by CK immunocytochemistry, was related to abaxial insertion of the tail, a larger and rounder sperm head size and to an increased proportion of amorphous sperm heads (Huszar and Vigue, 1993). Further, in a study of objective sperm morphometry, sperm midpiece shape, tail length and the ratio of tail length/large head axis were directly related to CK activity and HspA2 ratios within sperm fractions (Gergely *et al.*, 1999). Another supporting evidence for the relationship among sperm immaturity, sperm shape and chromosomal aneuploidies was provided by the demonstration of increased frequencies of sperm disomy and diploidy in teratozoospermic men (Harkonen *et al.*, 2001).

In summary of the two studies on the conventional sperm preparation, we have found that both gradient centrifugation and swim-up fractionation results in a decline in sperm having disomies and diploidies and in sperm with diminished maturity, but gradient centrifugation showed an even more efficient elimination of disomic sperm. Gradient centrifugation, in which density of the sperm cell is the major factor, is very efficient in reducing sperm with disomies, but is not as efficient for eliminating diploidies. On the other hand, the swim-up method is very efficient in reducing the proportion of diploid sperm: The large headed and double tailed diploid sperm remain preferentially in the lower phase, due to their swimming inefficiency. In addition to providing additional experimental support for the efficacy of swim-up, the present results, based on the assessment of 200,000 spermatozoa, have resolved the inconsistencies reported in the earlier publications which were reviewed in the Background. Our data suggest that the discrepancies were primarily related to inadequate numbers of sperm nuclei examined.

Based on these strong evidences, and on the data of the Population study detailed above we recommend, that (1) in case of samples with low sperm count with maintained motility, due to the risk of elevated frequency of sperm aneuploidy and immaturity the gradient centrifugation can be used efficiently to eliminate abnormal germ cells, and (2) if oligoasthenozoospermy, especially oligoasthenozoospermy (OAT) is present in the sample, the swim-up preparation is the choice of preparation due risk of elevated diploidy frequencies.

In addition to a better understanding of sperm cell biology and the genetic aspects of spermatogenesis, our studies is of particular interest for clinicians who practice IVF by ICSI. Immature sperm, which have a 2 to 4x higher rate of chromosomal abnormalities than mature sperm (based only on the X, Y and 17 chromosomes), have never before been part of the fertilizing pool, because immature spermatozoa, which have not completed the spermiogenetic plasma membrane remodeling, are deficient in the zona-binding site(s) (Huszar *et al.*, 1994, 1997). In ICSI, however, which is the most frequent treatment mode in severely oligospermic men, immature sperm

may be used for fertilization. One of the consequences is the about 3-4 x higher reported rate of sex chromosome aberrations in offspring of ICSI pregnancies (Bonduelle *et al.*, 1998, 2002).

ICSI as it is presently performed, is far from an ideal infertility solution, because sperm that are selected by embryologists may have genetic impairments. In our studies using objective morphometry and FISH on the same spermatozoa we addressed whether it is possible to predict the chromosomal status of a single sperm from its shape, thus allowing the ICSI embryologist to avoid selecting sperm with aneuploidies or diploidies by simply using their shape properties as a guide. Although several authors have reported the presence of sperm with abnormal morphology and increased frequency of aneuploidies in the same semen sample, the study of the common occurrence of these two factors within the same sperm is a novel finding of the present work.

The prerequisite for the morphometry study of the relationship between sperm morphology and disomies/diploidies in the same sperm was the confirmation that the original sperm shape remains conserved after the decondensation/denaturation steps, which are necessary for performing effective FISH on sperm. We utilized objective morphometry and the newly developed software, Metamorph™, to demonstrate that sperm shape was maintained, while the size increased. In order to validate our methods, we tested various aspects of our protocol. We examined the effects of DTT and LIS on the decondensation process and found that DTT and LIS work synergistically to effect optimal decondensation. We also investigated the effects of denaturation on sperm morphology. Denaturation of sperm DNA is also a prerequisite for performing FISH, since it is necessary to relax the DNA double helix, allowing probes to access and hybridize to the DNA. The decondensation process did enlarge sperm dimensions, but the denaturation step did not further increase sperm size.

The study of the normal, intermediate, abnormal, and amorphus sperm categories, which in general represent an increasing degree of diminished

maturity, indicated increases in aneuploidy frequencies in the four groups. Our hypothesis is further supported by the findings that regardless of the category of the shape classification, sperm which were decondensed maintained their shapes consistently (**Figure 9.**)**.** The percent increases in each of the four sperm shape categories, consisting of head area, perimeter, head long and short axis, were quite similar, except for a larger percent increase in the head area of the amorphus category (**Table 7.**). We suggest that this variation in decondensed sperm dimensions is caused by the maturation properties of individual sperm: Sperm with diminished maturity that are larger, due to cytoplasmic retention, either decondensed more extensively or over-decondensed, while sperm with average size decondensed within an even lower range.

Since we found that decondensation/denaturation tends to increase the cell size but does not affect cell shape, we were able to focus upon the question, using FISH, of whether sperm shape is associated with disomies/diploidies. The second phase of the morphology study revealed that among the 1073 selected, even the sperm with the most normal sperm population, which was classified as such by both visual evaluation or by objective morphometry, exhibited a 7% incidence of sperm with aneuploidies and diploidies. Both disomy and diploidy frequencies increased from the normal to the amorphus classes, and were highest among the amorphus, the most immature sperm population. Although our sample number, 1073, represents a substantial study population, selecting a different population of 1073 might result in a somewhat different distribution of sperm in the various categories, or in the incidences of aneuploid or diploid sperm in the various groups. However, the fact that sperm with chromosomal aberrations may occur among normal spermatozoa is now well established. Thus, selecting sperm for ICSI, based on shape properties alone, does not preclude the presence of chromosomal abnormalities, particularly disomies.

Regarding assisted reproduction, the presence of aneuploid sperm with diminished levels of plasma membrane remodeling and zona-binding sites in sperm preparations is not an important problem with conventional fertilization

via IUI or IVF, since these sperm also have diminished fertilizing capacity (Huszar et al., 1997). However, the issue has became prominent with the introduction of ICSI, in which the zona pellucida selection barrier is overridden upon fertilization. The power of zona sperm selection is well demonstrated by a small scale study (500 sperm per slide scored), in which disomy rates in spermatozoa from men treated with ICSI were determined both in the swim-up and hemizona-bound sperm fractions. As one might expect, based on the relationship among HspA2 expression, sperm membrane remodeling and formation of the zona-binding site(s), the combined aneuploidy frequency for 18, X, Y, and XY disomies was approximately 1.1% in semen and in the swim-up fractions, whereas in hemizona-bound sperm, the rates were < 0.4% (Van Dyk *et al*., 2000; Huszar *et al*., 1997; Huszar *et al*., 2000).

The relationship between the proportion of immature sperm with cytoplasmic retention and frequency of chromosomal aneuploidies in men is based on the dual role of the HspA2 chaperone, which supports meiosis as a component of the synaptonemal complex. It was also found, that along with cytoplasmic extrusion and the initiation of HspA2 synthesis, a developmentally regulated plasma membrane remodeling also occurs, which facilitates the formation of the zona pellucida-binding and HA-binding sites **(Figure 18.**) (Huszar *et al*, 1997). In the semen samples there are sperm with various degrees of cytoplasmic retention, but all sperm bound to the zona pellucida are mature as characterized with the absence of any cytoplasmic retention. Thus, immature sperm with retained cytoplasm, high CK content and low expression of HspA2 are apparently deficient in the zona-binding site (**Figure 19.**) (Huszar *et al*., 1994). Mature sperm selectively attach and remain bound to solid state HA, similarly to the zona pellucida (**Figure 20.**) (Huszar *et al.,* 2003). The data provided evidence that the sperm plasma membrane undergoes a remodeling process during spermiogenetic maturation which results in the formation of the zona- and HA-binding sites. The increased rate of chromosomal aberrations and other potential consequences of using immature sperm for ICSI is of major concern. The present data gave evidence that HA-selected mature sperm show low frequency of chromosomal aberrations comparable to that of sperm selected by the zona pellucida in

conventional fertilization. HA is a normally occurring component of the female reproductive tract, such as the cervical mucus or the cumulus oophorus, to which sperm are regularly exposed, thus there should not be any ethical concerns. Fertilization with HA selected sperm, and potential attachment of a few molecules of HA does not appear to be different from natural fertilization.

In addition to cytoplasmic retention as indicative of the relationship between HspA2 expression and sperm maturity, nuclear attributes such as aniline blue staining, which is a marker of persisting histones in immature spermatozoa have been explored previously. Selection of mature sperm, by binding to immobilized hyaluronan, eliminated sperm cells with aniline blue staining or with cytoplasmic retention (Huszar *et al.*, 2003). This result is in agreement with an earlier study, in which a relationship was found between frequencies of sperm with disomies and aniline blue staining in semen samples (Morel *et al.*, 1998).

As we hypothesized, HA selection eliminated sperm with disomy and diploidy. The 4-fold decline of sex chromosome disomies is consistent with the increase of chromosomal aberrations in ICSI children. In spite of the sample differences, the aneuploidy and diploidy rates in the HA-bound fraction declined to a narrow low 0.04-0.13% range, which comparable to that of sperm populations bound to the human hemizona (Van Dyk *et al.*, 2000). The similarly effective reduction in aneuploidy and diploidy frequencies to a very low level, based on selection by the sperm plasma membrane hyaluronic acid receptor, which is exclusively present only in mature sperm, gives a very promising perspective from the point of view of sperm selection for ICSI.

Conclusions of the Thesis

1. In patients with low sperm count who are candidates for ICSI, there is an increased frequency of sperm with sex chromosome aneuploidy, especially the XY disomy, with considerable inter-individual variations. Further, the diploidy frequency tends to increase in oligoastenozoospermic samples. Defective chromosome separation of either meiosis I. or meiosis II. can be responsible for the production of diploid spermatozoa, but in patients with low sperm count the failure of the nuclear cleavage during meiosis II. seems to be responsible to the elevated diploidy frequency. Conventional WHO parameters of semen analysis (sperm count and motility) do not correlate with the frequency of numerical chromosomal anomalies. The risk can be determined using fluorescence in-situ hybridization (FISH) on decondensed spermatozoa.
2. Due to the elevated risk of transmission of genetic disorders to the offspring with ICSI, there is a need to eliminate spermatozoa with numerical chromosomal anomalies before assisted fertilization. Presently used sperm preparation techniques (the gradient centrifugation and swim-up) are not sufficiently effective in eliminating both aneuploid and diploid sperm. The gradient centrifugation effectively decreases the frequency of immature and aneuploid sperm, while the swim-up preparation is able to reduce significantly the diploid sperm due to their defective motility. There is a correlation between the rate of immature sperm and aneuploidy frequency, but no relationship seems to exist among sperm motility and aneuploidy. Based on our large scale population data and strong evidences we recommend, that (1) in case of samples with low sperm count with maintained motility, regarding the risk of elevated frequency of sperm aneuploidy and immaturity the gradient centrifugation can be used efficiently to eliminate abnormal germ cells, and (2) if oligoasthenozoopermy, especially oligoasthenoteratozoospermy (OAT)

is present in the sample, the swim-up preparation is the choice of preparation due risk of elevated diploidy frequencies.

3. The study of the relationship between sperm morphology and disomy/diploidy within the same sperm is a novel finding of the present work. We proved, that regardless of the category of the shape classification, sperm which were decondensed maintained their shapes consistently. Using objective morphometry and FISH on the same sperm we gave evidence, that sperm with chromosomal aberrations may occur among normal spermatozoa. Thus, selecting sperm for ICSI, based on shape properties alone, does not preclude the presence of chromosomal abnormalities, particularly disomies.
4. We developed a sperm selection method based on the membrane properties and hyaluronic acid binding capacity of mature spermatozoa. Only mature sperm with low aneuploidy/diploidy frequencies are able to bind the solid state hyaluronan. As we hypothesized, HA selection eliminated sperm with disomy and diploidy. Thus, sperm selection with our experimental method may provide a new, safe and efficient solution for selection of individual mature sperm for ICSI with very low risk of numerical chromosome abnormalities.

