THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

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RELATIONSHIP AMONG ATTRIBUTES OF SPERM IMMATURITY, CHROMOSOMAL NON DISJUNCTION -RESULTS BY DOUBLE PROBING OF INDIVIDUAL SPERMATOZOON

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Abbreviations

AB–aniline blue

ABC – avidin-biotin complex

BSA – bovine serum albumin

CASA- computer-assisted semen analysis

CK- creatine N phosphotransferase - creatine-kinase

DAPI - 4'-6' diamino-2-phenyl-indole

DNA – deoxyribonucleic acid

dATP – deoxyadenosine triphosphate

dCTP – 2'-deoxycytidine 5'-triphosphate

dGTP – 2'-deoxyguanosine 5'-triphosphate

dUTP – deoxyuridil-triphosphate

FISH – fluorescence in-situ hybridization

FITC – fluorescein-isothiocianate

HA – hyaluronic acid, hyaluronan

HspA2 – heat-shock protein A2

ICSI – intracytoplasmic sperm injection

IVF – in vitro fertilization

LIS – lithium diiodosalicylate

NS – not significant

ROS-reactive oxygen species

SC- synaptonemal complex

SAIM-saline imidazol solution

SEM – standard error of mean

SSC – sodium chloride sodium citrate

WHO – World Health Organization

Introduction and Aims of the Thesis

Though the male contribution to the infertility of the couples is significant, there has been no comprehensive understanding of male infertility yet. The accurate assessment of the male factor, the estimation of fertilizing ability of a particular individuum is also difficult. In the clinical practice the routinely determined semen criteria of sperm concentration and motility seem to be with limited information only. Neither sperm count nor percentage of motility are with specific information in estimation whether or not the patient is able to father. Furthermore in the treatment of male infertility it is still a great challenges how one can select good quality spermatozoa for successful and safe ICSI.

In searching for reliable parameters of male fertilization potential much research has focused on the objective biochemical markers of sperm maturity and functions. Awareness of these biochemical markers may help to understand male infertility and can facilitate the diagnostic evaluation. Moreover the selection method has been developed on the knowledge of biochemical markers appears to be helpful in increase of the effectivity and safety in assisted reproduction (Jakab *et al.*, 2005).

In the meiosis from diploid spermatogonia in a two-round division process haploid spermatids are formed. In the spermiogenesis these haploid round spermatids undergo complex events including histone-transition protein-protamine replacement, acrosome development, cytoplasmic extrusion and sprouting of the sperm tail (Matzuk and Lamb, 2008). In unison with cytoplasmic extrusion, there is also a remodeling of the sperm plasma membrane in late spermiogenesis, a step that facilitates the formation of the zona pellucida and hyaluronic acid binding sites (Huszar *et al.*, 1998, 2003, 2007).

There are various nuclear and cytoplasmic markers that reflect to the degree of arrested maturation. However, the major hallmark of arrested sperm maturation is the low expression of the 70 kilodalton chaperon protein HspA2, which in normal sperm development is expressed in two waves (Huszar *et al.*, 2000). First, in round spermatocytes, HspA2 as a part of the synaptonemal complex (SC) supports meiosis (Dix *et al.*, 1997; Eddy, 1999). Second, a major expression of HspA2 occurs in elongating spermatids, which coincides with assembly, folding and delivery of various enzymes and

associated proteins that are necessary for sperm maturation (**Figures 1. and 2.**), such as DNA repair enzyme, and proteins necessary for sperm membrane remodeling (Aoki *et al.*, 2006; Carrell *et al.*, 2007). The HspA2 proved to be the main regulator of the production and transport of protamins also (Govin *et al.*, 2006), and seems to have anti-apoptotic function as well (Buzzard et al., 1998; Cayli et al., 2004).

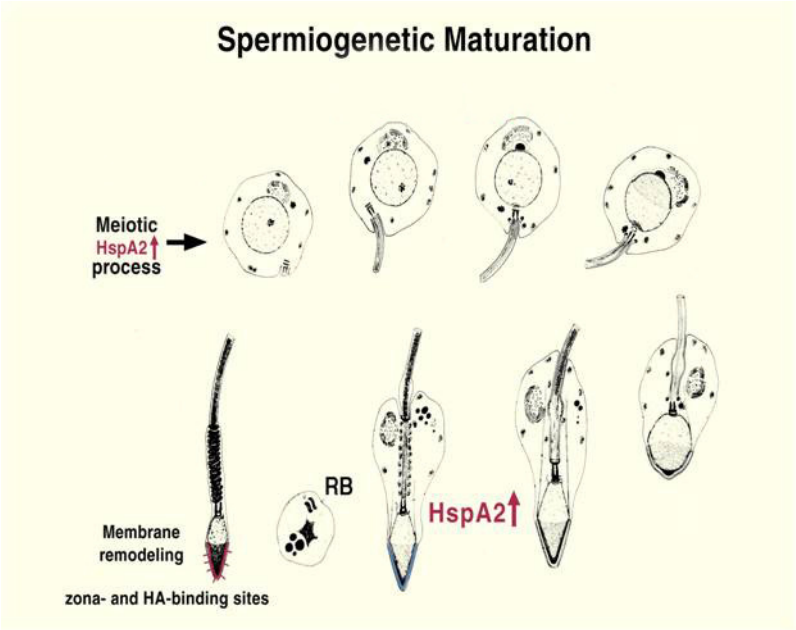

Figure 1. Schematic drawing of spermatogenesis, HspA2 expression occurs in two waves, the first lower peak in round spermatocytes, the second in elongating spermatids. RB is residual body that forms after cytoplasmic extrusion (RBMOnline, 2003)

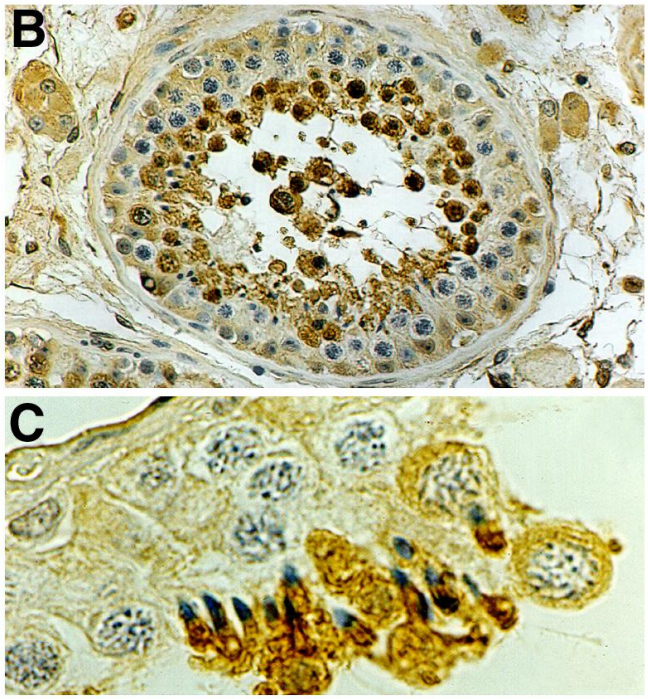

Figure 2. Immunhystochemistry for HspA2 in testicular tissue section. There is a marked expression (the second wave) of HspA2 in the elongating spermatozoa (RBMOnline, 2003).

The spermatogenetic defect of low HspA2 expression is related to various attributes of arrested sperm maturation. These attributes may be detected with biochemical markers, such as immunostaining for sperm creatine kinase (CK) content, a marker of surplus cytoplasm, aniline blue (AB) that points to persistent somatic type histones or testis specific transition protein, immunocytochemistry for apoptotic enzyme caspase-3 reflects to active apoptotic processes, and various approaches demonstrating DNA chain fragmentation, such as in situ nick translation (ISNT) (Irvine et al., 2000; Celik-Ozenci *et al.*, 2003; Huszar *et al.*, 2003; Cayli *et al.*, 2004; Seli and Sakkas, 2005). Because HspA2 is essential component of the SC and necessary for desynapsis of SC between homologous chromosomes pairs, features of arrested sperm maturity associated with low expression of HspA2 increased frequency of chromosomal aneuploidies (Guttenbach and Schmid, 1990; Wyrobek *et al.*, 1994; Moosani *et al.*, 1995; Blanco *et al.*, 1996; Bernardini *et al.*, 1997; Finkelstein *et al.*, 1998; Egozcue *et al.*, 2000; Kovanci *et al.*, 2001 Celik-Ozenci *et al.*, 2003).

In various studies of sperm with arrested maturity, relationships were established by objective biomarkers between HspA2 related spermatogenetic defects. For instance, in semen samples, where the proportion of spermatozoa of arrested maturity with high CK content, marking surplus cytoplasm was higher than in the 80% Percoll pellet of the same samples, and showed a 3x higher frequency of chromosomal aneuploidies. Further, there was a close relationship between frequency of sperm with cytoplasmic retention and sperm with aneuploidies ($r=0.72$, $P<0.001$, (Kovanci *et al.*, 2001). There was a relationship between increased sperm head size (related to surplus cytoplasm in sperm with arrested maturity) and higher frequencies of chromosomal aneuploidies (Gergely *et al.*, 1999; Prinosilova *et al.*, 2009)**.**

Regarding the nuclear compartment of sperm with arrested maturity, earlier studies reported diminished histone-transition protein-protamine exchange that may be detected by AB staining of the excess persistent lysine-rich histones (Dadoune *et al.*, 1988; Auger *et al.*, 1990; Foresta *et al.*, 1992; Morel *et al.*, 1998; Morel *et al.*, 2001)**.** Accordingly, based on the variations in sperm maturity, a polymorphic pattern of the sperm staining intensity was found by AB staining: light, intermediate, and dark patterns, that represent sperm with mature, moderately immature, and severely immature developmental status, respectively. In a study when correlation was tested between degree of nuclear immaturity expressed as percent of positive spermatozoa, and frequency of disomy in the same semen sample, there was close correlation ($P=.03$, (Morel *et al*., 1998). This latter study tested only correlation between these two biochemical markers without attempting to explain the common origin. We think that the correlation in this latter study between arrested nucleoprotein replacement and disomies can be explained also by our hypothesis of central role of HspA2; lower expression of the chaperone protein may disrupt the meiosis and can lead to abnormal transitional protein and protamine transport.

All of these aforementioned studies supported the theory on the central role of HspA2 in spermatogenesis; however the relationships among cellular and nuclear attributes of arrested sperm maturity and meiotic nondisjuntion were valid just for large number sperm populations, but individual spermatozoon has not been assessed whether these relationships may be pertinent in intracellular level.

My concern in the thesis work was as it follows; if lower expression of HspA2 is the cause of different forms of arrested maturation and meiotic nondisjunction by multiply

probing of the same individual spermatozoa one may expect co-occurrence of different nuclear and cellular signs of arrested maturity and meiotic nondisjunction in affected cells and not solitary abnormalities. In addition if the common causative factor of these defects the lower expression of the HspA2 one should expect nearly similar degree of collocating abnormalities in the same individual spermatozoon. If our concern is true and one can find collocating and similar degree immaturity abnormalities in individual spermatozoa that would further our consideration of HspA2 related development of male infertility.

In order to test the theoretical co-occurrence of different abnormalities related to low expression of HspA2 a technique of multiply, subsequent probing of individual spermatozoa has been developed. The AB that is a reliable marker for nucleoprotein maturity is a reversible staining and probed spermatozoa can be de-stained. It is fortuitous that acetic acid that is the solvent for AB, major component of fixatives used for many of the biochemical marker studies, such as FISH probing, immunocytochemistry for detection creatine-kinase (CK) and caspase-3 as well as in situ nick translation (ISNT). The double probing of individual spermatozoa in our experiments was commenced by evaluation of nucleoprotein replacement by AB and followed by a second probing like FISH, immunocytochemistry for CK, caspase-3 for apoptosis or ISNT. Localization-relocalization of individual spermatozoa was facilitated by Metamorph™ digital imaging computer program.

The perfect studying of central role of HspA2 in meiosis and spermiogenesis in individual spermatozoon would have been complete with the cotemporary detection of intracellular HspA2 expression together with probing cellular, nuclear maturity and chromosomal constitution. However in our attempts of double probing techniques of individual sperm, FISH probing became unreliable after immunostaining for HspA2.

The Metamorh™ digital imaging program that was used in the advanced localization and relocalization made it possible to examine sperm morphology also; therefore we combined the biochemical probing for attributes of immaturity of individual spermatozoa with the assessment of morphological attributes also. Moreover we have chance to examine in another study the dimensional, morphometrical attributes of spermatozoa with different chromosomal content also. Here in this thesis work we are to detail just our findings concerning differences of shape attributes between spermatozoa with haploid set

and aneuploidy as well as morphometrical differences between different aneuploidy forms. We pursued this study since previous studies evaluating dimensional attributes of disomic spermatozoa were with conflicting evidences and examined just not native, decondensed spermatozoa. We did attempts to examine native, non decondensed sperm also. We supposed that detailed morphometrical and morphological differences between mature / immature or haploid/disomic spermatozoa would increase the safety of sperm selection by micromanipulation technique for ICSI.

In my thesis I have focused on the issues as follows:

1. Can one detect co-occurrence of disomies, the failure of meiosis and arrested nucleoprotein replacement, the consequence of defected spermiogenesis in the same individual spermatozoa? Is there correlation between the degree of nucleoprotein arrest and frequency of aneuploidy?
2. Can different attributes of arrested nuclear and cytoplasmic maturity be related in same individual spermatozoa? If these abnormalities are present in the same spermatozoa is there any correlation among the degree of the different defects?
3. Can one detect morphometrical differences between spermatozoa with aneuploidy comparing to cells with normal chromosomal set? If there is difference between haploid and disomic cells can this difference is useful in selection euploid cells in ICSI treatment?