Summary

In oligospermic patients who are candidates for ICSI, there is an increased frequency of sperm with sex chromosome aneuploidy, especially the XY disomy. Further, the diploidy frequency is increased in oligozooastenospermic samples. Defective chromosome separation of either meiosis I. or meiosis II. can be responsible for the production of diploid spermatozoa, but in patients with low sperm count the failure of the nuclear ceavage during meiosis II. seems to be responsible to the elevated diploidy frequency. Conventional WHO parameters of semen analysis (sperm count and motility) do not correlate with the frequency of numerical chromosomal anomalies. The risk can be determined using fluorescence in-situ hybridization (FISH) on decondensed spermatozoa. Due to the elevated risk of transmission of genetic disorders to the offspring with ICSI, there is a need to eliminate spermatozoa with numerical chromosomal anomalies before assisted fertilization. Presently used sperm preparation techniques (the gradient centrifugation and swim-up) are not sufficiently effective in eliminating both aneuploid and diploid sperm. The gradient centrifugation effectively decreases the frequency of immature and aneuploid sperm, while the swim-up preparation is able to reduce significantly the diploid sperm due to their defective motility. There is a correlation between the rate of immature sperm and aneuploidy frequency, but no relationship seems to exist among sperm motility and aneuploidy. Also, selecting sperm for ICSI, based on shape properties alone, does not preclude the presence of chromosomal abnormalities, particularly disomies. Using objective morphometry and FISH on the same sperm we gave evidence, that sperm with chromosomal aberrations may occur among normal spermatozoa. We developed a sperm selection method based on the membrane properties and hyaluronic acid binding capacity of mature spermatozoa. Only mature sperm with low aneuploidy/diploidy frequencies are able to bind the solid state hyalunonan. Sperm selection with our experimental method may provide a new, safe and efficient solution for selection of individual mature sperm for ICSI with very low risk of numerical chromosome abnormalities.

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List of authors` publications in relation to the thesis

In-extenso Peer Review Publications

1. **Jakab A**, Kovacs T, Celik C, Huszar G. The origin of spermatozoa with extra chromosome set. Hum Reprod 2003; 18: 459-459. IF: 2.99
2. **Jakab A**, Kovacs T, Kovanci E, Vigue L, Borsos A, Ward DC, Huszar G. Számbeli kromoszóma eltérések humán spermiumban alacsony spermium-koncentráció esetén (Numerical chromosome aberrations in human sperm at low semen concentration) Orv Hetil 2003 (accepted for publication)
3. **Jakab A**, Kovacs T, Borsos A, Huszar G. Számbeli kromoszóma eltérések gyakorisága humán spermiumban és asszisztált reprodukciós vonatkozások (Numerical choromosome aberrations in sperm and their significance in assisted reproduction). Magyar Andrologia 2003; 8: 13-19.
4. **Jakab A**, Kovacs T, Zavaczki Z, Borsos A, Bray-Ward P, Ward D, Huszar G. Efficacy of the swim-up method in eliminating spermatozoa with diminished maturity and aneuploidy. Hum Reprod 2003; 18 (in press) IF: 2.99
5. Celik-Ozenci C, Catalanotti J, **Jakab A**, Aksu C, Demir R, Huszar G. Human sperm maintain their shape following decondensation and denaturation for FISH: shape analysis and objective morphometry. Biology of Reproduction 2003 (in press) IF: 3.51

Chapters in Books

1. Huszar G, **Jakab A**, Celik-Ozenci C, Sakkas D, Kovacs T, Vigue L. Sperm testing by hyalunonic acid binding: andrologic laboratory assessment and sperm selection for ICSI. In: Biotechnology of Human

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2. Kovács T, **Jakab A**, Kovanci E, Závaczki Z, Sakkas D, Huszar G. Preparation of sperm fractions and individual sperm with low levels of chromosomal aneuploidies for assisted reproduction. In: Essential IVF Volume 1. Van Blerkom J and Gregory L eds. Kluwer Academic Publishers, Norwell, MA, USA, in press.

Peer Rewiev Abstracts

1. Celik-Ozenci C, **Jakab A,** Vigue L, Demir R, Huszar G,. Mature and fertile sperm selectively bind to hyaluronic acid: cytoplasmic content, HspA2 levels, chromatin maturity, shape and ICSI sperm selection. J Soc Gynecol Investig Suppl. 2002; 9: Jan/Febr: p340A. Abstract No. 849. (SGI 49th Meeting, March 20-23, 2002, Los Angeles, CA) IF: 2.83
2. Huszar G, Celik-Ozenci C, **Jakab A**, Vigue L. A double chamber device for advanced sperm testing: semen analysis and an assay for sperm maturity by hilauronic acid (HA) binding. J Andrology Suppl. 2002: March/April: p55., Abstract No: 119. (27th Annual Meeting of the American Society of Andrology, April 24-27, 2002, Seattle, WA) IF: 2.13
3. **Jakab A**, Sakkas D, Celik-Ozenci C, Vigue L, Ward D, Bray-Ward P, Huszar G. Selection of sperm with low aneuploidy frequency for ICSI. (this paper was nominated for ESHRE Established Scientist Prize) Hum Reprod 2002; 17(Abstract Book 1): p35-36. (ESHRE 18th Meeting, July 1-3, 2002, Wien, Austria) IF: 2.99
4. Kovacs T, **Jakab A,** Borsos A, Vigue L, Huszar G. Association of diploidy and double tails in human sperm. Hum Reprod 2002; 17(Abstract Book 1): 104. (ESHRE 18th Meeting, July 1-3, 2002, Wien, Austria) IF: 2.99
5. Celik-Ozenci C, Catanalotti J, **Jakab A**, Kovacs T, Demir R, Huszar G. Can one visually select aneuploidy free sperm: a study of morphometry and fluorescence in-situ hybridization. (this paper was nominated for

ESHRE Young Investigator Prize) Hum Reprod 2002; 17(Abstract Book 1): 41. (ESHRE 18th Meeting, July 1-3, 2002, Wien, Austria) IF: 2.99

6. **Jakab A**, Kovacs T, Borsos A, Ward DC, Bray-Ward P, Huszar G. Association between diploidy and double heads in spermatozoa arising from meiosis I and meiosis II. Hum Reprod 2003; 18: (abstract, in press) (ESHRE 19th Meeting, July 29-June 2, 2002, Madrid, Spain) IF: 2.99

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Tables

Table 1. Sperm concentration, motility, number of cells evaluated, disomy and diploidy frequencies of the study subjects grouped according to the WHO definitions of spermatogram (±SD, range)

	NSp (N=5) *31252 sperm*	**ASP (N=6)** *34718 sperm*	**OSp (N=12)** *78684 sperm*	**OASp (N=9)** *55765 sperm*
Sperm concentration. (million/mL)	24.3±3.4 (20.2-27.9)	30.3±8.3 (22.3-45.5)	12.5±3.6 (8-19)	14.5±3.3 (10.4-19.9)
Motility (%)	54.9±5.2 (50.4-61.6)	44.3±3.5 (40-48.4)	56.6±6.5 (50-69.2)	39.9±5.9 (30-48)
No. of cells scored	6250±122 (6131-7415)	5786±930 (5030-7336)	6557±864 (5073-7243)	6196±921 (5071-7316)
Sex chromosome disomy (%)	0.37±0.13 (0.27-0.56)	0.27±0.14 (0.12-0.49)	0.40±0.19 (0.17-0.83)	0.34±0.10 (0.16-0.49)
Disomy 17 (%)	0.17±0.004 (0.11-0.22)	0.17±0.15 (0-0.42)	0.14±0.12 (0.06-0.49)	0.17±0.12 (0.05-0.35)
Diploidy (%)	0.52±0.52 (0.18-1.45)	0.48±0.48 (0.16-1.43)	0.33±0.25 (0.03-0.95)	0.96±0.65 (0.16-1.94)

Table 2. The Gradient Centrifugation Study: characteristics of the semen and gradient centrifugation pellet fractions

Man	Fraction	Concentration (million/mL)	Motility	Motile Sperm Concentration (million/ml)	Diminished maturity sperm	x Clearance of diminished maturity sperm
1	**initial**	10	50.0%	5	42.0%	
	pellet				28.0%	1.5 *p<0.001*
2	**initial**	8	60.0%	6	37.0%	
	pellet				20.0%	1.9 *p<0.001*
3	**initial**	12	40.0%	3.4	47.0%	
	pellet				30.0%	1.6 *P<0.001*
4	**initial**	17	63.0%	5.5	56.0%	
	pellet				23.0%	2.4 *P<0.001*
5	**initial**	19	40.0%	10.8	32.6%	
	pellet				19.3%	1.7 *P<0.001*
6	**initial**	19	60.0%	9	59.3%	
	pellet				39.5%	1.5 *p<0.001*
7	**initial**	10	50.0%	3.7	24.6%	
	pellet				21.0%	1.2 *NS*
8	**initial**	13	50.0%	6.3	51.0%	
	pellet				25.0%	2.0 *p<0.001*
9	**initial**	15	30.0%	7.3	49.5%	
	pellet				25.0%	2.0 *p<0.001*
10	**initial**	10	60.0%	4.9	55.5%	
	pellet				36.5%	1.7 *p<0.001*
Total	**initial**					
	Mean	**13.3**	**50.3%**	**6.72**	**45.5%**	
	SEM	**1.3**	**3.4%**	**0.8**	**3.6%**	
	pellet					
	mean				**26.7%**	**1.7**
	SEM				**2.5%**	**0.1** *p<0.001*

Table 3. The Gradient Centrifugation Study: disomy, diploidy frequencies and sex ratio in the semen and gradient centrifugation pellet fractions determined by using probes for chromosomes 17, X and Y

Man	Fraction	Sperm evaluated	X/Y ratio	Disomy X	Disomy Y	Disomy XY	Sex disomy	Disomy 17	Aggregate disomy (sex+17)	Diploidy
1	**initial**	7062	1.05	0.11%	0.06%	**0.68%**	**0.85%**	**0.17%**	**1.02%**	0.20%
	pellet	7007	1.03	0.03%	0.01%	**0.13%**	**0.17%**	**0.03%**	**0.20%**	0.10%
						p<0.001	*p<0.001*	*p<0.02*	*p<0.001*	
2	**initial**	7194	0.97	0.04%	0.17%	0.07%	0.28%	**0.10%**	**0.38%**	0.32%
	pellet	7087	1.03	0.03%	0.04%	0.08%	0.16%	**0.00%**	**0.16%**	0.16%
								p<0.02	*p<0.02*	
3	**initial**	7114	1.12	0.01%	0.15%	**0.27%**	**0.44%**	0.08%	**0.52%**	0.13%
	pellet	7097	1.19	0.03%	0.04%	**0.04%**	**0.11%**	0.03%	**0.14%**	0.06%
						p<0.001	*p<0.001*		*p<0.001*	
4	**initial**	7159	**1.03**	0.07%	0.17%	**0.22%**	**0.46%**	0.11%	**0.57%**	0.20%
	pellet	7067	**1.12**	0.03%	0.04%	**0.06%**	**0.13%**	0.07%	**0.20%**	0.08%
			p<0.01			*P<0.01*	*p<0.001*		*p<0.001*	
5	**initial**	7147	**1.05**	0.04%	0.11%	0.24%	**0.39%**	0.15%	**0.55%**	0.62%
	pellet	7079	**1.18**	0.04%	0.01%	0.08%	**0.14%**	0.08%	**0.23%**	0.41%
			p<0.001				*p<0.01*		*p<0.01*	
6	**initial**	7157	**0.98**	**0.17%**	0.18%	**0.20%**	**0.54%**	0.11%	**0.66%**	**0.28%**
	pellet	7072	**1.08**	**0.03%**	0.04%	**0.04%**	**0.11%**	0.07%	**0.18%**	**0.10%**
			p<0.01	*p<0.02*		*p<0.02*	*p<0.001*		*p<0.001*	*p<0.02*
7	**initial**	7116	1.05	0.11%	**0.14%**	0.11%	**0.37%**	0.08%	**0.45%**	**0.34%**
	pellet	7052	1.09	0.03%	**0.00%**	0.06%	**0.09%**	0.03%	**0.11%**	**0.11%**
					p<0.01		*p<0.001*		*p<0.001*	*p<0.01*
8	**initial**	7037	**1.08**	0.07%	0.13%	0.03%	0.23%	0.06%	**0.28%**	0.21%
	pellet	7147	**1.00**	0.01%	0.06%	0.03%	0.10%	0.00%	**0.10%**	0.17%
			p<0.1						*p<0.02*	
9	**initial**	7263	1.08	0.08%	0.08%	0.17%	**0.33%**	0.07%	**0.40%**	0.30%
	pellet	7033	1.09	0.00%	0.04%	0.07%	**0.11%**	0.04%	**0.16%**	0.17%
							p<0.02		*p<0.01*	
10	**initial**	7136	1.06	**0.22%**	0.17%	0.17%	**0.56%**	0.06%	**0.62%**	0.03%
	pellet	7042	1.00	**0.06%**	0.04%	0.10%	**0.20%**	0.04%	**0.24%**	0.04%
				p<0.01			*p<0.001*		*p<0.001*	
Total	**initial**	71385	**1.05**	**0.09%**	**0.14%**	**0.21%**	**0.44%**	**0.10%**	0.54%	**0.26%**
	pellet	70683	**1.08**	**0.03%**	**0.03%**	**0.07%**	**0.13%**	**0.04%**	**0.17%**	**0.14%**
	x clearance			*3.0*	*4.7*	*3.0*	*3.4*	*2.5*	*3.2*	*1.9*
			p=0.002	*p<0.001*	*p<0.001*	*p<0.001*	*p<0.001*	*p<0.001*	*p<0.001*	*p<0.001*

Bold numbers represent significantly different comparisons.

Table 4. The Swim-up Study: characteristics of the semen and swim-up fractions

Man	Fraction	Concentration (million/mL)	Motility	Motile Sperm Yield (%)	Diminished maturity sperm	x Clearance of diminished maturity sperm
1	**initial**	10	50.0%		51.7%	
	swim-up		90.0%	52.0	41.0%	1.3
						p=0.025
2	**initial**	45	42.1%		64.3%	
	swim-up		92.6%	43.4	35.7%	1.8
						p<0.001
3	**initial**	22	46.1%		29.0%	
	swim-up		76.1%	49.5	22.0%	1.3
						NS
4	**initial**	12	30.7%		43.0%	
	swim-up		30.0%	10.0	38.0%	1.1
						NS
5	**initial**	33	48.4%		21.0%	
	swim-up		90.0%	70.8	13.0%	1.6
						p=0.011
6	**initial**	16	42.3%		42.0%	
	swim-up		75.0%	51.5	29.0%	1.4
						p<0.001
7	**initial**	10	43.4%		62.0%	
	swim-up		70.0%	15.5	51.3%	1.2
						NS
8	**initial**	9	59.2%		47.7%	
	swim-up		50.0%	6.6	19.7%	2.4
						p<0.001
9	**initial**	15	50.0%		36.5%	
	swim-up		87.8%	67.1	18.7%	2.0
						p<0.001
10	**initial**	30	40.0%		46.7%	
	swim-up		80.0%	75.0	27.7%	1.7
						p<0.001
Total	**initial**					
	Mean	**20.2**	**45.2%**		**44.4%**	
	SEM	**3.9**	**2.4%**		**4.3%**	
	swim-up					
	mean		**74.2%**	**44.1**	**29.6%**	**1.5**
	SEM		**6.3%**	**8.0**	**3.7%**	**0.1**
						p<0.001

Table 5. The Swim-up Study: disomy frequencies and sex ratio in the semen and swim-up fractions determined by using probes for chromosomes 10, 11, 17, X and Y

		Probes 17,X, and Y (three-color FISH)								Probes 10 and 11 (two-color FISH)			
Man	**Fraction**	**Sperm evaluated**	**X/Y ratio**	**Disomy X**	**Disomy Y**	**Disomy XY**	**Sex disomy**	**Disomy 17**	**Aggregate disomy (sex+17)**	**Sperm evaluated**	**Disomy 10**	**Disomy 11**	**Aggregate disomy (10+11)**
1	**initial**	5281	1.09	0.04%	0.04%	0.11%	0.19%	0.06%	0.25%	5056	0.06%	0.20%	0.26%
	swim-up	5015	1.10	0.04%	0.04%	0.04%	0.12%	0.04%	0.16%	4827	0.17%	0.21%	0.37%
2	**initial**	7336	1.05	0.08%	0.07%	0.08%	0.23%	0.08%	0.31%	5147	0.33%	0.18%	0.51%
	swim-up	7009	1.01	0.06%	0.07%	0.07%	0.20%	0.04%	0.24%	5117	0.14%	0.20%	0.33%
3	**initial**	5032	1.12	0.08%	0.02%	0.10%	0.20%	0.14%	0.34%	5160	0.17%	0.14%	0.31%
	swim-up	5012	1.08	0.02%	0.06%	0.14%	0.22%	0.04%	0.26%	5021	0.06%	0.10%	0.16%
4	**initial**	5071	1.04	0.12%	0.04%	0.08%	0.24%	0.10%	0.34%	5056	0.12%	0.06%	0.18%
	swim-up	4872	1.03	0.06%	0.00%	0.08%	0.14%	0.02%	0.16%	5056	0.04%	0.08%	0.12%
5	**initial**	5054	1.05	0.02%	0.04%	0.06%	0.12%	0.00%	0.12%	5022	0.10%	0.12%	0.22%
	swim-up	5030	1.00	0.04%	0.02%	0.06%	0.12%	0.02%	0.14%	5040	0.06%	0.08%	0.14%
6	**initial**	5146	1.06	0.06%	0.06%	0.18%	0.29%	0.35%	**0.64%**	5104	0.14%	0.35%	0.49%
	swim-up	5087	1.05	0.04%	0.02%	0.08%	0.14%	0.20%	**0.33%**	5082	0.06%	0.20%	0.26%
									p<0.05				
7	**initial**	5128	1.11	0.06%	0.06%	0.04%	0.16%	0.18%	0.33%	5104	0.06%	0.22%	0.28%
	swim-up	5101	1.07	0.06%	0.04%	0.02%	0.12%	0.08%	0.20%	5072	0.00%	0.22%	0.22%
8	**initial**	5350	1.18	0.11%	0.02%	0.04%	0.17%	0.08%	0.24%	5356	0.06%	0.17%	0.22%
	swim-up	5219	1.10	0.04%	0.02%	0.00%	0.06%	0.02%	0.08%	5134	0.00%	0.14%	0.14%
9	**initial**	5073	**1.14**	0.08%	0.14%	0.06%	0.28%	0.12%	0.39%	5284	0.10%	0.17%	0.27%
	swim-up	5049	**1.04**	0.08%	0.12%	0.02%	0.22%	0.10%	0.32%	5213	0.04%	0.13%	0.17%
			p<0.05										
10	**initial**	5030	**1.10**	0.08%	0.04%	0.02%	0.14%	0.12%	0.26%	5129	0.10%	0.12%	0.21%
	swim-up	5047	**0.94**	0.08%	0.00%	0.04%	0.12%	0.08%	0.20%	5065	0.08%	0.08%	0.16%
			p<0.01										
Total	**initial**	53501	**1.09**	0.07%	0.05%	0.08%	**0.20%**	0.12%	**0.32%**	51418	0.12%	0.17%	**0.29%**
	Swim-up	52441	**1.04**	0.05%	0.04%	0.06%	**0.15%**	0.06%	**0.21%**	50627	0.07%	0.14%	**0.21%**
	x clearance			*1.4*	*1.3*	*1.4*	*1.4*	*1.9*	*1.5*		*1.8*	*1.2*	*1.4*
			p<0.001				*p<0.01*		*p<0.001*				*p<0.01*

Bold numbers represent significantly different comparisons.

Table 6. The Swim-up Study: diploidy frequencies in the semen and swim-up fractions

Man	Fraction	**Probes 17, X and Y** (three-color FISH)		**Probes 10 and 11** (two-color FISH)		**All diploidies detected**			
		Sperm evaluated	**Diploidy**	**Sperm evaluated**	**Diploidy**	**Sperm evaluated**	**Diploidy**	**Diploidy One-tailed**	**Diploidy Two-tailed**
1	**initial**	5281	0.21%	5056	**0.30%**	10337	**0.25%**	0.06%	**0.18%**
	swim-up	5015	0.06%	4827	**0.08%**	9842	**0.07%**	0.05%	**0.02%**
					p<0.05		*p<0.01*		*p<0.001*
2	**initial**	7336	**0.16%**	5147	0.18%	12483	**0.17%**	0.06%	**0.09%**
	swim-up	7009	**0.03%**	5117	0.04%	12126	**0.03%**	0.03%	**0.00%**
			p=0.02				*p=0.002*		*p<0.01*
3	**initial**	5032	0.42%	5160	0.35%	10192	**0.38%**	0.13%	**0.20%**
	swim-up	5012	0.20%	5021	0.16%	10033	**0.18%**	0.14%	**0.04%**
							p<0.02		*p<0.01*
4	**initial**	5071	0.41%	5056	0.32%	10127	0.36%	0.11%	**0.24%**
	swim-up	4872	0.21%	5056	0.24%	9928	0.22%	0.16%	**0.06%**
									p<0.01
5	**initial**	5054	0.16%	5022	0.10%	10076	0.13%	0.06%	0.06%
	swim-up	5030	0.04%	5040	0.06%	10070	0.05%	0.04%	0.01%
6	**initial**	5146	**1.59%**	5104	**1.92%**	10250	**1.76%**	0.66%	**0.74%**
	swim-up	5087	**0.57%**	5082	**0.67%**	10169	**0.62%**	0.45%	**0.10%**
			p<0.001		*p<0.001*		*p<0.001*		*p<0.001*
7	**initial**	5128	**1.40%**	5104	**1.72%**	10232	**1.56%**	0.67%	**0.60%**
	swim-up	5101	**0.49%**	5072	**1.08%**	10173	**0.79%**	0.57%	**0.14%**
			p<0.001		*p<0.01*		*p<0.001*		*p<0.001*
8	**initial**	5350	**0.95%**	5356	**1.20%**	10706	**1.07%**	**0.48%**	**0.48%**
	swim-up	5219	**0.27%**	5134	**0.21%**	10353	**0.24%**	**0.19%**	**0.05%**
			p<0.001		*p<0.001*		*p<0.001*	*p<0.001*	*p<0.001*
9	**initial**	5073	**0.47%**	5284	**0.65%**	10357	**0.56%**	0.20%	**0.28%**
	swim-up	5049	**0.12%**	5213	**0.10%**	10262	**0.11%**	0.10%	**0.01%**
			p=0.002		*p<0.001*		*p<0.001*		*p<0.001*
10	**initial**	5030	**0.28%**	5129	**0.29%**	10159	**0.29%**	0.13%	**0.13%**
	swim-up	5047	**0.06%**	5065	**0.08%**	10112	**0.07%**	0.07%	**0.00%**
			p<0.02		*p<0.05*		*<0.001*		*p<0.001*
Total	**initial**	53501	**0.59%**	51418	**0.70%**	104919	**0.65%**	**0.25%**	**0.30%**
	swim-up	52441	**0.20%**	50627	**0.27%**	103068	**0.24%**	**0.18%**	**0.04%**
	x clearance		*3.0*		*2.6*		*2.7*	*1.4*	*7.1*
			p<0.001		***p<0.001***		***p<0.001***	***p<0.001***	***p<0.001***

Interassay correlation r=0.992 (P<0.001, N=20) [initial r=0.992 N=10, swim-up r=0.893 N=10]
Correlation between diploid frequency in initial vs. swim-up: r=0.925 (P<0.001, N=10)
Bold numbers represent significantly different comparisons.