Background

Biochemical markers of sperm cellular maturation

In measurement of sperm creatine N-phosphotransferase or creatine kinase (CK) significantly higher CK activities have been found in man with diminished fertility (Huszar *et al*., 1998) The research has been addressed reasons underlying the sperm CK activity differences by direct visualization of the CK in individual spermatozoa with CK immunohistochemistry (Huszar and Vigue, 1993) The autoradiographic and CK immunostaining patterns indicated that the high sperm CK activity was direct consequence of increased cytoplasmic protein and CK concentrations in the spermatozoon. The combination of increased CK and protein concentration, coupled with the diminished fertility suggested the identification of sperm developmental defect in the last phase of spermiogenesis when the cytoplasm (unnecessary for the mature spermatozoon) normally is extruded (Clermont, 1963). Following electorphoretic analysis of human sperm extracts in addition to the CK-B isoform, another ATP-containing protein was found, which was proportional to the incidence of mature spermatozoa characterized by low CK activity and no cytoplasmic retention in semen samples (Huszar and Vigue, 1990) This developmentally regulated protein has been identified as the 70kDa testis expressed chaperone protein, which in human is called HspA2 (Huszar *et al*., 2000). The close inverse correlation between proportion of spermatozoa with cytoplasmic retention and low expression of HspA2 and those with lack of cytoplasmic retention and increased expression of the HspA2 indicated that the cytoplasmic extrusion and commencement of the HspA2 synthesis are related, developmentally regulated spermiogenetic events. In three independent studies, correlation between HspA2 levels and CK activity was r=-0. 69, -0.71, and -0.76 ($P<0.001$, n= 159, 134, and 119) (Huszar *et al.*, 1990; Lalwani *et al*., 1996; Ergur *et al.*, 2002). It was established that all sperm maturational evens related to decline of CK activity and increase in HspA2 expression are completed by time the spermatozoa enter the caput epididymis (Huszar *et al.,* 1998) . HspA2 which due to its electrophoretic properties and ATP content, was initially assumed to be an unusual form of sperm specific CK-M isoform proved to be a most useful objective biochemical marker. It has been shown that mature and immature

spermatozoa are different with respect to HspA2 ratio, as expressed by the concentration of sperm CK and HspA2[%HspA2/(Hspa2 + CK)], morphological and morphometrical attributes, zona pellucida binding properties and fertility (Huszar *et al.,* 1992, 1994). Furthermore, it has been established that in spermiogenesis, simultaneously with cytoplasmic extrusion and the commencement of HspA2 synthesis, the sperm plasma membrane also undergoes maturation-related remodeling. This remodeling step facilitates the formation of the sites and receptors for zona binding and for hyaluronic acid binding in mature spermatozoa.

Sperm maturity and fertilization function

The predictive value of CK activity, representing cytoplasmic retention, was tested in couples with oligozoospermic husbands treated with intrauterine insemination. In spite of identical sperm concentration and motility parameters in husband of those couples that have or have not achieved pregnancy, those with pregnancies had four times lower sperm CK activity, but not sperm concentration, contributed significantly to the predictive power (Huszar *et al*., 1990) The validity of HspA2 ratio in the assessment of male fertility was tested in two blinded studies of couples undergoing IVF. In the first, 84 husbands from two different IVF centers were classified without any information on their semen parameters or reproductive histories) based only their sperm HspA2 ratios into "high likelihood" (>10% HspA2 ratio) and "low likelihood" (<10% HspA2 ratio) for fertility groups. All pregnancies occurred in the "high likelihood" group.

Morphometrical differences have also been demonstrated between mature and diminished maturity spermatozoa (Gergely *et al.,* 1999).

It has also been established that sperm samples with high CK activities and cytoplasmic retention have proportionally higher levels of lipid peroxidation (Aitken *et al.,* 1994; Huszar and Vigue, 1994) due to high level of reactive oxygen species; there is increased degradation of DNA, which contributes to diminished ability of the spermatozoon to provide the paternal contribution to the zygote. The high level of lipid peroxidation has not affected normal spermatozoa without cytoplasmic retention, even if incubated in sperm pellets with high reactive oxygen species producing spermatozoa. For this reason,

it has been concluded that high level of lipid peroxidation in spermatozoa is an in born error rather than an acquired property (Huszar and Vigue, 1994)

To identify the steps in the fertilization process at which the low HspA2 immature spermatozoa are deficient, human sperm-oocyte binding was explored. With the study of sperm-hemizona complexes, it was established that only the clear headed (low CK), mature spermatozoa were able to bind to the zona **(Figure 3**.) (Huszar *et al., 1994*)

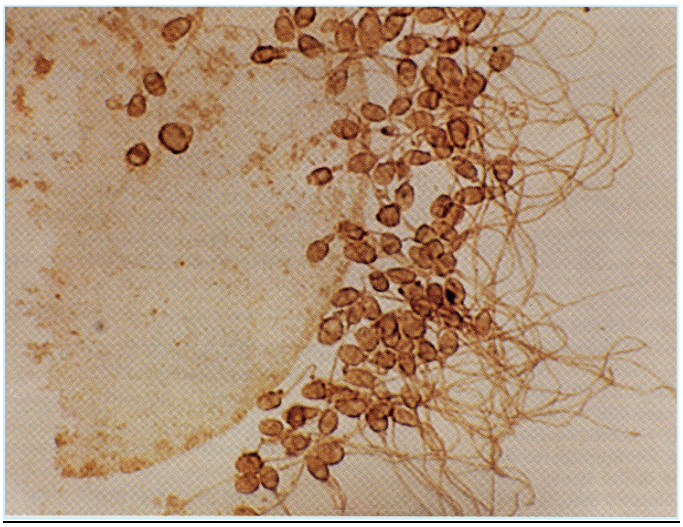

Figure 3. Microscopic picture of hemizona - spermatozoa complex after immunocytochemistry for creatine kinase (CK). All the spermatozoa bind to the hemizona are CK negative, mature cells (RBMOnline, 2003).

Spermatozoa with retained cytoplasm were deficient in the oocyte binding site. In further study, it was confirmed that plasma membrane remodeling occurs in human spermatozoa, simultaneously with cytoplasmic extrusion, during spermiogenetic maturation. This was demonstrated by the close correlation (r=0.8) between CK concentration or the HspA2 ratio and density of the sperm plasma membrane specific enzyme β2 – galactosyltransferase in sperm fractions of various maturities (Huszar *et al*., 1997). Such remodeling apparently facilitates the formation of zona pellucida and hyaluronic acid binding sites. These finding explains two major characteristics of spermatozoa with diminished maturity: cytoplasmic retention and deficiency in zona pellucida binding (Huszar and Vigue, 1993, 1994; Huszar *et al.*, 2000) In general chaperone proteins facilitate the assembly and intracellular transport of proteins. In deed the expression of HspA2 is simultaneous with major sperm protein movements underlying cytoplasmic extrusion and remodeling of the human sperm membrane. This in turn facilitates the

development of zona pellucida binding sites. It is thought that retention of cytoplasm, and the lack of zona-binding sites in immature spermatozoa, is probably related to the diminished expression of HspA2, and also to the diminished DNA integrity, as a consequence of the impaired delivery of DNA repair enzymes during and following meiosis. In order to confirm a finding regarding the expression of HspA2 during terminal spermiogenesis, the expression pattern of HspA2 in human testicular tissue was also examined (**Figure 2**). Varying low levels of immunostaining was particularly striking in the cytoplasm of elongating spermatids and mature spermatozoa about to be released from adluminal compartment (Huszar and Vigue, 1990 Huszar *et al.,* 2000) From the perspective of male infertility, it is important that synthesis of the 70 kDa family of testis specific chaperone proteins is developmentally regulated. The apparent functions of HspA2 are maintaining the synaptonemal complexes and assisting chromosome crossing over during meiosis and spermatocyte development (Allen *et al.,* 1996). These events could be related to faulty meiotic cell cycle regulatory machinery, or perhaps to a more direct disruption of apoptotic machinery in spermatocytes or even in spermatids or ejaculated immature spermatozoa. (Dix, 1997)

Diminished sperm maturity and chromosomal aneuploidy

Because HspA2 is a component of the synaptonemal complex, it was postulated that the frequency of chromosomal aneuploidies will be higher in immature versus mature spermatozoa (Kovanci *et al.,* 2001). This question was examined in spermatozoa from semen and from 80% Percoll pellets of the same ejaculate in 10 oligozoospermic men. Immature spermatozoa with retained cytoplasm, which signifies spermiogenetic arrest, were identified by immunocytochemistry. Around 7000 sperm nuclei were evaluated with FISH in each of 20 fractions using centromere-specific probes for X, Y and 17 chromosomes. The proportions of immature spermatozoa were 45.4±3.4% vs. 26.6±2.2% in the two groups. There was also a concomitant decline in total disomy in 80% Percoll vs. semen fractions (0.17 vs. 0.54% respectively $P<0.001$). Regarding the hypothesis that aneuploidies are related to sperm immaturity, there was a close correlation between the incidence of immature spermatozoa and disomies ($r=0.7$ $P<0.0001$, indicating that disomies originate primarily in immature spermatozoa.

Estimation of attributes of arrested spermatogenesis in the nuclear compartment, persistent nucleoproteins by AB, DNA chain brakes by ISNT and chromosomal nondisjunction by multicolour FISH probing

In the meiotic process from diploid spermatogonia by a two-round division four haploid spermatids are formed. During spermiogenesis the somatic type histone is replaced gradually first by testis specific type transition proteins than by protamine-1 and 2 that are in normal case expressed in almost equal volume (Aoki et al). Because of high quantity of arginin in protamins intra and interchain disulfide bridges are created that facilitates the chromatin to obtain a highly condensed form. This high degree of compaction in mature spermatozoa provides sufficient defense to the chromatin against environmental damaging factors (Dadoune 1995).

To test the sperm nucleoprotein replacement AB staining is generally accepted. Acidic AB is a simple staining method that binds to the basic lysine rich somatic type histones and testis specific transition protein, hence indicates arrested nucleoprotein replacement (Dadoune, 1995; Morel et al.2001). The staining intensity is proportional to the extent of nucleoprotein replacement arrest. The most immature cells bind AB in high amount and appear as “dark” cells, less immature so called “intermediate” cells take up dye just partially; mature cells do not bind the stain at all and occur as “light” (Huszar *et al*., 1997). The AB staining indicating the degree of nucleoprotein maturity in serial of studies proved to be good indicator of sperm fertility function also (Hammadeh et al. 1996, Henkel *et al*., 2001). In our experiments the AB was chosen because of its reliability in assessment of nucleoprotein state, on the other hand because of its favorable chemical property for double probing study. The binding of AB to histones is reversible and moreover its solvent the acetic acid is component of fixatives in probing for DNA chain brakes by ISNT, immunostaining for CK and for FISH probing.

DNA integrity is an attribute of paramount importance in spermatozoa, because fragmented DNA in spermatozoa adversely affects the paternal contribution to fertilization and conception, and to zygote development (Aitken *et al.,* 2003. 2004; Seli *et al.,* 2005; Borini *et al,* 2006; Tarozzi *et al.,* 2007). DNA chain fragmentation is thought to

be related to the various nuclear attributes of arrested/diminished sperm maturity, as shown by excessive persistent histones, which indicate a lower efficiency of histone-protamine replacement (Dadoune *et al.* 1988; Foresta *et al*., 1992; Hammadeh *et al*., 1996; Morel *et al*., 1998). Lower concentrations of the protamine-DNA complex render the DNA chains more vulnerable to damage due to inappropriate DNA folding and packaging (Spano *et al.,* 2000; Steger *et al.,* 2003; Ozmen *et al.,* 2007). Further, studies on sperm maturity and expression levels of the heat shock protein (HspA2) chaperone protein indicated that the low expression of HspA2 in diminished maturity spermatozoa contribute to a reduced DNA repair capacity (Eddy, 1999; Huszar *et al.,* 2000). Another relevant aspect of DNA damage is the relationship between production of sperm reactive oxygen species (ROS) and arrested sperm maturation.

In the assessment of DNA degradation the nick-end labeling or TUNEL, in-situ DNA nick translation with or without prior chemical decondensation of the chromatin as well as single sell electrophoresis COMET assay proved to be effective(Irvine *et al.,* 2000). The ISNT assay that we used with prior chemical decondensation in our experiment quantifies the incorporation the biotinylated dUTP – deoxyuridil-triphosphate at strand DNA breaks in a reaction that catalyzed by the template-dependent enzyme DNA polymerase I. It specifically stains spermatozoa that contain appreciable and variable levels of endogenous DNA damage. The ISNT assay indicates anomalies that have occurred during remodeling of nuclear DNA in spermatozoa.

In the natural conception the paternal contribution to numerical chromosomal abnormalities are insignificant compared with that of oocyte origin (Hunt and Hassold, 2002). The aneuploidy rate in healthy men is as high as 6-7% for the whole chromosomal set. But the spermatozoa with numerical chromosomal anomaly are rarely father in natural conception. Reproductive difficulties are associated intimately with cytogenetic abnormalities. The numerical chromosomal abnormalities of spermatozoa are more frequent in men with male infertility (Shi and Martin, 2001). However in intrauterine insemination and IVF because of the natural selection of zona binding spermatozoa with numerical chromosomal anomaly are eliminated. By advent of ICSI techniques the sperm aneuploidy gained interest, because artificially one may by-pass genetic defect into the oocyte. Beside this clinical aspect the knowledge of aneuploidy also remains important in the comprehensive understanding of male infertility. The technique however labor

intensive and time consuming since the frequency of disomy of a particular chromosome is low usually. The mean disomy frequency is 0.15% for each of the autosomes and 0.26% for the sex chromosomes (Shi and Martin, 2000). Therefore the adequate evaluation requires studying of at least 5000 spermatozoa. There is also respectable inter and intraindividual variability also. The multicolour FISH gives possibility to evaluate the chromosomal constitution of thousands of cells of the same individuum but only for two or three chromosomes of our interest and not the whole set. In our research we used centromere-specific probes for sex chromosomes together with autosomal control using probe for chromosome 17. The reason why we chose sex chromosomes in the assessment was their higher frequency among other disomies. This is related to single almost terminal chiasma between X and Y chromosomes at meiosis I. (Hassold, 1991). The autosomal control is necessary to identify diploidy. A locus specific probe in case of an inadequate decondensation may lead to false detection of aneuploidy. With the use of centromere-specific probe major diagnostic problem can be the artifacts by α satellite polymorphisms. But using strict scoring criteria one may avoid artefactual mistakes. The interphase chromatin in sperm is in highly compacted form and steps necessary to take the DNA accessible to the in situ hybridization. The first step, the decondensation starts with DTT treatment that brakes up disulphide bridges what is followed by application of LIS (Wyrobeck *et al*., 1990, Robbins *et al.*, 1993). Denaturation of DNA double strands for singe strands takes carried out at high temperature. This process denaturates nucleoproteins also therefore makes it impossible to test the nuclear immaturity after FISH. Double testing for nuclear immaturity and numerical chromosomal testing the aniline should be the first step. Another issue is the type of probes used in FISH procedure.

HspA2 and apoptosis of spermatozoa

Apoptosis is a mode of programmed cellular death based on a genetic mechanism that induces a series of cellular, morphological and biochemical alterations, leading the cell to suicide without eliciting an inflammatory response. During spermatogenesis and spermiogenesis, apoptosis, the controlled degradation of sperm DNA, has been suggested to play a key role in adjusting the appropriate number of proliferating germ cells

associated with the surrounding Sertoli cells. (Kaufmann *et al*., 2001). The apoptosis is based on the intracellular dominance of various proteins that induce or inhibit the apoptotic process, such as BAX, Bcl-$_{XL}$, caspase-3 and several key enzymes. (Sakkas *et al*., 2002).