Table 7. The Morhology Study: mean values of pre-decondensation (native) and post-decondensation parameters. Values are indicated as Mean ± SEM. Bold numbers in the decondensed sperm indicate the significance level p<0.001 when compared to native sperm.

	Normal	Intermediate	Abnormal	Amorphous	**Normal**	Intermediate	Abnormal	Amorphous
	INITIAL				**DECONDENSED**			
Area (μm²)	14.3±0.2	15.4±0.3	21.2±0.6	24.7±1.5	**21.7±0.6**	**22.9±0.7**	**33.1±1.1**	**46±5.3**
Perimeter (μm)	15.0±0.1	16.4±0.2	19.4±0.3	21.2±0.8	**18.7±0.3**	**19.7±0.3**	**24.1±0.4**	**26.4±1.1**
Long Axis (μm)	5.8±0.1	6.6±0.1	7.5±0.2	7.6±0.3	**7.0±0.1**	**7.6±0.1**	**8.8±0.2**	**9.2±0.4**
Short Axis (μm)	4.7±0.03	4.6±0.1	5.6±0.1	6.0±0.2	**5.7±0.01**	**5.6±0.1**	**6.9±0.1**	**7.4±0.3**
Shape Factor	0.89±0.01	0.80±0.01	0.79±0.01	0.76±0.02	**0.85±0.01**	0.80±0.01	0.78±0.01	0.75±0.02
Roundness Ratio	0.82±0.01	0.71±0.01	0.79±0.02	0.83±0.03	0.82±0.01	0.74±0.01	0.81±0.02	0.83±0.02
Tail / Long Axis	10.3±0.2	9.1±0.2	7.4±0.2	7.7±0.4	8.6±0.2	7.9±0.2	6.5±0.2	6.8±0.3

Table 8. The Morphology Study: Percent increases in mean values after decondensation, N=8 men studied.

	Normal n=115		**Intermediate** n=115		**Abnormal** n=115		**Amorphous** n=50	
	% Increase	**P value**	**% Increase**	**P value**	**% Increase**	**P value**	**% Increase**	**P value**
Area (μm^2)	52	<0.001	49	<0.001	56	<0.001	87	<0.001
Perimeter (µm)	25	<0.001	20	<0.001	24	<0.001	25	<0.001
Long Axis (µm)	21	<0.001	15	<0.001	17	<0.001	21	<0.001
Short Axis (µm)	21	<0.001	22	<0.001	23	<0.001	21	<0.001
Shape Factor	**4**	**<0.001**	0	**NS**	1.2	**NS**	1.3	**NS**
Roundness Ratio	**0**	**NS**	**4**	**<0.001**	**3**	**<0.001**	**0**	**NS**

Table 9. The Morphology Study: distribution of disomies and diploidies within the shape categories according to their morphometrical measurements.

Sorting Categories	Area		Perimeter		Long Axis		Short Axis	
	Disomy	Diploidy	Disomy	Diploidy	Disomy	Diploidy	Disomy	Diploidy
Small Heads (N=300)	18%	2%	17%	1%	20%	3%	19%	2%
Intermediate Heads (N=300)	16%	5%	25%	9%	27%	14%	17%	5%
Large Heads (N=300)	66%*	93%*	58%*	90%*	53%*	83%*	64%*	93%*

* p<0.01 Large heads vs. Small and Intermediate heads in all categories.

Table 10. The Morphology Study: FISH data: incidence of disomies/diploidies within each morphological category, N=15 men studied.

		Diploidy	**Disomy**	**No defects**
Normal	n=326	1%	6%	93%
Intermediate	n=337	3%	9%	88%
Abnormal	n=357	8%	13%	79%
Amorphus	n=53	45%	25%	30%

Table 11. The HA-binding Study: disomy, diploidy frequencies and sex ratio in the semen and HA-bound fractions determined by using probes for chromosomes 17, X and Y in 12 olygospermic men (serm concentration±SEM: 20.^{6}±1.7x10^{6}/ml, motility: 54.1±2.5%)

Man	Fraction	Sperm evaluated	X/Y ratio	Disomy X	Disomy Y	Disomy XY	Sex disomy	Disomy 17	Aggregate disomy (sex+17)	Diploidy
1	**initial**	4259	1.12	0.03%	0.12%	0.09%	0.52%	0.17%	0.68%	1.39%
	HA-bound	696	1.28	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.14%
2	**initial**	4177	1.31	0.21%	0.12%	0.12%	0.12%	0.07%	0.50%	2.06%
	HA-bound	477	1.39	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.63%
3	**initial**	4072	1.06	0.10%	0.05%	0.22%	0.22%	0.37%	0.73%	1.52%
	HA-bound	691	1.14	0.00%	0.00%	0.00%	0.00%	0.29%	0.29%	0.15%
4	**initial**	4090	1.09	0.22%	0.12%	0.07%	0.73%	0.12%	0.54%	0.15%
	HA-bound	988	1.12	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
5	**initial**	4452	1.05	0.09%	0.09%	0.18%	0.18%	0.56%	0.92%	0.27%
	HA-bound	1128	0.99	0.09%	0.09%	0.00%	0.00%	0.00%	0.17%	0.18%
6	**initial**	4043	1.10	0.32%	0.07%	0.07%	0.07%	0.30%	0.77%	0.37%
	HA-bound	393	0.97	0.50%	0.00%	0.00%	0.00%	0.00%	0.51%	0.00%
7	**initial**	4191	1.07	0.17%	0.09%	0.00%	0.26%	0.14%	0.41%	0.31%
	HA-bound	683	0.99	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
8	**initial**	4258	1.02	0.09%	0.09%	0.09%	0.28%	0.16%	0.45%	0.26%
	HA-bound	518	0.98	0.19%	0.00%	0.00%	0.19%	0.00%	0.19%	0.00%
9	**initial**	4066	1.01	0.07%	0.05%	0.20%	0.32%	0.20%	0.52%	0.66%
	HA-bound	224	0.98	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
10	**initial**	4094	1.02	0.10%	0.10%	0.07%	0.27%	0.22%	0.49%	0.08%
	HA-bound	1092	1.09	0.00%	0.00%	0.00%	0.00%	0.09%	0.09%	0.18%
11	**initial**	4137	0.97	0.12%	0.05%	0.10%	0.27%	0.31%	0.58%	1.43%
	HA-bound	1004	1.03	0.00%	0.10%	0.10%	0.19%	0.10%	0.30%	0.30%
12	**initial**	4071	0.98	0.22%	0.15%	0.05%	0.27%	0.15%	0.42%	0.32%
	HA-bound	1142	0.94	0.06%	0.08%	0.00%	0.08%	0.00%	0.09%	0.00%
Total	**initial**	49910	1.07	**0.16%**	0.09%	**0.11%**	**0.35%**	**0.23%**	**0.59%**	**0.81%**
	HA-bound	9036	1.06	**0.04%**	0.03%	**0.01%**	**0.09%**	**0.04%**	**0.13%**	**0.13%**
	x clearance			*4.0*	*3.0*	*9.6*	*4.0*	*5.3*	*4.4*	*6.11*
			NS	*p=0.013*	*NS*	*p=0.01*	*p<0.001*	*p<0.001*	*p<0.001*	*p<0.001*

Bold numbers represent significantly different comparisons.

Table 12. The HA-binding Study: disomy, diploidy frequencies and sex ratio in the semen and HA-bound fractions determined by using probes for chromosomes 17, X and Y in 12 gradient treated normospermic samples (serm concentration±SEM: 121.3±21.4x10^6/ml, motility: 59.5±4.9%)

Man	Fraction	Sperm evaluated	X/Y ratio	Disomy X	Disomy Y	Disomy XY	Sex disomy	Disomy 17	Aggregate disomy (sex+17)	Diploidy
1	**initial**	4257	1.06	0.16%	0.12%	0.02%	0.31%	0.23%	0.54%	0.61%
	HA-bound	1955	1.00	0.05%	0.00%	0.00%	0.05%	0.05%	0.10%	0.15%
2	**initial**	4037	1.05	0.25%	0.12%	0.05%	0.42%	0.20%	0.62%	0.30%
	HA-bound	1084	1.11	0.00%	0.00%	0.09%	0.09%	0.37%	0.46%	0.00%
3	**initial**	4046	1.07	0.07%	0.05%	0.10%	0.22%	0.17%	0.40%	0.33%
	HA-bound	569	1.00	0.18%	0.00%	0.00%	0.18%	0.18%	0.35%	0.18%
4	**initial**	4157	1.02	0.07%	0.05%	0.07%	0.19%	0.05%	0.24%	1.11%
	HA-bound	1310	1.07	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.15%
5	**initial**	4102	1.03	0.12%	0.22%	0.15%	0.49%	0.07%	0.56%	0.15%
	HA-bound	504	1.07	0.00%	0.00%	0.20%	0.20%	0.00%	0.20%	0.00%
6	**initial**	4141	1.03	0.12%	0.17%	0.05%	0.34%	0.02%	0.36%	0.17%
	HA-bound	373	1.08	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
7	**initial**	4211	1.04	0.07%	0.10%	0.10%	0.26%	0.07%	0.33%	0.62%
	HA-bound	1129	0.99	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
8	**initial**	4153	1.05	0.07%	0.02%	0.00%	0.10%	0.39%	0.48%	2.07%
	HA-bound	647	1.03	0.00%	0.00%	0.00%	0.00%	0.16%	0.16%	0.62%
9	**initial**	4148	1.06	0.07%	0.05%	0.02%	0.15%	0.10%	0.24%	0.46%
	HA-bound	1446	1.11	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
10	**initial**	4088	1.03	0.12%	0.07%	0.05%	0.25%	0.07%	0.32%	0.22%
	HA-bound	451	0.99	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
11	**initial**	4155	1.00	0.02%	0.05%	0.02%	0.10%	0.12%	0.22%	0.70%
	HA-bound	1003	1.13	0.00%	0.10%	0.00%	0.10%	0.10%	0.20%	0.00%
12	**initial**	4119	1.05	0.05%	0.29%	0.07%	0.41%	0.05%	0.46%	0.44%
	HA-bound	1195	1.01	0.00%	0.17%	0.00%	0.17%	0.08%	0.25%	0.17%
Total	**initial**	51678	**1.13**	**0.10%**	**0.10%**	0.06%	**0.26%**	0.12%	**0.38%**	**0.58%**
	HA-bound	11666	**1.05**	**0.02%**	**0.03%**	0.02%	**0.06%**	0.08%	**0.14%**	**0.10%**
	x clearance			*5.6*	*4.1*	*3.3*	*4.3*	*1.6*	*2.8*	*5.6*
			P<0.01	*P<0.02*	*P<0.05*	*NS*	*p<0.001*	*NS*	*p<0.001*	*p<0.001*

Bold numbers represent significantly different comparisons.

Figures

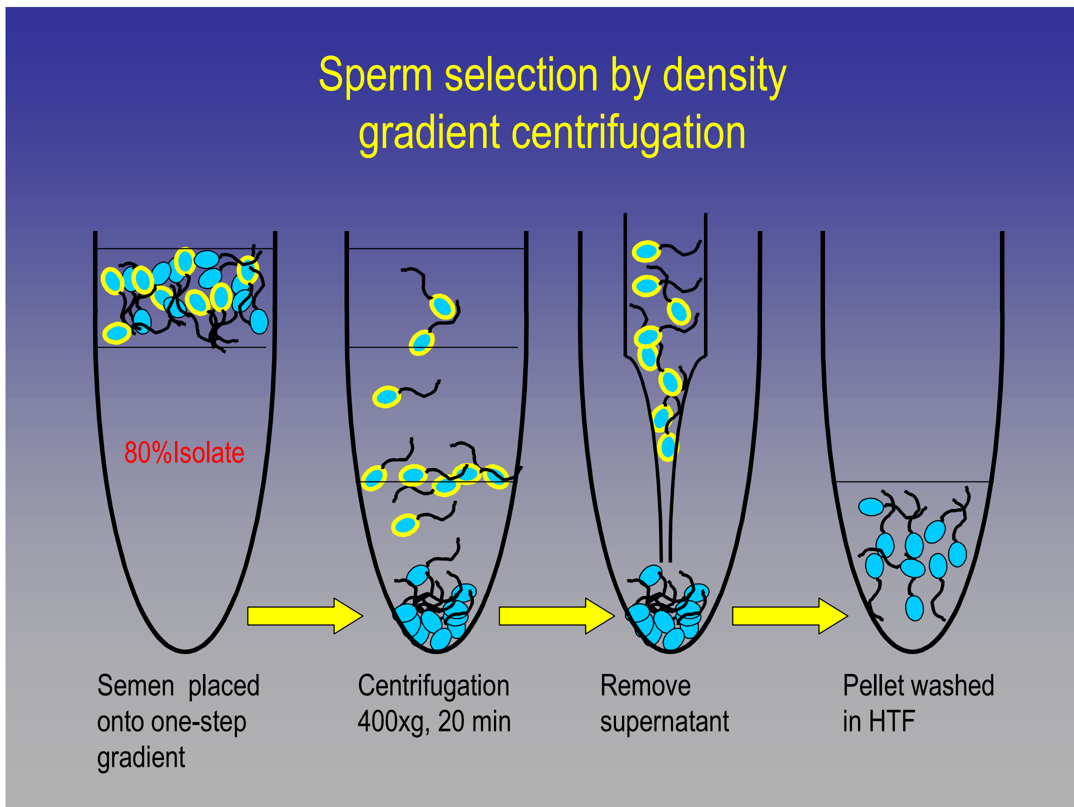


Figure 1. The gradient centrifugation method.

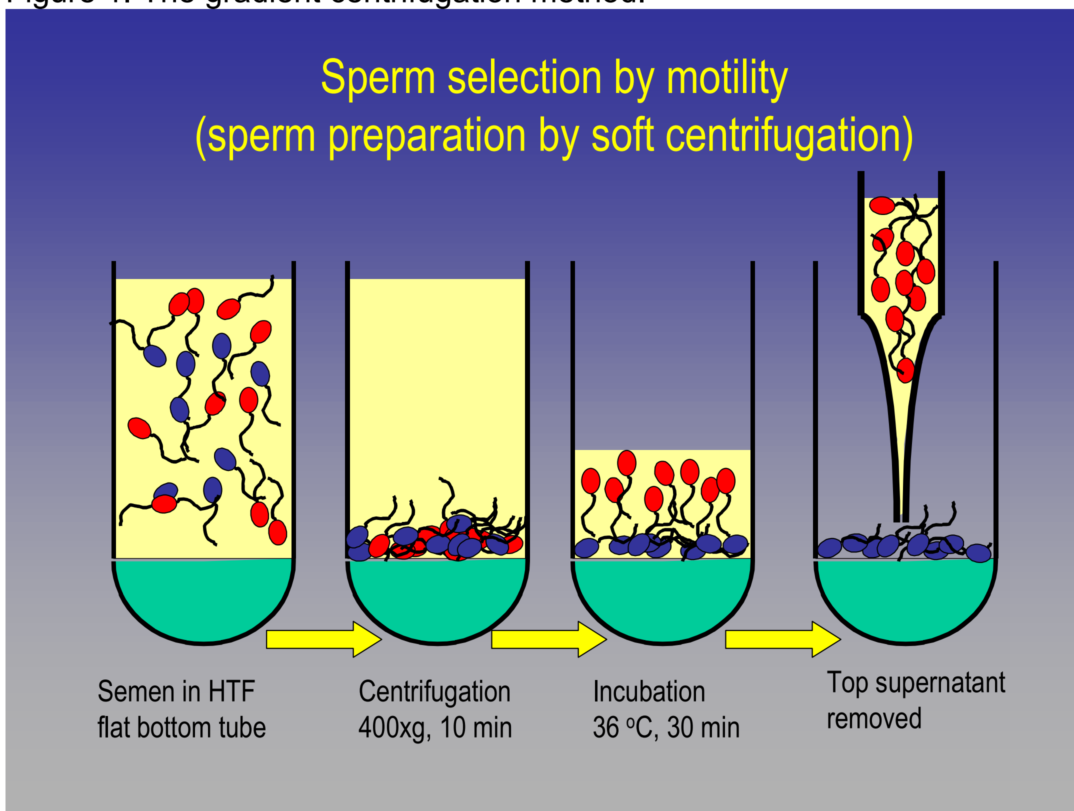


Figure 2. The swim-up technique

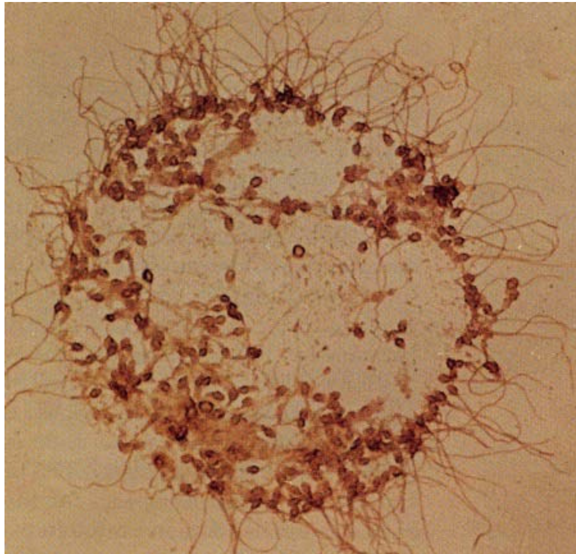


Figure 3. Sperm-hemizona complex.

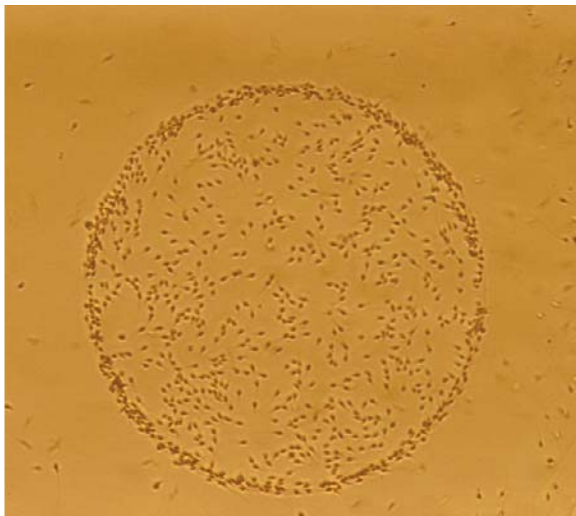


Figure 4. Sperm-HA spot complex.

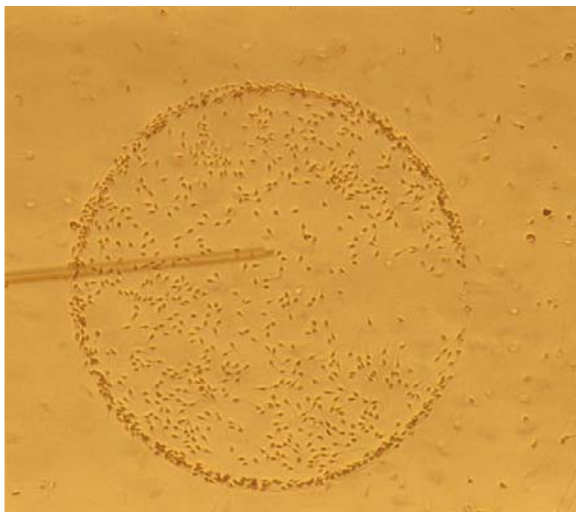


Figure 5. The HA-binding experiment. Collection of HA-bound spermatozoa with ICSI micropipette.

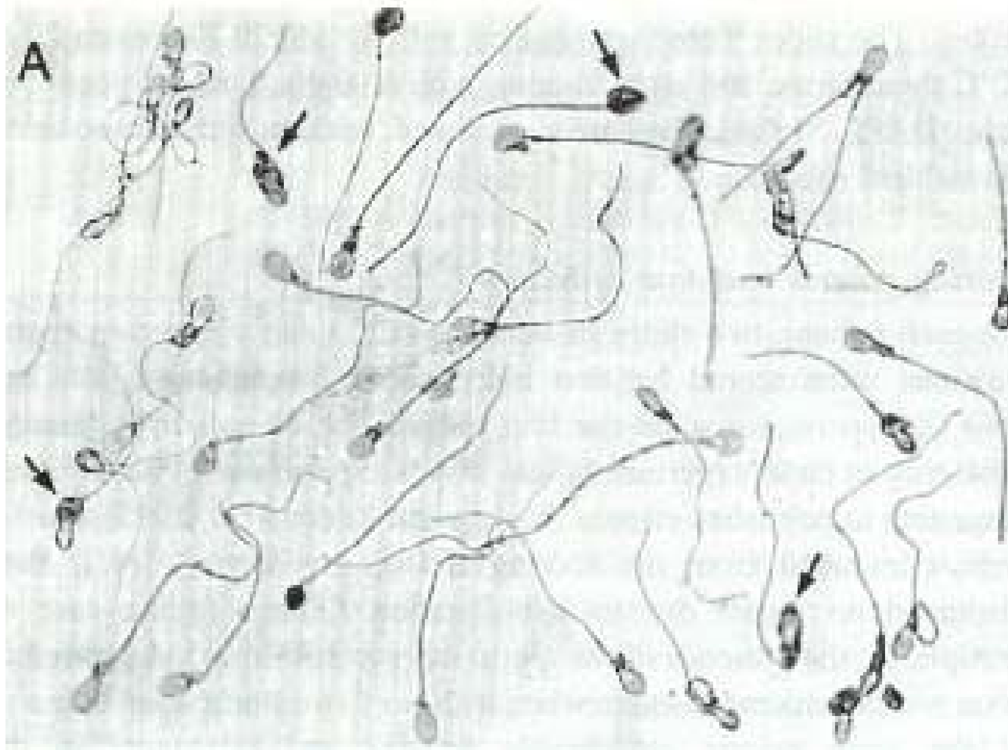


Figure 6. CK-immunocytochemistry on spermatozoa. Dark cells (arrows) represent immature sperm with retained cytoplasm.

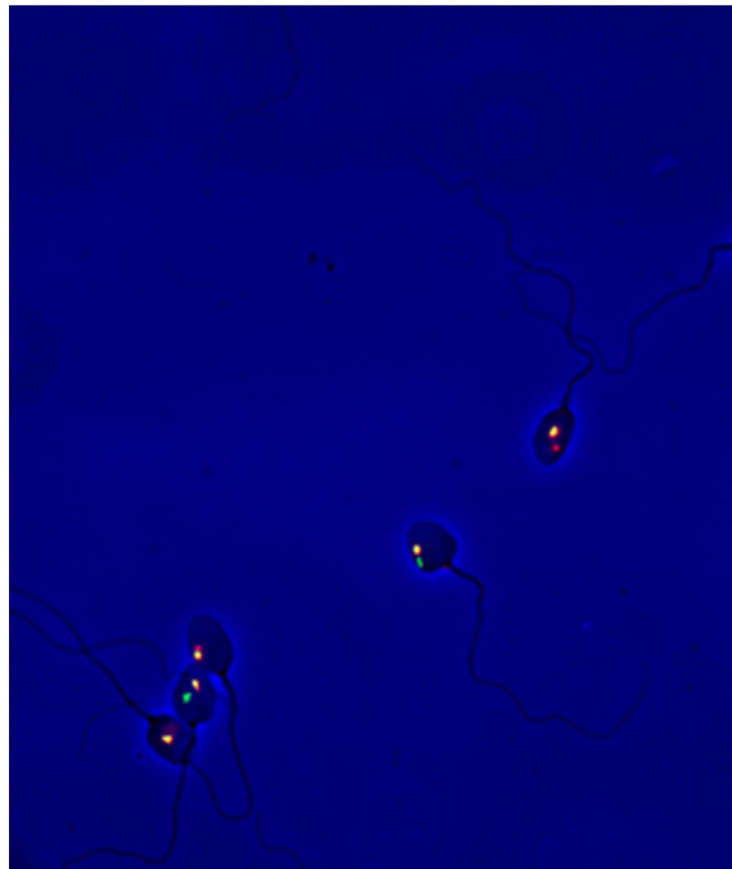


Figure 7. FISH on spermatozoa. Color signals represent chromosomes (green: X, red: Y, yellow: 17).