In mouse HSP70-2 exhibits an anti-apoptotic function by interfering with apoptotic programs (Buzzard *et al.*, 1998; Gariddo *et al.*, 2001; Parcellier *et al*., 2003). Indeed, in Hsp70-2 knockout mice, in addition to arrested meiosis, there is increased germ cell apoptosis with the characteristic apoptotic DNA degradation pattern (Dix *et al*., 1996).

The relationship between human sperm maturity and apoptosis is of interest because of the persistence of immature sperm in ejaculates in spite of various apoptotic processes. From the same laboratory in a previous study Cayli *et al.* (2004) found that immature sperm show a proportionally higher level of Bcl-XL and caspase-3 expression. Up to their data it was suggestible that in faulty spermiogenesis there are three options concerning apoptosis: there is early apoptosis in developing germ cells within the adluminal area that sperm are eliminated and are not present in the ejaculate. The second way of immature cells, caspase-3 is activated, and in response Bcl-$_{XL}$ is also expressed which provides a protective effect that substitutes the presence of HspA2. Other immature sperm (caspase-3 only) may survive without any compensatory Bcl-$_{XL}$ expression, most likely because these cells contain HspA2. In a third type of sperm with diminished maturity that proceeds to elongated spermatids, there are secondary effects of diminished HspA2 chaperone activity, such as cytoplasmic retention, larger and amorphous heads, lack of sperm membrane remodeling and retarded tail sprouting. These immature sperm are severely affected; there is CK retention, in addition to the caspase-3 and Bcl-$_{XL}$ expression.

Materials and Methods

The researches that served basis for this thesis were pursued in a collaborative work between the Department of Obstetrics and Gynaecology, Debrecen University, Hungary and Sperm Physiology Laboratory, Department of Obstetrics and Gynaecology, Yale University, USA

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Sperm preparation

Aliquots of liquefied semen were assayed for sperm concentration and motility, and the semen samples were diluted with SAIM up to a final volume of 10 ml. The semen samples were then centrifuged at 500x *g* for 18 min at room temperature. After the supernatant was discarded, each sperm pellet was re-suspended in the SAIM solution to a concentration of 30-40x10^6sperm/ml. The sperm slides were prepared by smearing 5-10 µl of sperm onto clean precleaned, poly-lysine coated glass slides and were allowed to air-dry.

Design of experiment of double probing same individual spermatozoa by aniline blue staining and FISH

We have studied samples of seven moderately oligozoospermic men. The study population was selected because of the known association between increased rates of aneuploidies in men with lower sperm concentration. After CASA determination of sperm concentration and motility the sperm was washed in SAIM solution. A consecutive double treatment was performed with AB staining followed by evaluation of approximately 8000 sperm in each man. The sperm field were localized and relocalized by diamond-scratched lines on the slide and by microscope coordinates. The stained sperm cells according to staining intensity reflecting sperm maturity and persistent histones were assigned as light (mature), intermediate (moderately immature), and dark (arrested maturity) spermatozoa. Following assessment of spermatozoa and recording individual sperm fields, the AB was de-stained, and sperm were subjected to fluorescent in-situ hybridization with three chromosome probes **(Figure 8.)**. The order of AB

staining and FISH procedure was important because after decondensation and denaturation steps, necessary for FISH, the chromatin is affected and AB staining is not reliable.

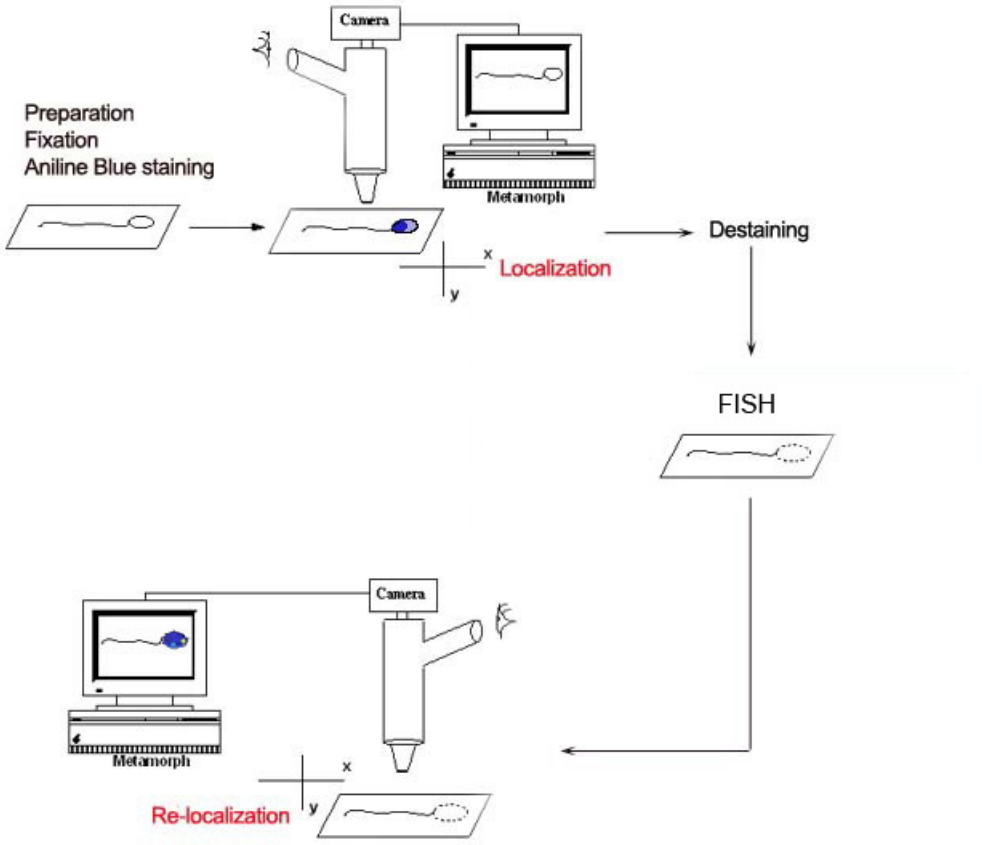

Figure 8. Flow chart of the experimental design of double probing of the same individual spermatozoa by aniline blue and fluorescent in-situ hybridization (FISH).

Aniline blue staining of sperm chromatin

After air-drying slides were stained by 5% AB solution (Sigma Co., St. Louis, MO) acidified to approximately pH: 3.5 with acetic acid for 5 min, then washed with tap water and allowed to completely air-dry. One drop of 5% aqueous glacial acetic acid and a cover slip was placed over the slide regions to be studied. The sperm were evaluated at a magnification of 600x, and images of random microscopic fields were recorded (**Figure 9**.).

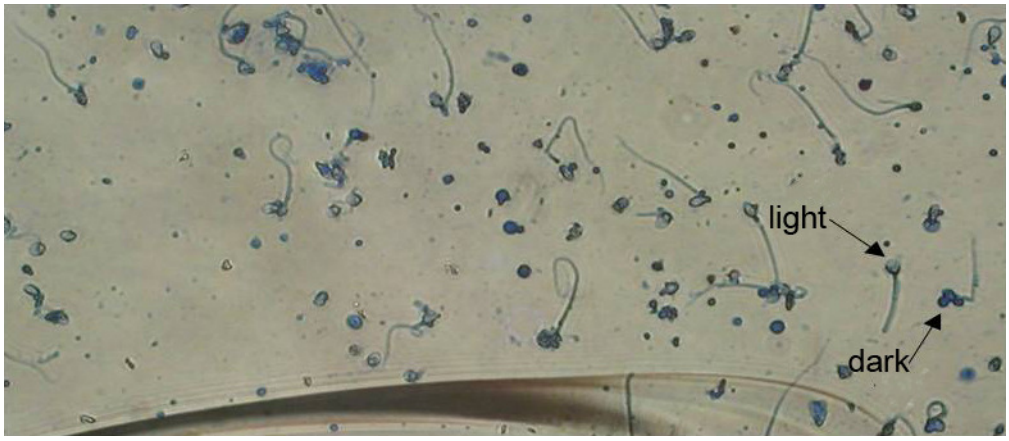

Figure 9. Microscopic field of spermatozoa stained by aniline blue (magnification is 600X). Down the scratch line that helps the relocalization of the same field. Arrows indicate a spermatozoon with "light" head that has complete nucleoprotein replacement and another cell with arrested nucleoprotein replacement contains persistent histones scored as "dark".

Destaining of the AB staining, prior to the FISH step has occurred in 5% aqueous acetic acid, the same solvent that was used for staining with AB. Further, the slides were dehydrated in an ethanol series for 5 min. each, and were stored at room temperature.
Mature sperm, having completed histone-transition protein-protamine replacement, stained very lightly with AB (*Light*, mature sperm), the slightly immature sperm are stained more extensively (*Intermediate*, diminished maturity), and immature sperm with substantial degrees of persistent histones stain darkly (*Dark*, immature sperm (Huszar *et al.*, 2003).

FISH procedures

The DNA decondensation treatment of the destained spermatozoa was performed using a modification of the method from previous studies (Robbins *et al.*, 1993; Kovanci *et al.*, 2001; Celik-Ozenci *et al.*, 2003; Jakab *et al.*, 2005). The cells were treated by 10-mmol/l dithiothreitol (DTT, Sigma) in 0.1-mol/l Tris-HCL buffer, pH 8.0 for 30 min on ice.

Afterwards, the sperm slide was covered with 4-mmol/l lithium 3, 5-diiodosalicylic acid (LIS; Sigma) in 0.1-mol/l Tris-HCl buffer, pH 8.0 for additional 90 min at room temperature. Slides were rinsed for 2 min in 0.1-mol Tris-HCl, pH 8.0 buffer, allowed to air-dry then fixed in methanol: acetic acid (3:1) for at least 10 min.

Directly labeled alpha satellite centromere-specific probes were used for FISH assay in case of chromosome X (Vysis, Downers Grove, Il, USA: Spectrum Green CEP X) and chromosome Y (Vysis, Downers Grove, Il, USA: Spectrum Orange CEP Y). Chromosome 17 was hybridized by an indirectly, biotin labeled centromere-specific probe (SPOT-Light chromosome 17 centromeric probe, Zymed, San Francisco, Cal, USA). The sperm DNA was denaturated in a solution of 70% formamide and 2X standard saline citrate (SSC) at 75°C for 6 min. Further slides were snap cooled and dehydrated in an ethanol series of increasing concentration at –20° C, and air-dried then placed onto a slide warmer at 37°C.

The hybridization solution was prepared according to the manufacturer's instructions. The hybridization solution was dropped onto the slide, and then it was covered by 22x22 mm cover slip and sealed by acid free rubber cement (Ross, OH, USA).That is what rubber cement contains.

The hybridization was conducted in dark humidity chamber for overnight at 37 °C. Post-hybridization washing was accomplished by 50 % formamide in 2X SSC at 42 °C for 15 minutes followed by treatment in 2X SSC for 15 min at 42 °C. After a blocking step of 3% bovine serum albumin for 30 min, in order to visualize the indirect biotin-labeled probe 17, slides were incubated with avidin-rhodamine (fluorescent red, Roche Biochemicals): avidine-FITC (fluorescent green, Roche Biochemical). Slides were washed with a solution of 4 X SSC, 0,1% Teen 20 at 42°C for 15 min, and counter stained by 4,6 diamidino-2-phenylindol (DAPI, Roche Biochemical's) and mounted with an antifade solution (Vectashield;Vector) and coverslipped.

Scoring criteria -FISH

For the evaluation of the FISH patterns, an Olympus AX 70 epifluorescence microscope was used primarily with a triple pass filter for DAPI, fluorescent isothyocyanate (FITC), and tetramethyl-rhodamine isothyocyanate (rhodamine). For improving signal resolution, separate single pass filters were also used for DAPI, FITC and rhodamine (**Figure 10**.)

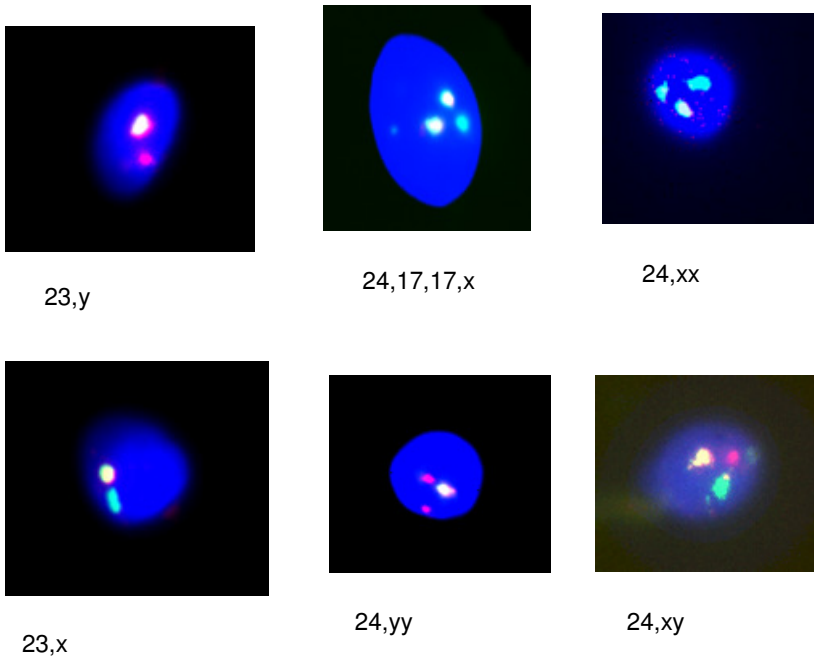

Figure 10. Multicolor FISH labeling on decondensed sperm nuclei (centromere-specific probes for X, Y and 17). Chromosome X appears as green, chromosome Y as red, meanwhile chromosome 17 as yellow. Nucleuses counterstained by DAPI appear as blue. On the left two pictures of nucleuses with haploid set. On the other pictures variable forms of disomies can be demonstrated.

To decrease the subjectivity of the observations in scoring the chromosomal integrity, we used following criteria, (Martin *et al.,* 1996) overlapped spermatozoa or sperm heads without a well defined boundary were not evaluated; (ii) in cases of disomy or diploidy, all signals should have the same intensity, and be separated from each other by a distance longer than the diameter of each signal; and (iii) nullisomies were not directly scored and were conservatively considered as equivalent to the incidence of disomies (Blanco *et al.*,

1996; Martin *et al.*, 1996; Kovanci *et al.*, 2001; Celik-Ozenci *et al.*, 2003; Celik-Ozenci *et al.*, 2004; Jakab *et al.*, 2005). We detected the presence of disomy 17, XX, YY, XY and diploidy and the nuclear maturity by AB in each cells, scoring at least 8.000 spermatozoa per case.