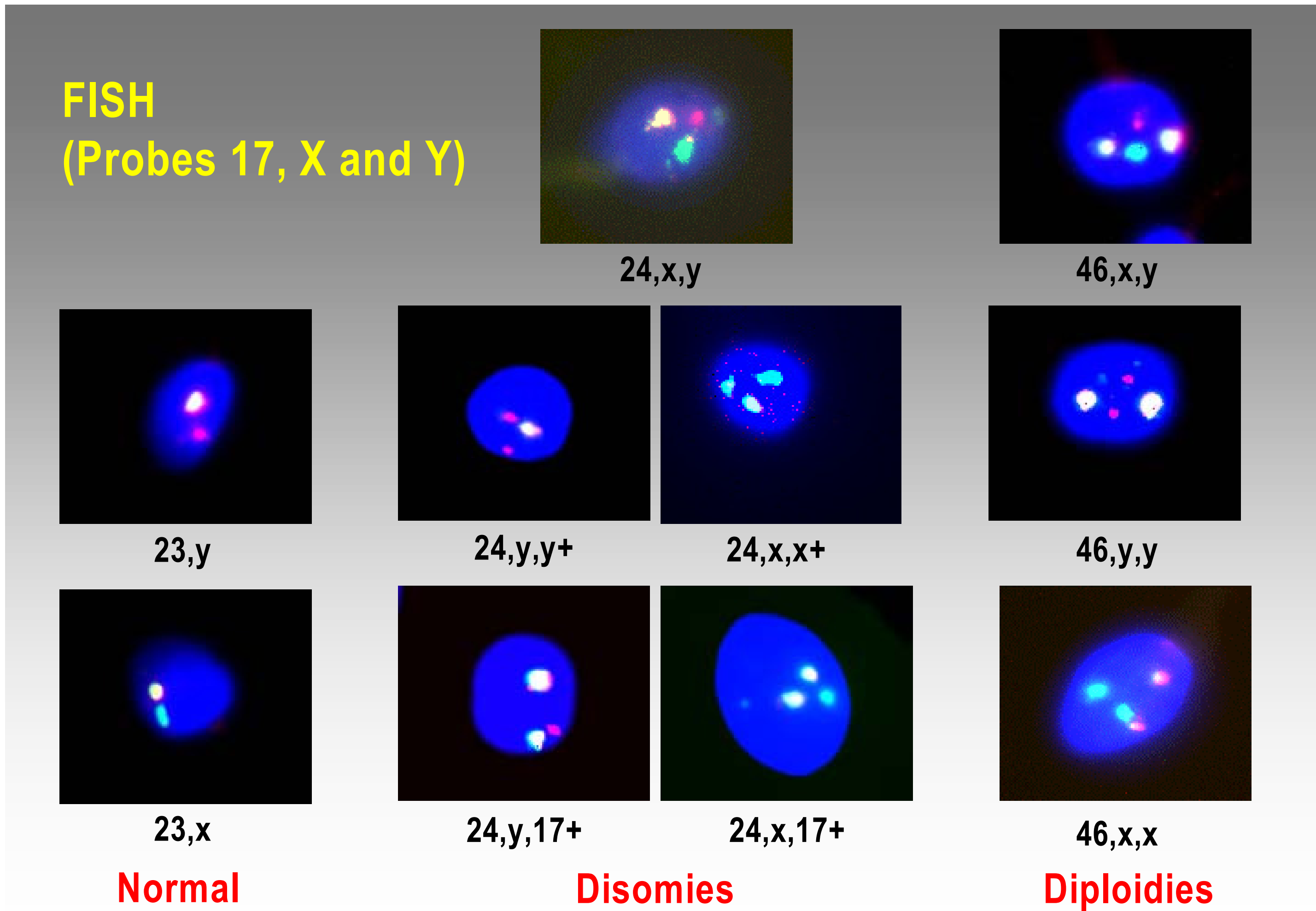


Figure 8. Normal and abnormal spermatozoa detected by three-color FISH using chromosome probes X (FITC, green), Y (rhodamine, red) and 17 (FITC and rhodamine combined, yellow).

Figure 9: Native and decondensed sperm shape categories. Please observe the maintained shape properties of the each sperm shape category.

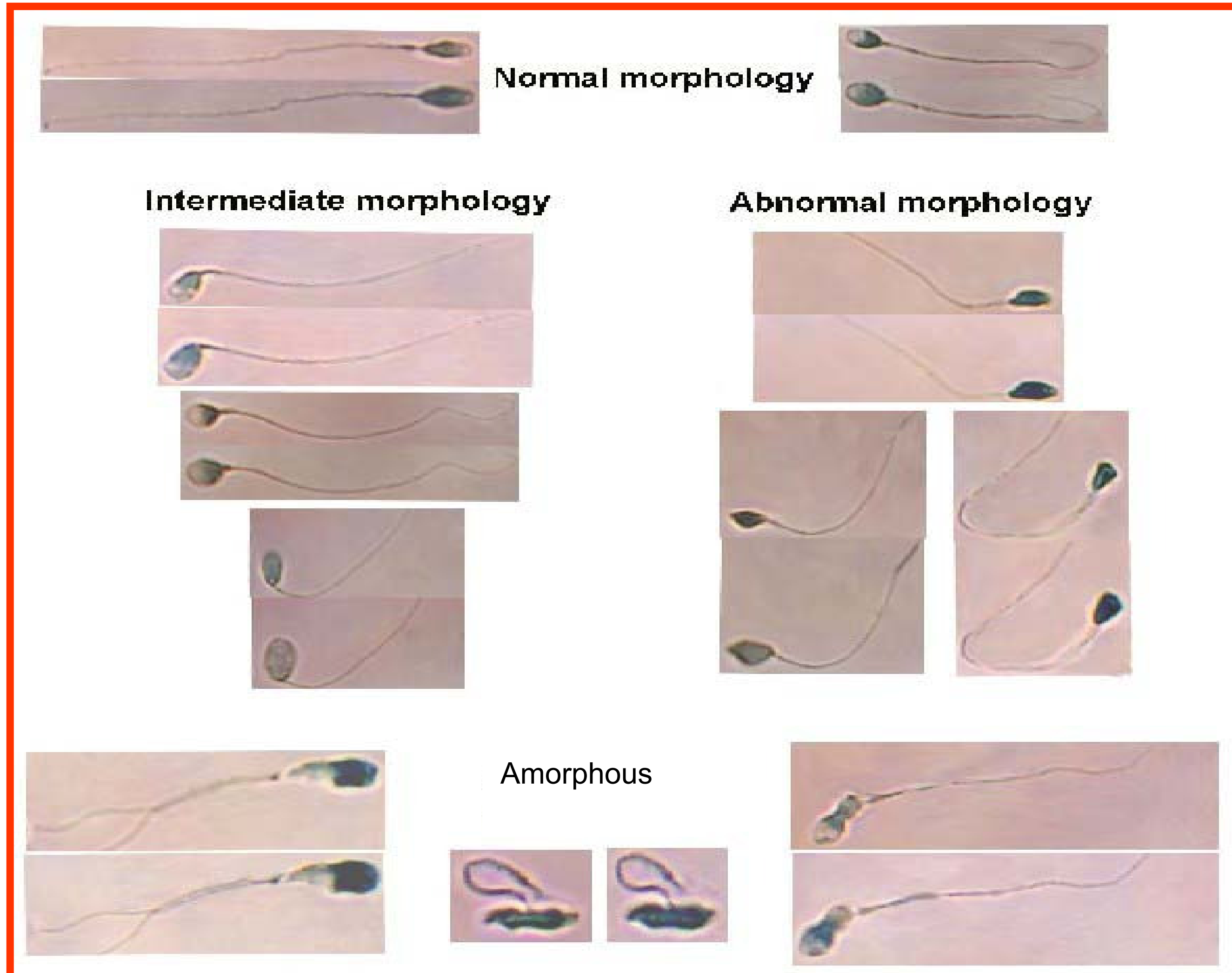
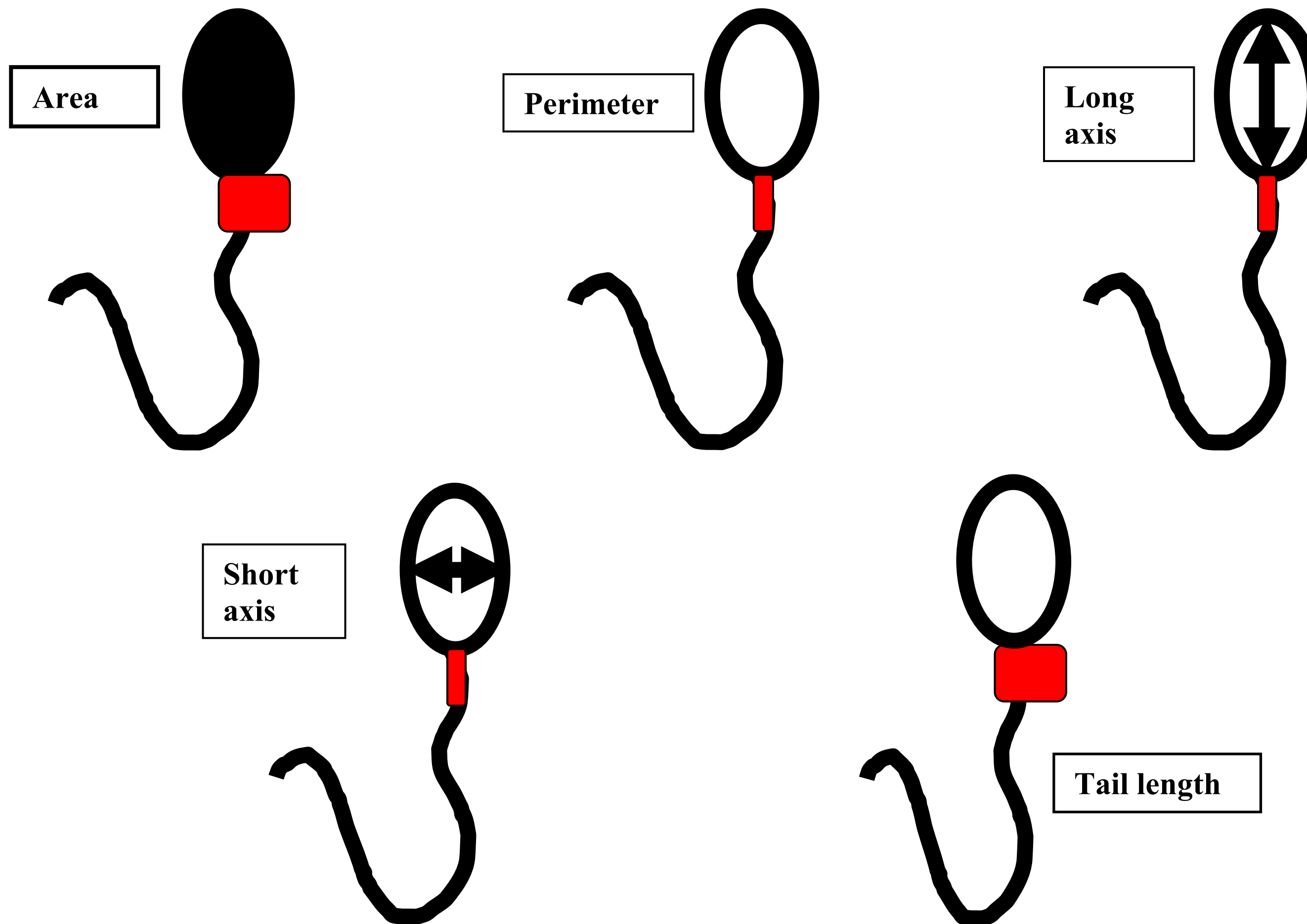


Figure 10: Sperm head and tail parameters measured by the Metamorph™ program.