Designs of the experiment of double probing same individual spermatozoa for persistent histones, surplus cytoplasm, apoptosis and DNA fragmentation

For this experiment, oligozoospermic or low normal sperm concentration samples were selected in order to achieve an adequate representation of arrested/diminished maturity spermatozoa. Approximately 5600 double stained spermatozoa were studied, originating from semen samples from 13 men (mean sperm concentration: 25.0 ± 6.2 x 10^6 sperm/ml, motility: 36.2 ± 6.5 %).

In series of studies, two biochemical probes were applied on the same sperm cell following the experimental scheme of the steps were as follows. The spermatozoa, smeared onto glass slides, were fixed with methanol-acetic acid, and the sperm cells were stained with AB to detect the presence and degree of persistent histones. Images of sperm fields and individual spermatozoa were then captured using the Metamorph™ imaging program (Universal Imaging Co. Downingtown, PA, USA) (Celik-Ozenci *et al.,* 2003). Subsequently, with the slides still on the microscope platform, the X-Y co-ordinates of the fields were determined, in order to facilitate the re-localization of the same sperm fields. Following this step, the sperm slides were de-stained from AB by overnight incubation in the fixative solutions that were appropriate for the second biochemical markers (methanol for ISNT, and paraformaldehyde for the immunocytochemistry studies). In further steps, the de-stained spermatozoa were treated with one of the second biochemical probes: (I) CK immunocytochemistry to demonstrate cytoplasmic retention in diminished-maturity spermatozoa (Huszar *et al.,* 1993); (ii) ISNT for detection of DNA chain breaks (Irvine *el al.,* 2000); and (iii) caspase-3 immunostaining to detect the apoptotic process in spermatozoa (Cayli *et al*., 2004). Following the staining steps with the second probes, the previously noted X-Y co-ordinates were used to re-locate the same fields that were captured by the Metamorph ™ program following the AB staining. Next,

the images of the same spermatozoa treated with the second probe were captured, in order to compare side-by-side the same spermatozoa stained with AB and the second probe applied.

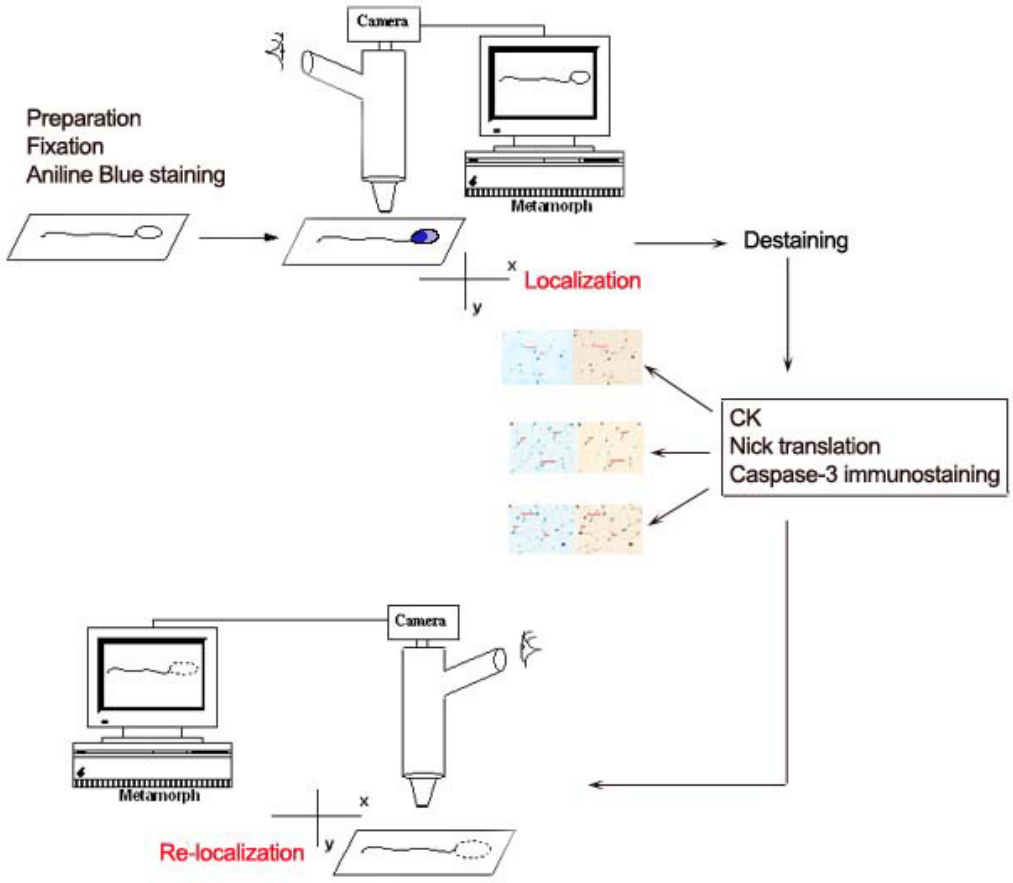

Figure 4. Flow chart of the experimental design of double probing for aniline blue staining followed by in situ nick translation, caspase-3 and creatine kinase of the same spermatozoa

Immunostaining of spermatozoa for CK

After recording the sperm fields, the AB stained sperm cells were destained with 0.5% paraformaldehyde incubation in phosphate buffer/sucrose (PB-suc) overnight at ambient temperature. All de-staining procedures were carried out on a shaking platform. The

formaldehyde was removed by three washing steps with PB-suc, the slides were allowed to air dry, and the spermatozoa were exposed to a 3% bovine serum albumin (BSA) blocking solution in PB-such at room temperature. After further washing, the spermatozoa were overlaid with a 1:1000 dilution of polyclonal anti-CK-Bantisenim (Chemicon Co, Temecula, CA, USA). Following further PB-suc washes, the slides were treated with a biotinylated second antibody (Sigma-Aldrich. Milwaukee. WI) at a 1:1000 dilution and were exposed to a Vector horseradish peroxidase/ABC kit (Vector Laboratories, Buriingame, CA, (USA) according to the manufacturer's instructions. The avidin-biotin complex (ABC)-treated slides were further processed with diaminobenzidine and hydrogen peroxide (Sigma, St Louis, MO, USA). The developed brown color highlighted spermatozoa with various degrees of cytoplasmic retention (**Figure 5.**). The specificity of the CK staining was established by using pre-immune serum (Sigma-Aldrich, Milwaukee, WI, USA) in place of the first antibody, or by applying the second antibody only. In these experiments 1284 spermatozoa were studied (four men, concentration: 10.9 ± 2.2 x 10^6 /ml, motility: 32.5 ± 4.8%, 321 sperm/man, range: 209-610).

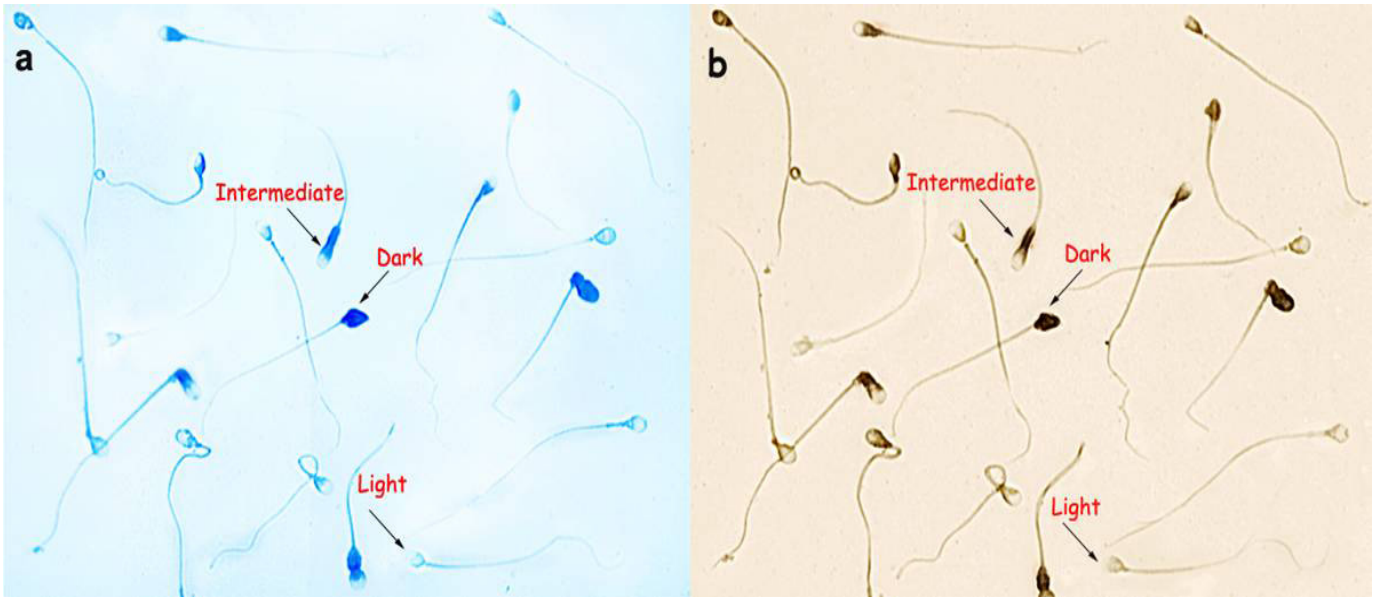

Figure 5. (a) Acidic aniline blue staining and (b) creatine kinase (CK)-immunostaining of the same spermatozoa. Note the substantial degree of similarity in the light-, intermediate- and dark-staining patterns with aniline blue and CK.

Immunostaining of spermatozoa for caspase-3

The de-staining and other procedures for caspase-3 immunocytochemistry were carried out similarly to those described for CK immunostaining (Cayli *et al., 2004*). However, after the spermatozoa were exposed to the *We* BSA blocking solution, they were treated with a 1:300 dilution of active caspase-3 (PharMingen, San Diego, CA. USA) antibody overnight at 4°C. Further, the slides were processed with 1:1000 dilution of a biotinylated anti-rabbit second antibody (Vector Laboratories). The brown color representing the caspase-3 content of spermatozoa was developed by the ABC method (Vector and Sigma; **Figure 6.**). The specificity of staining was established by applying the secondary antibody only. These studies were carried out in 2101 spermatozoa (4 men, sperm concentration: 11.9 ± 1.4 x 10^6 sperm/ml, motility: 34.0 ± 6.6%).

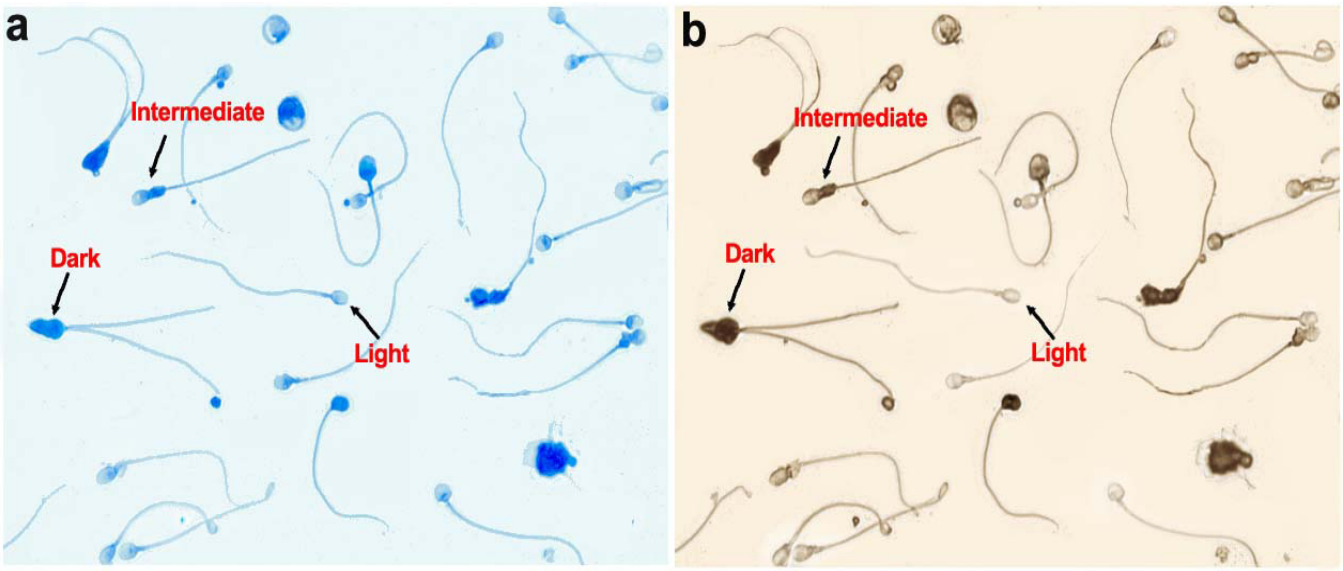

Figure 6. (a) Acidic aniline blue staining and (b) caspase-3 immunostaining of the same spermatozoa. Note the similarity in the light-, intermediate- and dark-staining patterns of the aniline blue and caspase-3 panels. Also, caspase-3 immunostaining is present in the mid-piece of intermediate-type spermatozoa, whereas in dark spermatozoa with more extensive maturity arrest both the head and the mid-piece are stained (a and b).