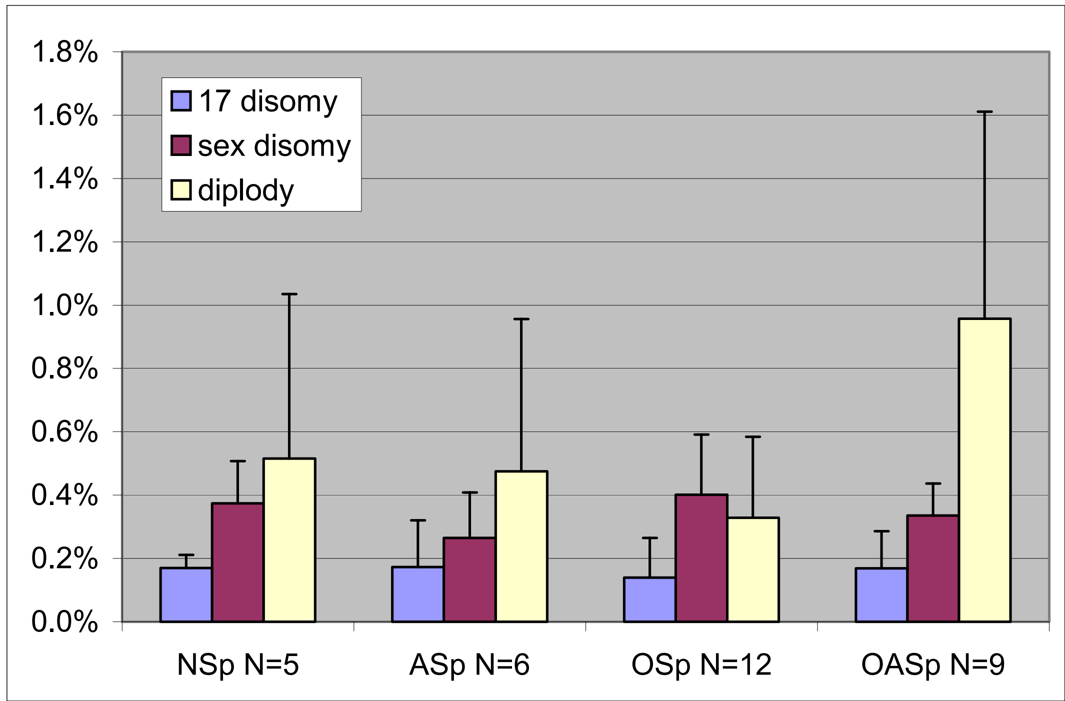


Figure 11. Frequency of disomy sex, disomy 17, and diploidy, in the WHO spermatogram groups of patients (N=32) (NSp: normozoospermy, Asp: asthenozoospermy, OSp: oligozoospermy, OASp: oligoasthenozoospermy)

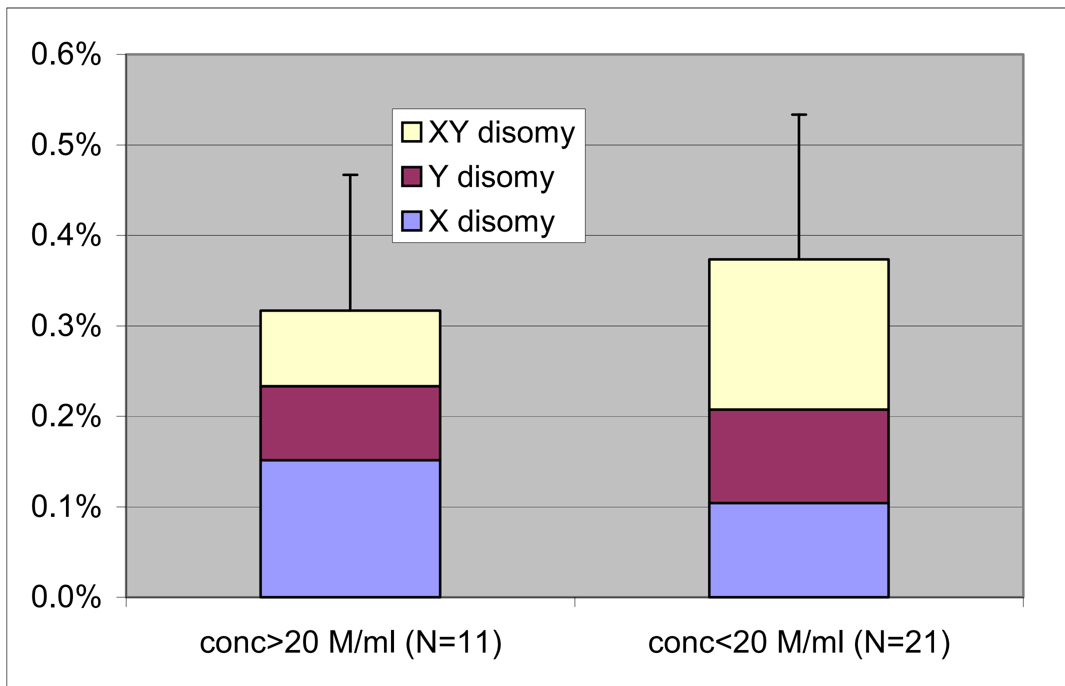


Figure 12. Aggregate sex chromosome disomy frequency and the distribution of XX, YY and XY disomy in oligospermic and normospermic patients of the studies (N=32).

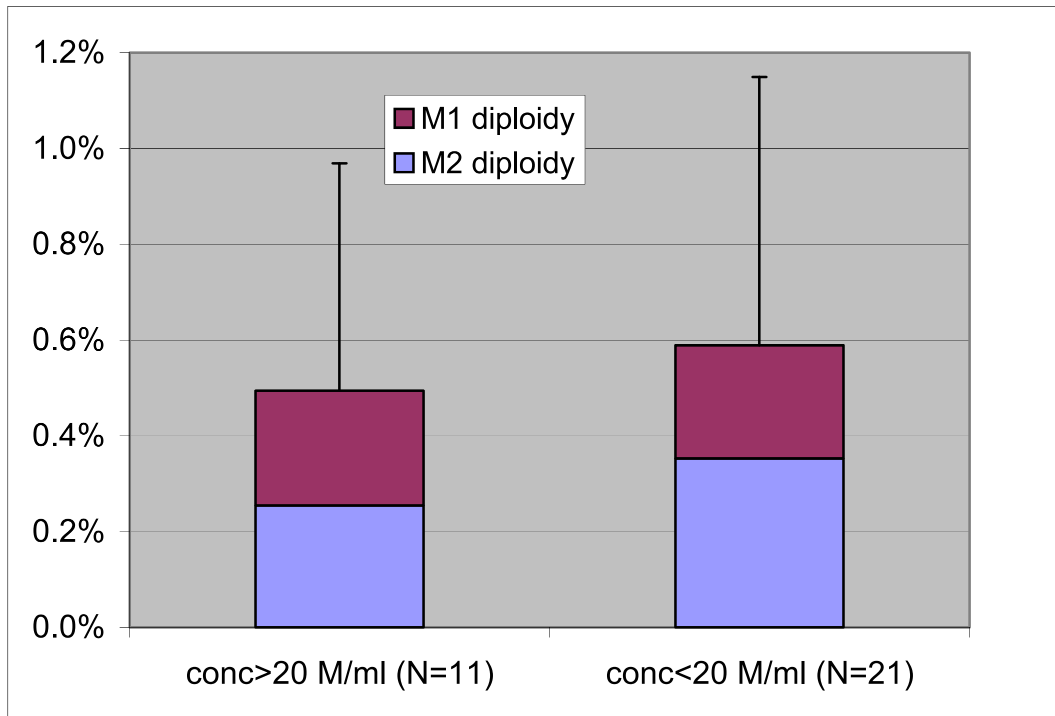


Figure 13. Diploidy frequency and the distribution of meiosis I. and meiosis II. origin diploids in oligospermic and normospermic patients of the study

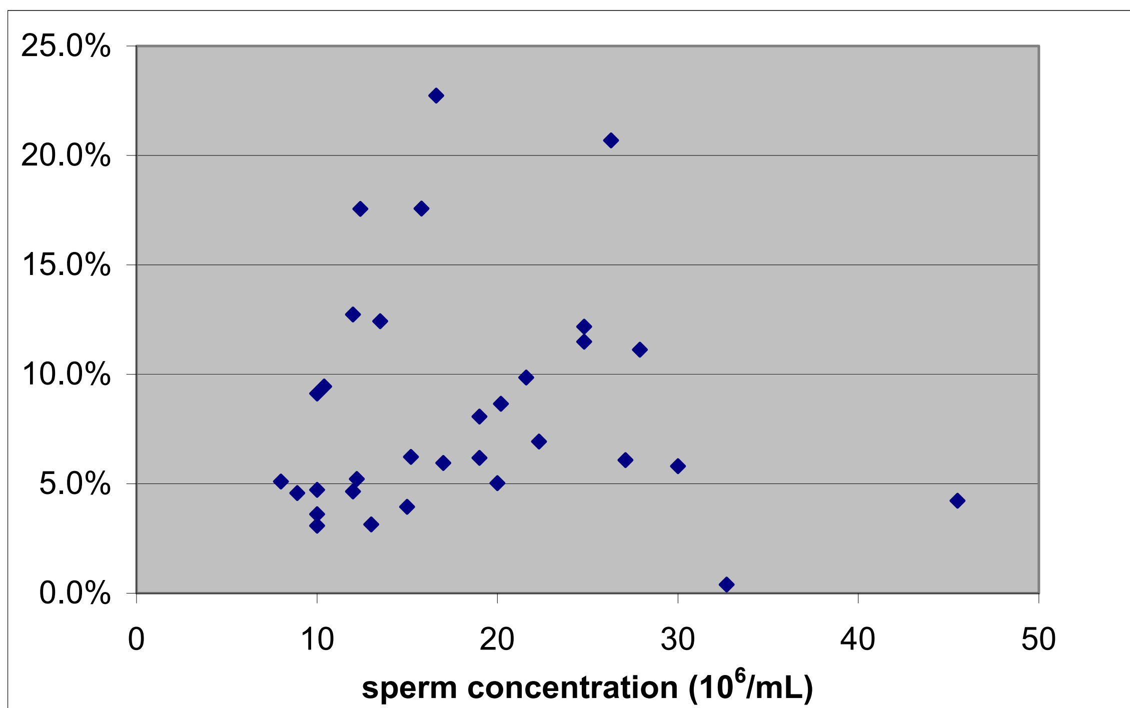


Figure 14. Scatter plot diagram of the estimated frequency of the numeric chromosome anomalies in relation to the sperm concentration in the study patients (N=32)