Assessment of DNA integrity by nick translation

The basic concept of the assay is as follows. The enzyme DNA polymerase repairs DNA strand breaks by incorporation of nucleotides. However, one of the repair elements is biotin-labeled dUTP which serves as the anchor for avid in conjugated horseradish peroxides. Thus, after the assay, the horseradish peroxidase-generated color in a spermatozoon is proportional to the extent of DNA breaks and repair (Irvine *et al. 2000).* All steps were carried out at room temperature. After recording of the fields, the AB stained sperm cells were destained in 30% methanol overnight. Further, the slides were covered with 20 mmol/l of imidazole buffer pH: 7.0 for 1 h, and 30% methanol applied for 15 min. followed by air drying. The slides were further treated with methanol-glacial acetic acid (3:1) for 15 min. After exposure to a dehydrating ethanol series (70, 85. 100%). the slides were air dried and treated with 10 mmol/l dithiothreitol (DTT) (in 100 mmol/l Tris, pH: 7.2) for 30 min to initiate DNA decondensation. After a washing step

with 100 mmol/l Tris, pH 7.2, the slides were exposed to 10 mmoI/1 lithium diiodosalicylic acid in 100 mmol/l Tris. pH 7.2 for 3 h. Following further washing with PBS the slides were blocked with biotin (1:10 dilution of a 0.01% stock) for 20 min, washed again, and then blocked with avidin (1:10 dilution of a 1 mg/ml solution) for 20 min. Further, the slides were exposed to a solution containing 1 mmol/l DTT. 10 mmol/l MgSO4. 50 mmol/l Tris-HCI. pH 7.2. 0.01 mmol/l biotin-16-dUTP and a 0.01 mmol/l mix of dGTP, dCTP, dATP and DNA polymerase I (0.025 IU/ml) for 30 min. The repair with biotin-labeled nucleotides was detected by avidin-biotin horseradish peroxidase using the Vector ABC method (Vector Laboratories: all other chemicals were from Sigma). After further washing, the slides were developed in 3, 3-diaminobenzidine solution for 10-15 min. The slides were counterstained with Coomassie Blue (0.04%. in 25% isopropanol. 10% glacial acetic acid) for 30 s. in order to optimize visualization of the sperm contours. The slides were then washed, and mounted with Permount (Sigma-Aldrich, Milwaukee, WI) (**Figure 7.**). In these experiments 2446 spermatozoa were studied (5 men, sperm concentrations: 17.6 ± 1.1 x 10^6 sperm/ml, motility: 38.4 ±5.9%, 489 sperm/ man; range; 276-602).

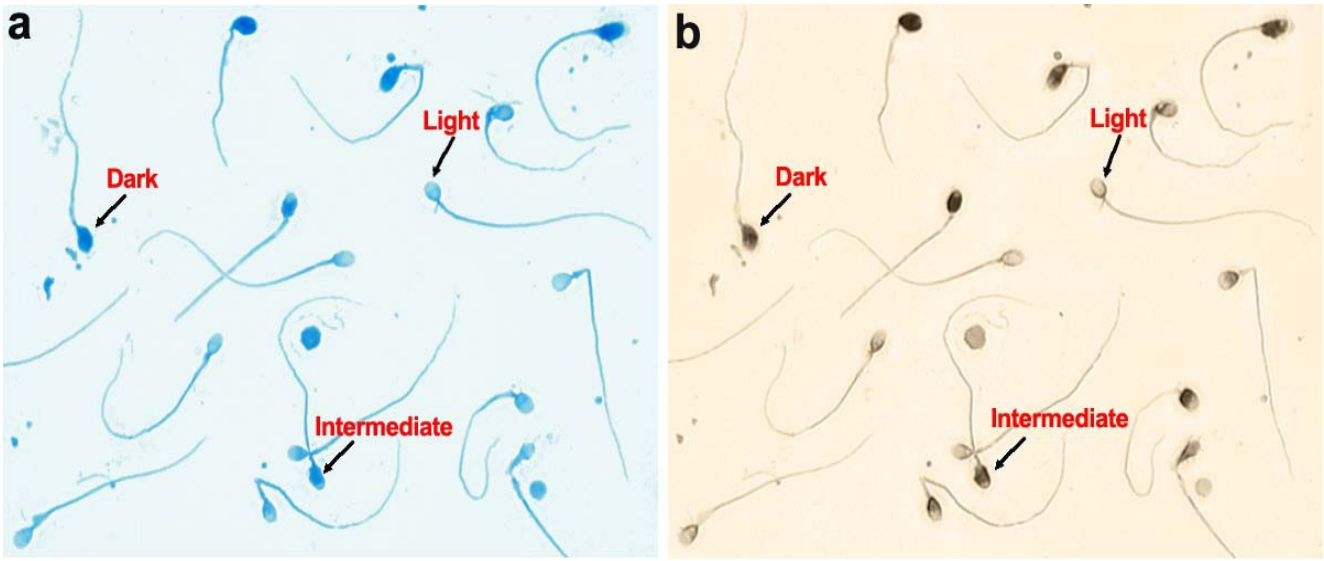

Figure 7. Aniline blue staining (a) and (b) DNA in situ nick translation of the same spermatozoa. Note the substantial degree of similarity in the light-intermediate- and dark-staining patterns with the two methods.

Evaluation of sperm morphology

Sperm smears were dried on glass slides and stained with Diff-Quik (Dade-Behring, Newark. USA) according to the manufacturer's instructions. The slides were scored by two investigators in a blinded manner according to the Tygerberg criteria (Kruger *et al.,* 1986. 1988: Menkveld *et al.,* 1990; WHO. 1999). The results were averaged for each slide.

Experimental design of dimensional assessment of haploid and disomic sperm with use of FISH and objective morphometry

The first group of six subjects who were studied specifically for this morphology experiment was composed of three oligozoospermic men (sperm concentrations 9.3±2.3 x 10^6 sperm/ml range (5-13); motility 39.7%-5.5% (range 30%-49%) and three normozoospermic men (sperm concentrations 46.5±21.0 x 10^6 sperm/ml range (25-135)); motility 46.7%-4.6% (range 40%-50%). Approximately 200 sperm from each of the six men (n: 1,227 in all) were subjected to digitalization and images were evaluated by using morphometry with computer-based Metamorph program in native nondecondensed state. Subsequently sperm were subjected to FISH for identification to determine whether the chromosomal constitution haploid or disomic. We found 24 disomic spermatozoa in this group of 1,227 sperm. (**Figure 11**, **12**)

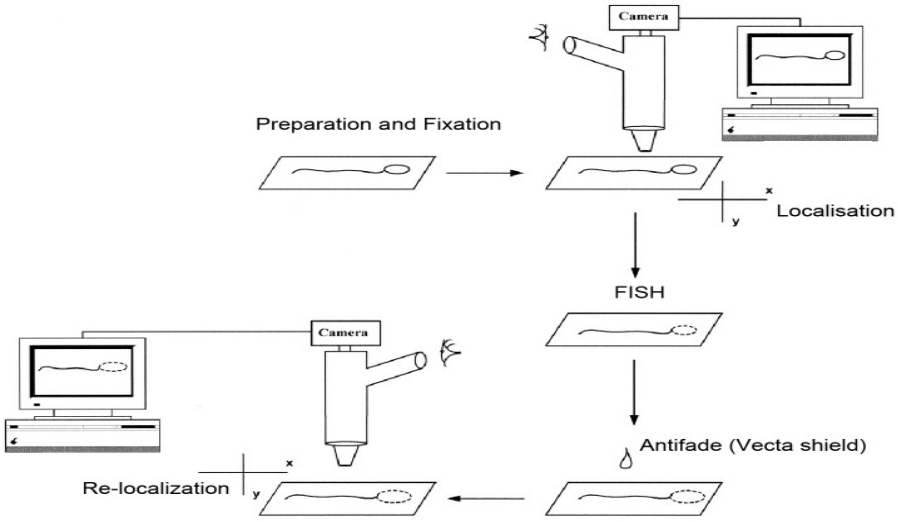

Figure 11. Flowchart of the experimental design in the study on differences of sperm morphology between haploid and disomic spermatozoa.

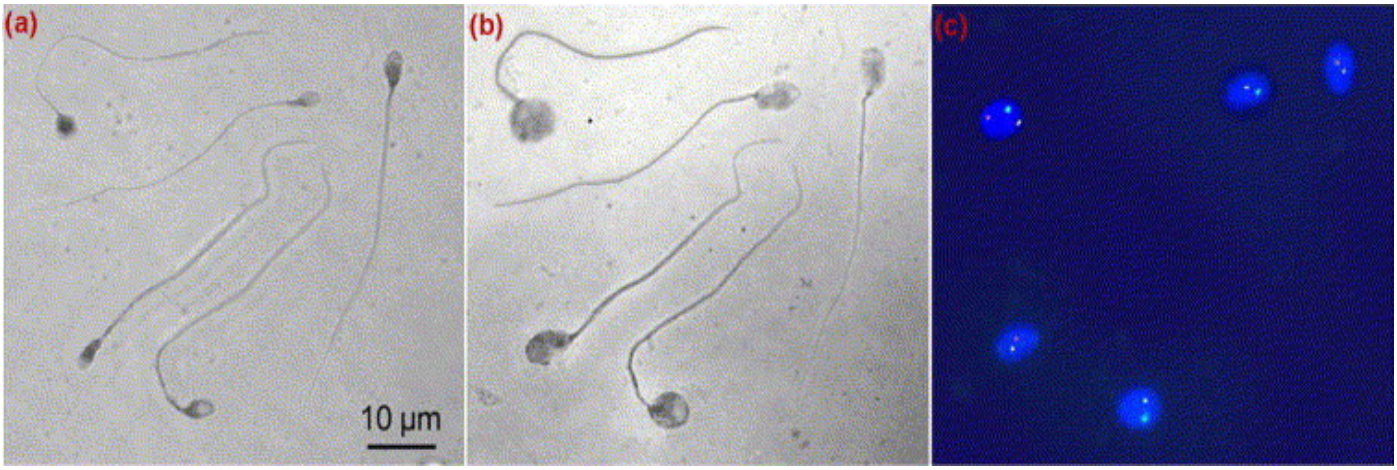

Figure 12. Phase-contrast images of (**a**) nondecondensed and (**b**) decondensed spermatozoa. (**c**) FISH labeling of X (*green*), Y (*red*), and somatic 17 (*yellow*) chromosomes.

In the second phase of the studies, sperm slides originating from 20 men (concentration 24 ± 2.5 x 10^6 sperm/m (range: 8.9–51); motility 52.5% ± 2.5% (range 39%–84%)) used in earlier studies of Kovanci *et al.,* 2001 and Jakab *et al.,* 2005 were reused for this dimensional studies in order to find more disomic spermatozoa in the decondensed sperm populations. In all, 178 disomic sperm images were found (75 XX, 63 XY, and 40 YY nuclei).

Phase-contrast imaging of Individual sperm Cells

In the first step of the phase contrast and FISH procedure, sperm in the native non decondensed state were photographed by Hammamatsu C 4880 water–cooled CDD camera attached to an Olympus AX-70 microscope. Approximately 1000 sperm images from each patient were captured and recorded using phase-contrast microscopy at 1,000x magnification and the computer-based Metamorph program. We studied all sperm in each randomly selected field. The 1,000 sperm captured from each man yielded approximately 2-300 properly evaluated sperm, which showed no overlap of the sperm head, clearly visible tails, and all parts of the image in the same planes. In order to relocalize the sperm after FISH procedure used orientation scratches on the slides and noted horizontal and vertical coordinates of microscope stage. Following the first capture step, the slides were then dehydrated in a series of 70%, 80%, 100% ethanol and stored for FISH identification.

Metamorph Evaluation of Sperm Dimensions

Metamorph evaluation is initiated by a thresholding feature adjusted for each sperm image, which distinguishes sperm from the background. The sperm tail is delineated manually using an optical mouse device. The measurements of individual spermatozoa were carried out according to the following parameters: *Head area:* the total number of pixels within the head area multiplied by the pixel size in microns as determined by camera calibration; *perimeter:* the distance around the edge of the object, measuring from the mid-points of each pixel that defines its border; *long axis:* the longest diameter through the head; *short axis:* the longest diameter through the head perpendicular to the long axis; *tail length:* the length of the tail, including the midpiece; *shape factor:* defined as a value from 0 to 1 representing how closely the sperm head represents a circle, calculated as (4π &times area)/perimeter^2, where a value of 1 indicates a perfect circle; *Elliptical form factor*: calculated as long axis/short axis.

Relocalization and identification of formerly photographed fields with characteristic sperm configurations were performed by referring to the orienting scratches on the slides, and to the previously noted stage coordinates of the microscope. Each cell was labeled directly next to the computer image as X-bearing or Y-bearing haploid or as XX, XY, or YY disomic.

Calibration of the system was carried out by photographing a 0.01-mm grid micrometer scale with the optics utilized for the morphometry studies; 1 pixel was set as 0.1367 µm.

Reproducibility of the measurements was tested by measuring 20 individual cells (4 sperm from each of 5 patients) five times. Repeated measures analysis of variance indicated no detectable differences between the measurements. Average coefficient of variation percentages (range) were: head area 1.1% (0.1%–2.6%); perimeter 0.9% (0.02%–1.8%); long axis 1.3% (0.1%–2.5%); short axis 1.0% (0.02%–2.5%); shape factor 1.3% (0.4%–2.1%); elliptical form factor 1.5% (0.7%–2.8%); tail length 0.7% (0.3%–1%).

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FISH procedure and scoring

The FISH procedure and scoring of FISH signals in this study were similar done as it was detailed formerly in the “aniline blue-FISH study” design.

Statistical analysis of the three experiments

In the study of consecutive double probing same individual spermatozoa by AB and FISH for analysis of significance between disomy rates in the clear cells vs. intermediate cell group the Mann-Whittney U test was applied. For evaluation the relationship between the frequency of the intermediate cells and the frequency of cell with disomies in particular semen samples the Pearson Correlation test was used.

In the study where spermatozoa were double probed by AB, CK and NT differences among groups, and the various morphometric parameters, were compared using one-way analysis of variance (ANOVA) on normally distributed data, and one-way ANOVA on ranks test on data that were not normally distributed. Following ANOVA Dunn's post hoc test was performed. Data analysis was carried out using R statistical software (R Core Development Team. Vienna, Austria) and Sigma-Stat 2.0 (Jandel Corporation). All data are presented as mean ± SEM. In order to compare the level of agreement between probe pairs in the same spermatozoa, weighted kappa analysis with quadratic weights was used. A rating scale for kappa, suggested by Altman (1991) indicates that a kappa value >0.8 represents an excellent level of agreement.

In the study where dimension differences was assessed, for the evaluation of the significance of the difference between haploid and aneuploid cells the Mann-Whitney U test, Student's t test with Tukey post hoc test was used. The level of significance was set at $P<.05$.

Results

Aniline blue and FISH study

In the 7 moderately oligozoospermic men, we have evaluated a total of 58,793 spermatozoa (**Table 1.**).

Men	Sperm Conc. 10^6/ml	Motility (%)	Cells scored No.	AB Light (%)	AB Intermediate (%)	AB Dark (%)
1	21,0	38,0	8174	84.6	11.5	3.9
2	18,0	38,0	9155	75.5	20.9	3.6
2	10,0	49,0	8193	73.9	21.8	4.2
4	10,0	25,0	8322	69.5	23.9	6.7
5	16,0	47,0	8372	69.6	26.2	4.2
6	16,0	49,0	8721	75.0	19.0	6.0
7	13,0	29,0	7856	63.8	29.0	6.5
means±SEM	14.8 ±1.5	39.2 ± 3.6	8399 ±159.6	73.1 ±2.3	21.8 ±2.1	5.0 ±0.5

Table 1. Sperm attributes of the seven semen samples evaluated in study of double probing of individual spermatozoa by aniline blue and Fluorescent in-situ hybridization. Fertility and Sterility 2009.