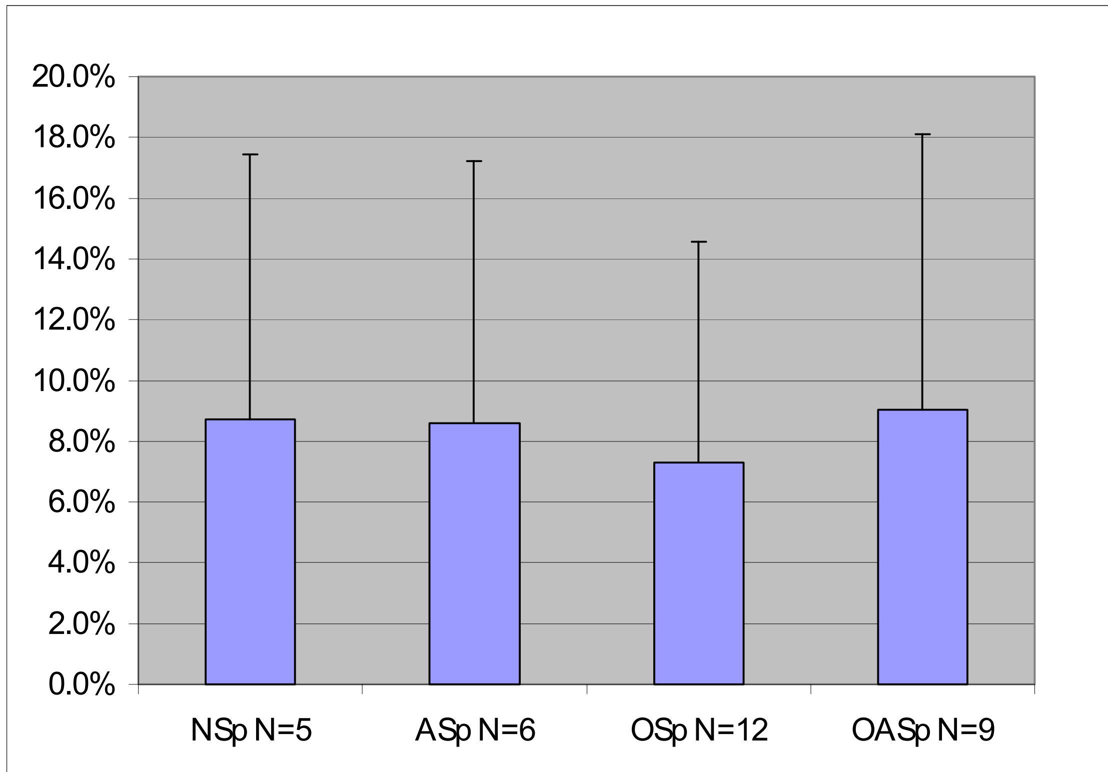


Figure 15. Mean estimated numerical chromosome anomalies of the WHO spermatogram groups in the studies (N=32) (NSp: normozoospermy, Asp: astenozoospermy, OSp: oligozoospermy, OASp: oligoasthenozoospermy).

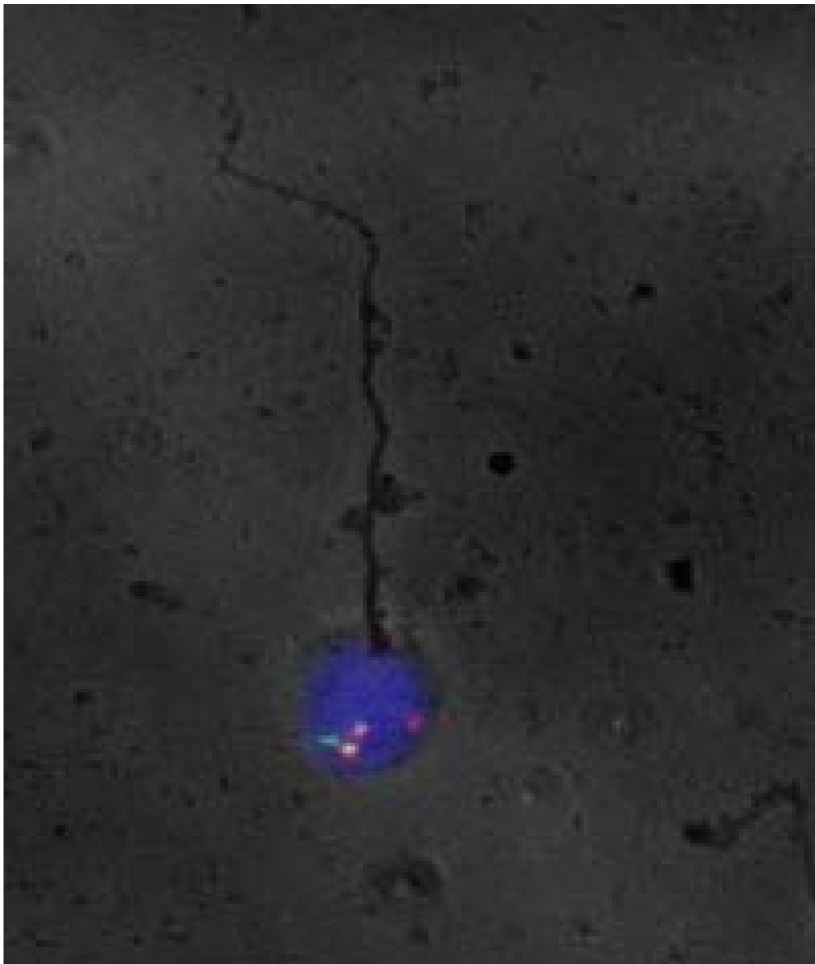


Figure 16. Diploid sperm with one tail.

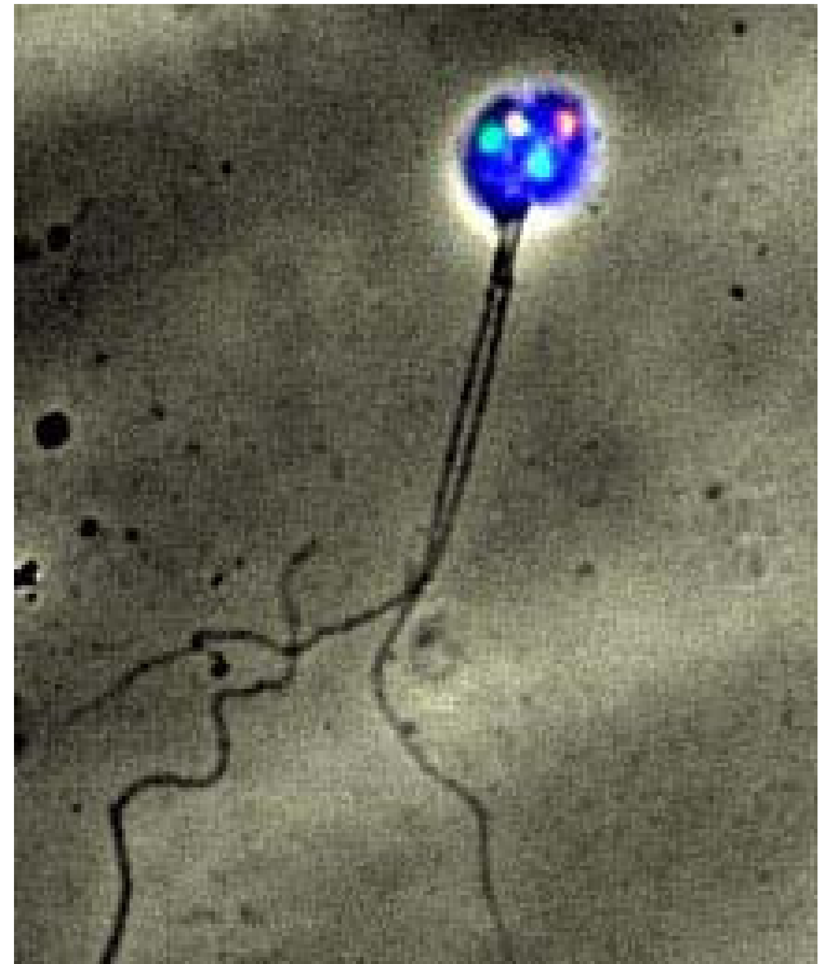


Figure 17. Diploid sperm with two tail

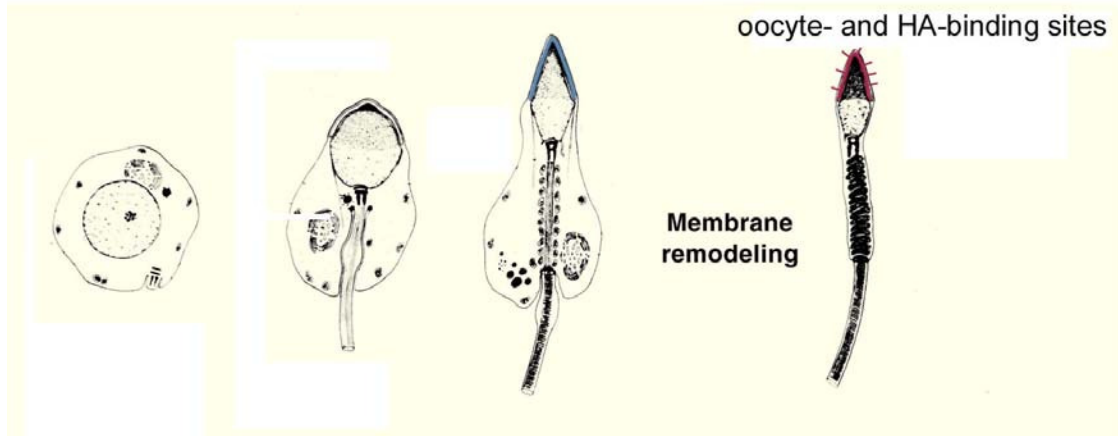


Figure 18. Progress of spermiogenesis. In the last phase of maturation, along with the cytoplasmic extrusion the plasma membrane remodeling takes place resulting in the occurence of the oocyte- (zona-) and HA-binding sites.

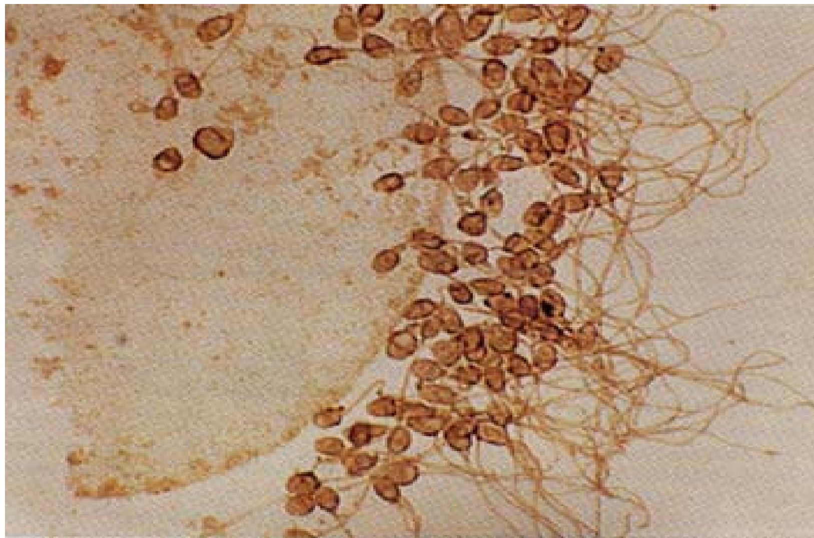


Figure 19. CK-immunochemistry on spermatozoa attached to the hemizona. Note that only clear headed sperm without cytoplasmic retention are able to bind to the zona.

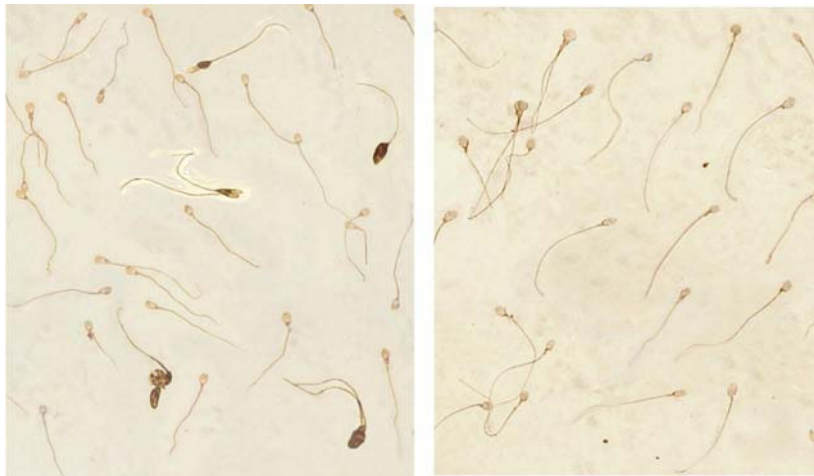


Figure 20. CK-immunochemistry on native sperm and sperm that bound to the HA-coated surface. Clear headed mature sperm without cytoplasmic retention bind selectively to the HA.

Appendix