Regarding nuclear maturity, the proportion of the light, intermediate and dark AB stained sperm (representing mature, intermediate maturity, and arrested maturity spermatozoa) were 73.1±2.3%, 21.8±2.1% and 5.0±0.5%, respectively. The distribution of sperm with various staining intensity was fairly uniform. Assessment of the sperm population with FISH indicated that the mean frequency of aneuploidies follows: disomy X: 0.07±0.01%, disomy Y: 0.09±0.02%; disomy XY: 0.18± 0.03%, and disomy 17: 0.16±0.03%. The frequencies of diploidy were 0.17± 0.04%.

In the sperm groups of various AB staining intensity or nuclear maturity, the frequency of each of the four disomies and total disomy was higher in the intermediate compared to the light staining (mature) sperm. Indeed, the increase of frequencies were 3.9x in X disomy, 5.2x in Y disomy, 6.1x in XY disomy, 4.5x in 17 disomy, and 5.3x higher in the aggregate or total disomy rates (all comparisons, except X disomy, are $P<0.001$) (**Table 5.**). There was also a higher incidence of diploidy in the intermediate compared to the light cells, but it did not approach significance.

In light of our hypothesis, regarding the correlation between the variations of disomy frequencies vs. the AB attributes, the findings were confirmatory**:** (1) There was a relationship between the frequencies of various disomies and sperm with light and intermediate AB staining, particularly between the proportions of intermediate sperm and frequencies of disomy XY (r=0.82, $P<0.05$), disomy 17 (r=0.70, $P< 0.05$), and total disomy frequencies (r=0.76, $P<0.05$)**. Figure 14.** is a graphic presentation of these correlations. There was no correlation between AB staining and diploidies.

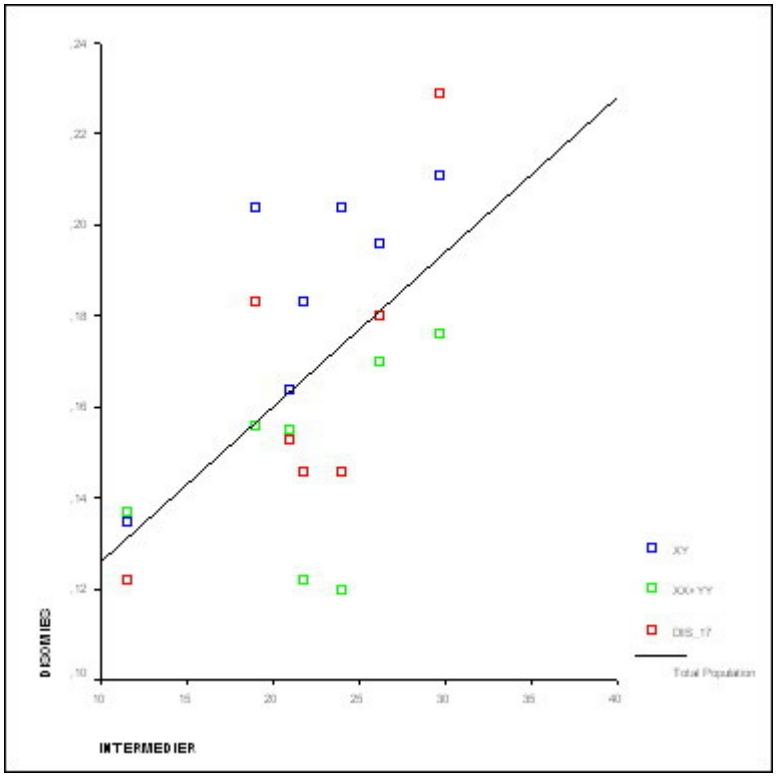

Figure 14. Correlation of frequency of disomies and spermatozoa with intermediate nuclear immaturity in the same semen sample (r=0.6, P =.002)

There was an unexpected finding with respect to the FISH patterns in the case of AB dark, the arrested maturity spermatozoa. There was no chromosome signal with the locus specific FISH probes. Further, some of the dark staining sperm also lacked DAPI nuclear staining, indicating a diminished presence of DNA **(Figure 13.)**.

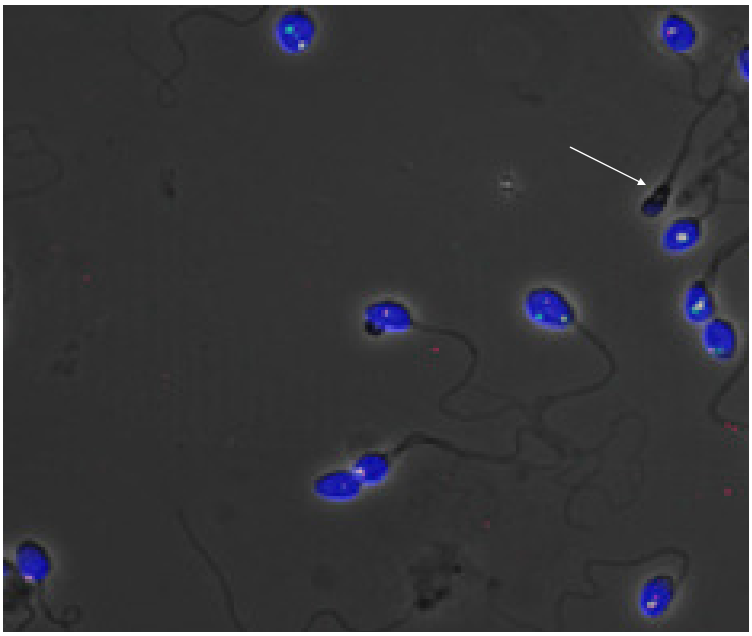

Figure 13. FISH image of spermatozoa after aniline blue staining. Arrow indicates a spermatozoon that shows no hybridization and scarcely uptakes DAPI counter staining; that cell was scored as "dark" by aniline blue staining. FISH labeling of gonosomal X (appears *green*), Y (*red*), and autosomal 17 (*yellow*) chromosome.

Multiply probing of same spermatozoa by CK immunohistochemistry, aniline blue staining and nick translation

Approximately 5600 double stained spermatozoa were studied, originating from semen samples from 13 men (mean sperm concentration: 25.0 ± 6.2 x 10^6/ ml. motility: 36.2 ± 6.5%).

Double probing of spermatozoa with CK-immunocytochemistry and AB staining

In the first part of this experiment spermatozoa were double stained with nuclear and cytoplasmic markers of arrested sperm maturation. Initially, AB was applied to highlight the presence of persistent histones, and this step was followed by CK immunocytochemistry. As **Figure 5.** indicates, the spermatozoa stained light, intermediate or dark, represent spermatozoa that are mature, intermediate maturity or diminished maturity respectively. There was good agreement between the two staining patterns with the AB and CK probes. The dark spermatozoa showed high degrees of persistent histones as well as retained cytoplasm. A quantitative analysis of the 1284 cells evaluated is presented in **Table 2**.

	CK staining		
Aniline blue staining	Light	Intermediate	Dark
Light	511 (39.8)	117(9.1)	3 (0.2)
Intermediate	34 (2.6)	313(24.4)	34 (2.6)
Dark	1 (O.l)	47 (3.7)	224 (17.4)

Table 2. Double probing of spermatozoa with aniline blue and creatine kinase (CK) immunocytochemistry. Total of 1284 spermatozoa studied from four men (sperm concentration: 10-9 ± 2.2 X 10^6/ml. motility: 32.5 ± 4.8%). Values are actual numbers of spermatozoa (% of total). RBMOnline 2008.

The actual number of spermatozoa with light-light, intermediate-intermediate and dark-dark staining, and the proportion of spermatozoa expressed as a percentage of the total, is presented. The majority of the spermatozoa follow a light-light (approximately 40%), intermediate-intermediate (approximately 25%) and dark-dark (approximately 18%) pattern. The representation of intermediate-light or intermediate-dark is approximately 18%, whereas spermatozoa with the discordant staining pattern of light-dark and dark-

light occur only in <1% of the cells. Thus, 82% of the spermatozoa showed conforming staining patterns with the biochemical markers of nuclear (persistent histones) and cytoplasmic (cytoplasmic retention) probes of sperm maturity/arrested maturity.

Double probing of the same spermatozoa with AB and for DNA chain fragmentation with ISNT

In this study, similar to the previous experiment, the sperm fields were stained first with AB, ISNT was subsequently performed on the same cells. **Figure 7.** demonstrates a substantial agreement between the staining patterns with the two nuclear markers. In **Table 3.** the distribution of the 2446 spermatozoa studied with both probes is presented.

	DNA nick translation		
Aniline blue staining	Light	Intermediate	Dark
Light	1261 (51.6)	162 (6.6)	20 (0.8)
Intermediate	80 (3.3)	498 (20.4)	71 (2.9)
Dark	8 (0.3)	55 (2.2)	291 (11.9)

Table 3. Double probing of spermatozoa with aniline blue and nick translation. Total of 2446 spermatozoa studied from five men (sperm concentration: 17.6 ± 1.1 x 10^6 /ml. motility: 38.4 ± 5.9%). Values are actual numbers of spermatozoa (% of total). RBMOnline 2008.

The overwhelming majority of spermatozoa showed staining patterns that were conforming, as light-light (n=1261), intermediate-intermediate (n= 498), and dark-dark (n = 291).The data are also expressed as a proportion of all spermatozoa, as light-light (approximately 52%), intermediate-intermediate (approximately 20%) and dark-dark (approximately 12%), 84% in all. Sperm cells with heterogeneous staining of light-intermediate, dark-intermediate, intermediate-light or intermediate-dark, were approximately 15% of the population, whereas the discordant light-dark or dark-light

spermatozoa represented approximately 1.2%. As with the AB-CK experiment, 84% of the spermatozoa showed an identical staining pattern between AB and DNA fragmentation. Thus, spermatozoa with arrested maturity and persistent histones also exhibited a high level of DNA degradation (about 12% of the population), and about 52% of the sperm population tested normally developed mature cells, devoid of both persistent histones and DNA fragmentation.

Double probing of spermatozoa with AB and caspase-3

In **Figure 6.** fields of spermatozoa double stained with AB and caspase-3 immunostaining can be seen. As **Table 4.** indicates 2101 spermatozoa were evaluated for the staining patterns.

	Caspase-3 staining		
Aniline blue staining	Light	Intermediate	Dark
Light	1047 (49.8)	123 (5.9)	3 (0.1)
Intermediate	77 (3.7)	483 (23.0)	46 (2.2)
Dark	0 (0.0)	61 (2.9)	261 (12.4)

Table 4. Double probing of spermatozoa with aniline blue and caspase-3. Total of 2101 spermatozoa studied from four men (sperm concentration: 11.9 ± 1.4 x 10^6/ml, motility: 34.0 ± 6.6%).Values are actual numbers of spermatozoa (% of total). RBMOnline 2008.

There was light-light staining in 1047 spermatozoa, intermediate-intermediate pattern in 483 spermatozoa and dark-dark staining in 261 cells. Light-light pattern occurred in approximately 50% of the spermatozoa, intermediate-intermediate pattern in 23% and dark-dark diminished maturity pattern in about 12% of spermatozoa. Thus, as with the other cytoplasmic and nuclear markers **(Tables 2. and 3.**), approximately 85% of the double stained spermatozoa showed conforming staining patterns with AB and the apoptotic marker. Similarly to the other markers, intermediate-light or intermediate-dark

sperm staining occurred in about 14% of the cells, and the discordant pattern of dark-light and light-dark staining was <1.0%.

Sperm shape and sperm maturity

Sperm morphology evaluated according to the Tygerberg strict criteria seems to be related to the sperm biochemical maturity because the abnormal head and mid-piece shapes, and abaxial insertion of the tail occur as a consequence of cytoplasmic retention in spermatozoa with arrested spermatogenetic maturation. The shorter sperm tail, characteristic for spermatozoa with arrested maturity, is also a component of the Kruger strict morphology evaluation.

The relationship between Tygerberg normal morphology and AB staining intensity was studied in five samples by counting approximately 400 spermatozoa for each patient (n = 3882 in all, evaluated by two investigators independently). Within the groups of spermatozoa that stained light (normal spermatozoa), intermediate (intermediate maturity) and dark (diminished maturity), the proportion of Tygerberg normal spermatozoa were 9.5 ± 1.3, 2.3 ± 0.9 and 0.5 ± 0.3% respectively. There were significant differences in the light versus intermediate ($P< 0.05$), and intermediate versus dark groups ($P< 0.01$). Thus, there is a relationship between the proportion of spermatozoa with various degrees of arrested maturity and abnormal Tygerberg morphology.

Inter-subject variation in staining patterns with the various probes

In order to better evaluate the validity of the two probe staining pattern data, variations among the men studied with each probe combination are presented. Within the group of four men whose spermatozoa were studied with the AB-CK immuno-probe combination, the proportion of light-light spermatozoa was 36.0 ± 4.4% (mean ± SEM), and in the individual men 34.4, 30.3, 30.4 and 48.9%, respectively, the mean incidence of intermediate-intermediate staining was 24.9 ± 4.1% (25.8, 15.8, 35.4 and 22.8%), whereas spermatozoa with dark-dark pattern were present in 21.1 ±5.9% (28.7, 33.3, 12.7

and 9.5%). In the group of five men whose spermatozoa were studied with aniline blue-DNA nick translation, the mean light-light pattern was 52.0 ± 6.7% (54.8, 66.4, 32.9. 55.2 and 50.8%), the mean intermediate-intermediate pattern was 19.7 ± 1.9% (20.6, 16.6, 25.9, 20.5 and 15.0%). The dark-dark pattern was 12.2 ± 1.7% (12.5, 7.2, 16.1, 9.8 and 15.4%). Finally, within the group of four men whose spermatozoa were double stained with aniline blue-caspase 3, the proportion of light-light spermatozoa was 49.4 ± 6.7% (44.5, 56.7, 33.2 and 63.3 %) and the intermediate-intermediate stained spermatozoa were 23.3 ± 3.5 (30.3, 26.4, 20.9 and 14.4%), Further, the dark-dark spermatozoa were 12.7 ± 5.5% (11.3, 3.5, 28.3 and 7.6%).

Interpretation of the staining patterns: a close relationship among probes

The consistently high agreement (83-85% overlap) of probe signal within the mature, intermediate and arrested/diminished maturity spermatozoa indicates that cytoplasmic retention. DNA chain fragmentation and the presence of the apoptotic process are all related to persistent histones. This relationship among the various nuclear and cytoplasmic probes was quantified with regression analysis based on the weighted kappa method. There was a very close correlation (kappa= 0.8) among the attributes of arrested maturation within the same sperm cell. Thus, double probing of the same spermatozoa is an extremely sensitive demonstration of the proportion of mature and immature spermatozoa in semen.

Dimensions of Haploid vs. Disomic Spermatozoa

The comparison between haploid and disomic native nondecondensed spermatozoa is based on the 1,227 versus 24 spermatozoa (**Table 6.**).

	Sperm	**Head area (μm^2)**	**Perimeter (µm)**	**Long axis (µm)**	**Short axis (µm)**	**Shape factor**	**Elliptical form factor**	**Tail length (µm)**
Native								
Haploids	1,227	16.6 ± 0.1	16.2 ± 0.1	6.09 ± 0.02	3.80 ± 0.01	0.79 ± 0.01	1.62 ± 0.01	53.3 ± 0.2
Disomies	24	21.9 ± 1.8	18.3 ± 0.7	6.59 ± 0.2	4.63 ± 0.2	0.79 ± 0.01	1.47 ± 0.05	52.0 ± 0.6
Differences		32%	13%	+8.2%	+21.8%	0%	−10.2%	−2.5%
P value		.001	.001	.04	<.001	NS	.01	NS
Decondensed								
Haploids	600	30.9 ± 0.3	22.1 ± 0.1	7.66 ± 0.04	6.41 ± 0.03	0.79 ± 0.01	1.20 ± 0.01	60.9 ± 0.4
Disomies	178	34.2 ± 0.9	22.8 ± 0.3	7.85 ± 0.09	6.77 ± 0.08	0.81 ± 0.01	1.41 ± 0.01	58.7 ± 0.6
Differences		+10.6%	+3.1%	+2.5%	+5.6%	+2.5%	+17.5%	−3.7%
P value		.02	NS	NS	.001	NS	.02	.002

Table 6. Dimensions of haploid and disomic sperm in the native nondecondensed (n = 1,251 sperm; 6 men) and decondensed (n = 778 sperm; 20 men) states. All values are mean ± SEM. Fertility and Sterility 2006

It is of note that, as described in Methods, we have surveyed 6,000 spermatozoa for FISH in order to find these 1,251 morphometrically evaluable sperm cells. The 24 sperm with disomic nuclei (9, 10, and 5 sperm with XX, XY, and YY disomies, respectively) represent a 0.15%, 0.16%, and 0.08% (aggregate 0.39%) frequency of sex chromosome disomies. This proportion is in agreement with data from our own and other laboratories

In comparing the X- and Y-bearing decondensed spermatozoa, the 600 haploid and 178 disomic spermatozoa collected from previous studies provided a solid basis for comparison (**Table 6.**). As in the nondecondensed state, the mean dimensions of the disomic sperm were larger. However, a detailed comparison of size distributions indicated that the dimensional differences were inconsistent (**Figure 15.**).

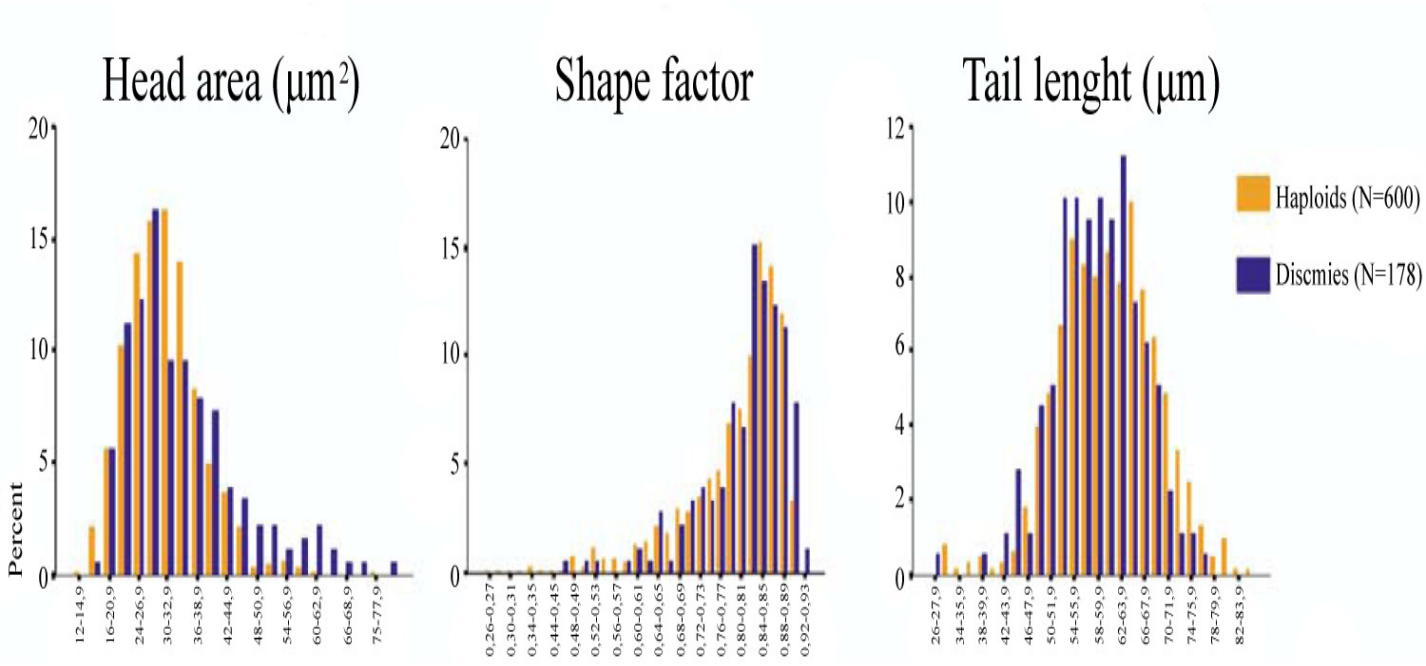

Figure 15. Dimensional distribution of haploid and disomic decondensed spermatozoa. Data of haploid spermatozoa are demonstrated in brown, disomies are blue.

For instance, in the head area category it is clear that the majority (>60%) of the disomic sperm overlap in size with the haploid sperm. However, some disomic sperm are larger and others are smaller than haploid sperm. The latter group indicates the occurrence of disomies in microcephalic sperm. The right side of the curve suggests that approximately 20% of disomic spermatozoa show head sizes larger than haploid sperm. A similar difference in size distribution may also be detected in the other sperm shape attributes, for instance, in the shape factor or tail length. Conversely, approximately 70% of the haploid and disomic sperm coincide in size ranges. The distribution of the nondecondensed haploid and disomic sperm follow the same patterns.

We have also addressed potential dimensional differences between sperm with YY, XX, and XY disomies (**Table 7.**). In the decondensed sperm population, The 40, 75, and 63 disomic sperm indicated dimensional differences among sperm with the three types of disomies. Assuming that the chromosomal mass would play a role in sperm head dimensions, one would expect that sperm with the XX-bearing nuclei would have the largest head, followed by the XY and then the YY-bearing spermatozoa. However, in all four head dimension parameters the YY-bearing sperm were larger by approximately 10% (6%–12%, bold entries in **Table 7.**) compared to the dimensions of XY and XX

sperm, which were similar. Thus, it is likely that the variations are consequences of diminished sperm maturity and the decondensation process and not related to the chromosomal mass. This notion is further supported by the finding that the sperm maturity marker, tail length/long head axis ratio of disomic sperm, is significantly lower compared to that of haploid spermatozoa (in nondecondensed sperm 7.9±0.3 vs. 8.8±0.04; in decondensed sperm 7.4±0.13 vs. 8.0±0.06 (*P*=.02 and .005, respectively).

	Sperm	Head area (μm^2)	Perimeter (µm)	Long axis (µm)	Short axis (µm)	Shape factor	Elliptical form factor	Tail length (µm)
Native								
YY-bearing	5	19.8 ± 2.5	17.1 ± 0.9	5.9 ± 0.2	4.6 ± 0.5	0.83 ± 0.01	1.34 ± 0.08	50.4 ± 1.7
XX-bearing	9	21.1 ± 3.5	17.9 ± 1.4	6.4 ± 0.4	4.6 ± 0.5	0.78 ± 0.02	1.42 ± 0.08	49.3 ± 1.7
XY-bearing	10	23.7 ± 2.8	19.1 ± 1.0	7.1 ± 0.3	4.6 ± 0.4	0.79 ± 0.02	1.57 ± 0.09	55.2 ± 3.2
Decondensed								
YY-bearing[a]	40	**38.3 ± 1.9**	**24.5 ± 0.6**	**8.20 ± 0.2**	**7.22 ± 0.2**[b]	0.79 ± 0.01	1.15 ± 0.02	59.9 ± 1.1
XX-bearing	75	32.1 ± 1.3	22.0 ± 0.4	7.70 ± 0.2	6.51 ± 0.1	0.82 ± 0.01	1.19 ± 0.01	59.1 ± 0.9
XY-bearing	63	34.1 ± 1.5	22.7 ± 0.5	7.8 ± 0.2	6.78 ± 0.1	0.82 ± 0.01	1.16 ± 0.01	57.5 ± 0.9

Table 7. Dimensions of disomic native nondecondensed (n = 24 sperm; 6 men) and decondensed (n = 178 sperm; 20 men) sperm. All values mean ± SEM.

Note: One-way ANOVA, Tukey post hoc test

[a] Bold numbers represent statistical differences $P < .01–.05$ vs. XX and XY.

[b] $P < .05$ vs. XX only. Fertility and Sterility 2006.

Discussion

Attributes of sperm with arrested maturity maybe detected by various nuclear and cytoplasmic probes that were shown to be approximately 70% inter-related in individual spermatozoa, thus in most sperm the nuclear and cytoplasmic aspects of arrested maturity occur concurrently (Carrell *et al.*, 2007). It is also well established that there is a polymorphic pattern in sperm development; some of this polymorphism is due to the fact that arrest of sperm may occur at differing spermatogenetic stages. Association was also reported between sperm maturational arrest, as demonstrated by aniline blue staining of persistent histones, and numerical chromosomal aberrations the sign of disrupted meiosis in semen samples (Dadoune, 1995; Morel *et al.*, 1998; Morel *et al.*, 2001).

We think that the common contributory factor to the relationship among the cytoplasmic and nuclear features of arrested sperm maturity and aneuploidy is the diminished level of the HspA2 chaperon protein during spermatogenesis.

The developmentally regulated HspA2 is expressed in two waves; the first expression occurs in round spermatocytes, related to meiosis. Regarding the function of HspA2 it is a component of the SC, necessary for desynapsis of homologous pairs and hence for successful completion of meiosis I (Eddy, 1999). Lower expression of HspA2 may lead to meiotic arrest resulted in nondisjunction of homologous pairs therefore in aneuploidies. The second expression wave of HspA2 occurs in late spermiogenesis, simultaneously with the major maturational changes in elongated spermatids, including gradual replacement of somatic type histones to protamins, cytoplasmic externalization, remodeling of the sperm plasma membrane that facilitates the formation of hyaluronic acid receptors (Huszar *et al.*, 1998; Huszar *et al.*, 2000; Huszar *et al.*, 2007). In addition HspA2 has antiapoptotic effect (Cayli *et al.,* 2004). Lower expression of HspA2 may disrupt cellular maturation processes in spermatids and resulted in surplus cytoplasm, abnormal zona binding, and nucleoprotein replacement arrest. Additionally disrupted chaperone function may result in inadequate expression and transport of the DNA repair enzymes. The surplus cytoplasm, the fragmented DNA induces apoptotic activity also (Kaufman *et al*., 2001; Cayli *et al*., 2004).

In line with this theory several researches found correlation between of different sperm immaturity attributes studying the frequencies affected spermatozoa (Huszar and Vigue,

1994; Gergely *et al.,* 1998; Cayli *et al.*, 2003). Later studies tested the correlation between meiotic arrest and defected spermiogenesis both consequences of low expression of HspA2 of semen samples. There was strong correlation between disrupted cytoplasmic extrusion and frequency of chromosomal aneuploidy in same semen sample (Kovanci *et al.,* 2001). In another study published by Jakab *et al*. (2005) correlation was found in semen samples of oligozoospermic men between good binding features and frequency of aneuploidy.

However the intracellular correlation among different spermiogenetic abnormalities and arrested meiosis-all related to lower expression of HspA2-remained unclear. It was not known whether the arrested cellular, nuclear maturity attributes and disrupted meiosis affect the same cells.

The aim of my thesis was to evaluate this "intracellular" aspect to the HspA2 theory whether the defects related to the diminished level of the chaperon protein occur within the same individual spermatozoon. We studied the relationships between cytoplasmic and nuclear features of arrested spermiogenesis together with chromosomal nondisjunction the consequence of arrested meiosis in the same individual spermatozoon. We have also studied whether degrees of the different attributes are in agreement or there is polymorphic appearance.

In order to validate the hypothesis we pursued two experiments of consecutive double probing of individual spermatozoa. In the first experiment we evaluated the relationship between abnormalities of the nuclear compartment, between arrested nucleoprotein replacement and chromosomal nondisjunction examining individual spermatozoa. The hypothesis of the potential relationship between diminished maturation of the nucleoprotein replacement pathway, and occurrence of aneuploidies has been based on two aspects: a) In spermatozoa with arrested maturity, the low expression levels of HspA2 would adversely affect histone-transition protein-protamine replacement as well as the meiotic process (Dix *et al.*, 1996; Aoki *et al.*, 2006; Govin *et al.,* 2006; Carrell *et al.*, 2007). b) In mature sperm that underwent plasma membrane remodeling, and are able to selectively bind to solid state hyaluronic acid, there is a lack of persistent histones, as well as a low normal frequency of chromosomal aneuploidies, independently from the respective frequencies in the original semen sperm population.

In the second experiment we studied correlation between abnormalities of arrested maturation in the nuclear and cellular compartment together with abnormal morphological appearance. Our hypothesis concerning the supposed combination of various developmental defects in individual spermatozoa was as follows. The HspA2 as chaperone facilitates the assembly, folding and transport of proteins related to the sperm maturations, like protamine, DNA repair enzyme as well facilitate main protein movements necessary for the cytoplasm extrusion, therefore low expression of HspA2 expectably lead to co-occurrence of arrested nucleoprotein replacement, DNA chain brakes and surplus cytoplasm in the same cells. The surplus cytoplasm and DNA chain brakes induce apoptotic process in the same spermatozoa. The abnormal protein synthesis and movements of these cells may also result in abnormal shape development. We hypothesized also that the degree of the arrest and therefore intensity of the staining should be conforming. Additionally from technical approach we were interested whether single probing of spermatozoa is enough to evaluate maturity processes.

Technically in both experiments we employed the possibility that AB-which is a reliable probe for arrested nucleoprotein replacement-may be removed from the marked spermatozoa by simple destaining techniques. The AB stain and its solvent the acetic acid as well as destaining techniques do not have influence on the results of consecutive probing for CK, ISNT, caspase-3 and FISH. The localization-relocalization was facilitated by Metamorph™ digital imaging programs that offered possibility to evaluate the sperm Tygerberg morphology also.

We faced the technical problem that after HspA2 immunostaining of spermatozoa the FISH was unreliable hence we tested the correlation among different forms of arrested maturity and aneuploidy only, without determination of actual HspA2 expression level.

The AB staining and FISH results of > 58,000 individual spermatozoa evaluated in the first study has confirmed the validity of our hypothesis on the central role of HspA2. Regarding nucleoprotein maturity, there were sperm with light, intermediate and dark staining, in proportions reported previously (**Table 1.**). Considering the relationship between nuclear maturity and aneuploidies, in sperm with intermediate vs. light staining

pattern (or mature or moderate arrested maturity sperm, respectively), there was a 4-6x higher frequency of chromosomal aneuploidies (**Table 5.**), affecting across the board the sex and the autosomal chromosomes. There was also a correlation between disomy frequencies and AB staining of disomy 17, disomy XY and total disomy (**Figure 14.**), but not in case of disomy Y and disomy X. These findings are in agreement with previous work in which an increased frequency of chromosomal aneuploidies were found in sperm with arrested maturity, identified with a probe for surplus cytoplasm (Kovanci *et al.*, 2001).

There was also a slight increase in the frequency of diploidies in sperm with arrested maturity, but it did not follow the relationship of disomies and arrested sperm maturity, because diploidies arise from a different mechanism unrelated to sperm maturation. The disomies originates from the disrupted desynapsis of the synaptonemal complex-prophase of meiosis I., meanwhile diploidy is the consequence of meiotic I disorder affecting anaphase I checkpoint (Egozcue *et al.*, 2000; Kovanci *et al.*, 2001).

The most interesting and unexpected finding in this study was related to spermatozoa with severe arrest of nucleoprotein maturity and dark AB staining, thus high level of persistent histones. These cells showed no FISH signal, and sometimes the DAPI counter painting of sperm DNA was also missing (**Figure 13.**). We propose two potential mechanisms for the lack of FISH probe binding: 1) The low levels of HspA2 expression causes a combination of retarded histone- transition protein - protamine replacement and delayed delivery of DNA-repair enzymes, thus sperm DNA chain integrity is diminished (Aitken *et al.*, 1994; Huszar and Vigue, 1994; Aitken and Baker, 2006). 2) Sperm with arrested maturation exhibit cytoplasmic retention and increased ROS production with a combined effect of DNA chain degradation. The fragmented DNA is held in place by the secondary helical structure in intact sperm. However, following the decondensation and denaturation steps necessary for FISH, the denatured DNA fragments are released from sperm.

The close relationship between occurrence of persistent histones and chromosomal disomies support the theory of the common origin of the defects: the low expression of the HspA2 chaperon protein family affecting both meiosis and the spermiogenetic events. Our experimental findings suggest that in sperm with severe maturation arrest, both of the

proposed mechanisms may lead to the loss of FISH signal. Thus, one may surmise that the FISH results and frequencies of disomies in oligozoospermic men, with a higher proportion of arrested maturity sperm, are likely to be underestimated.

In the other experiment individual spermatozoa have been studied with probes for persistent histones combined with other probes for cytoplasmic retention, for apoptotic processes, and for DNA fragmentation with in-situ nick translation. In this experiment we also used the possibility of destaining and consecutive staining of the same cell after aniline blue probing. The other fortuitous recognition was that all these probes we chose provide light, intermediate and dark staining patterns representing mature, intermediate maturity and arrested/ diminished maturity spermatozoa respectively (Huszar *et al.,* 2003). This recognition gives rise to test the correlation among different quantity of abnormalities. In the experiment we evaluated the relationshin among the degree of arrested maturity and the sperm degree of abnormal morphology also.
In the evaluation of data approximately 84% of the spermatozoa studied showed maturity levels with AB that were consistent with the staining patterns for cytoplasmic retention, DNA chain fragmentation, and the presence of the apoptotic process. These attributes are also related to persistent histones, and presumably also to lower expression of transition proteins and protamine (Steger *et al.,* 2003; Aoki *et al*., 2006). Thus, there is a substantial agreement between the cytoplasmic and nuclear probes of maturity in individual spermatozoa. Confirming that the cytoplasmic and nuclear attributes of arrested sperm maturity are related, there was >80% agreement in the presence or absence of the cytoplasmic and nuclear biochemical marker combinations within individual spermatozoa. Thus, the majority of sperm cells with DNA fragmentation also showed diminished sperm maturity, as detected by cytoplasmic retention, AB staining and apoptotic processes. In **Figure 6**. one can observe fields of spermatozoa double stained with AB and caspase-3 immunostaining. Caspase-3 is an important marker because it is a probe of active apoptotic processes in conjunction with cytoplasmic retention, and in some spermatozoa with the expression of the anti-apoptotic protein, Bclx2 (Carrell *et al.,* 2003; Cayli *et al.,* 2004; Seli *et al.,* 2005). In addition to the relationship between arrested sperm maturity and DNA integrity, the data also contribute to the compensatory concepts of sperm DNA fragmentation, and to the limitations of antioxidant therapy and similar strategies. It is likely that a substantial proportion of spermatozoa with arrested maturation and related defects of spermatogenesis, as well as the consequential

infrastructure deficiencies, would not and could not respond to such therapeutic interventions (Gil-Guzman *et al.,* 2001; Aitken *et al.,* 2003; Steger *et al*., 2003; Suzuki *et al.,* 2003; Agarwal and Said, 2005; Comhaire *et al.,* 2005; Greco et al., 2005; Lewis and Aitken, 2005; Aitken and Baker, 2006; Ménézo *et al.,* 2007). Considering the nuclear probes, the AB-DNA fragmentation and the AB-caspase-3 combinations indicated that approximately 12% of spermatozoa showed arrested maturity, whereas with the AB-cytoplasmic retention probes there was a slightly higher, approximately 17%, incidence of spermatozoa with dark and intermediate staining. It is unclear whether these differences arises from the probe specific attributes alone, or because spermatozoa that show nuclear impact of immaturity survive at a lower rate. Approximately 15% of spermatozoa tested with any probe combination showed an intermediate-light or intermediate-dark maturity pattern. In these spermatozoa one can identify signs of arrested maturity with one probe that is not detected to the same extent with another probe. This is an important finding for two reasons. First, this heterogeneity in staining intensity or in the regional distribution of markers in the head and midpiece is in line with the polymorphic attributes of spermatozoa. Thus, even in arrested maturity the sperm cell may follow a variety of pathways {Huszar *et al.,* 2003; Huszar *et al.,* 2007). Second due to this polymorphism, it has become apparent that one can not determine sperm maturity reliably with a single probe. However, the quick AB test is very useful in detecting the proportion of spermatozoa that are suspected to be of arrested maturation in semen (Dadoune *et al.,* 1988; Foresta *et al.,* 1992; Liu and Baker, 1992; Morel *et al.,* 1998). The association between AB and caspase-3 in the same spermatozoa suggests that DNA degradation is also related to arrested maturity. An earlier study investigated why these spermatozoa with increased levels of cytoplasmic retention, caspase 3 content and DNA fragmentation survive to be ejaculated rather than, as one would expect, being eliminated by apoptosis within the adluminal area or in the epididymis (Cayli *et al.,* 2004). The probe studies demonstrated the simultaneous presence of caspase-3 and the anti-apoptotic Bclx2 protein in these surviving arrested maturity cells. Further, caspase-3 and Bclx2 were located almost exclusively together in the mid-piece. These data suggest that these spermatozoa overcome apoptotic decay due to the protective Bclx2. Additional factors that may lead to DNA strand breaks in arrested maturity spermatozoa include an impaired capacity for DNA repair due to the low levels of HspA2 chaperone, which would normally deliver the enzymes and other DNA repair components. Low HspA2 chaperone levels cause meiotic errors and chromosomal aneuploidies, diminished zona pellucida

binding, low fertilization and oocyte activation rates, and increased miscarriage rates due to defects of paternal contribution of spermatozoa (Aitken and Baker, 2006; Huszar *et al.,* 2007). Whatever cause may prevail, in this study it has been established that DNA chain breaks are related to arrested sperm maturity, whether shown by one or more of the nuclear and cytoplasmic probes. Additional adverse effects related to persistent histones may reflect the upstream events of spermatogenesis, as indicated by aberrant protamine-l / protamine-2 ratios at both the mRNA and protein levels in spermatozoa with arrested maturity (Steger *et al.,* 2003: Aitken *et al.,* 2004; Agarwal *el al.,* 2005; Seli and Sakkas, 2005). Another relevant aspect is that spermatozoa with intermediate or dark staining with probes of arrested maturity may also exhibit shape properties that reflect the spermiogenetic defects with respect to cytoplasmic retention and tail sprouting, such as lower proportion of spermatozoa with normal head shape, abaxial insertion of the tail, shorter tail length, or lower tail length/head long axis ratio (Gergely *et al.,* 1999; Celik-Ozenci *et al.,* 2003; Chemes and Rawe, 2003). The shorter sperm tail, characteristic for spermatozoa with arrested maturity, is also a component of the Tygerberg strict morphology evaluation. (Menkveld *et al.,* 1990; Gergely *et at.,* 1999; WHO, 1999; Celik-Ozenci *et al.,* 2004). Mature spermatozoa present in sperm-hemizona complexes are clear headed. This supports the idea that sperm shape may also be considered as a biochemical parameter (Huszar *et al.,* 2007). The study of individual spermatozoa with the various probes provides enhanced levels of statistical power in the analysis of mature and immature spermatozoa, and with this approach, a close correlation was demonstrated between the maturity attributes (kappa = 0.8). It is suggested that this type of analysis and the use of biomarkers will be useful in men exposed to environmental reproductive toxicity. It is further suggested that this may be particularly true in the early stages of exposure, when the toxic agents interfere only with sperm maturation, but have not yet placed stress on daily sperm production to cause a decline in sperm concentrations. The presence of spermatozoa with various degrees of maturity in semen samples further supports the day-to-day and man to-man variations in sperm maturity, independent of sperm concentrations (Huszar *et al.,* 1992, 2003, 2007). The pregnancy success in couples treated with intrauterine insemination or IVF was inversely related to the proportion of spermatozoa with arrested maturation, as indicated by sperm cytoplasmic retention and HspA2 concentrations (Huszar *el al.,* 2007). Furthermore IVF fertilization rates are inversely related to DNA damage (Sakkas *et al.,* 1999; Greco *et al.,* 2005). Although intracytoplasmic sperm injection fertilization rates seem to be normal with

spermatozoa with damaged DNA, this discrepancy may be due to the fact the paternal genome is only expressed at around the 4- to 8-cell stage of human embryos; thus, the DNA breaks may not have an effect in fertilization or in very early development, but the negative effect becomes apparent in the later embryonic stages. Indeed, the proportion of spermatozoa with DNA fragmentation was related to the time needed to achieve pregnancy, and DNA fragmentation was more extensive in men whose wives suffered recurrent pregnancy loss compared with sperm donors in the general population. Also, the incidence of miscarriage is higher with the less mature testicular spermatozoa, compared with ejaculated spermatozoa (Evenson *et al.,* 1999; Spano *et al.,* 2000; Virro *et al.,* 2004). The present data are also relevant to therapies directed to improve male subfertility, including several lines of research exploring new strategies or therapeutic interventions, i.e. antioxidant therapy, for the prevention or repair of sperm DNA fragmentation which causes a decline in fertility and the paternal contribution of spermatozoa to the zygote (Aitken *et al.,* 2003; Suzuki *et al.,* 2003; Agarwal and Said, 2005; Comhidre *et al.,* 2005; Greco *et al.,* 2005; Lewis and Aitken, 2005; Tesarik *et al.,* 2006). However, intermediate and diminished maturity spermatozoa, approximately 40% of spermatozoa in this study, in addition to DNA chain fragmentation, show cytoplasmic retention, persistent histones, apoptotic processes, and structural defects of spermatogenetic and spermatogenetic origin. Those cells with a defective infrastructure are not likely to be amenable to repair thus; the study of sperm maturity is relevant to assessment of the potential sperm pool that would be available for improvement by therapeutic interventions. Otherwise, the proportion of spermatozoa that are subject to rescue might be overestimated (Ménézo *et al.,* 2007).

By the use of Metamorph™ imaging program we could not only pursue localization-relocalization of individual spermatozoa so that side-by-side comparison of results in consecutive double probing but we were able to evaluate the morphology in relation to degree of nuclear immaturity. The possibility of capturing native spermatozoa together with shape evaluation facilitated by the Metamorph™ program followed by FISH probing gave the idea to assess the correlation among attributes of sperm morphology and different chromosomal content. There can be two reasons to test shape attributes and chromosomal set; first to select X and Y bearing spermatozoa, we do not detail our results here. Second is to examine morphometric differences of spermatozoa with haploid

vs. disomic chromosomal set. The possible morphologic difference between haploid and disomic cells has been of interest to increase of the safety of sperm selection in ICSI.

The micromanipulation techniques of sperm injection in assisted reproduction increased the effectivity of interventions. However the sperm selection depends on the visual judgment of the embryologist, and there is a fear that by an inadequate selection one may bypass numerical chromosomal abnormality with spermatozoa into the oocyte (Bonduelle et al.,1998; Gianaroli et al., 2000; Van Steirteghem et al., 2002). Data of studies evaluating possible prognostic value of shape attributes in relation to spermatogenetic arrest were with conflicting results (Gergely *et al*., 1999; Celik-Ozenci C *et al.,* 2004, Prinosilova *et al.*, 2009). However regarding the negative study between shape attributes and disomies (Celik-Ozenci C *et al.,* 2004) data was collected by evaluation of non native, decondensed sperm. Therefore in our third experiment we evaluated morphometric data: head area, perimeter, short and long axis, tail length of native nondecondensed disomic and haploid spermatozoa. After multicolor FISH probing for centromere specific probes for 17 and sex chromosomes we determined the chromosomal content of the same individual cells. The morphometrical differences between haploid and disomic spermatozoa were compared. We studied 1,227 haploid spermatozoa versus 24 disomic spermatozoa (**Table 6.)**. The differences concerning the mean value of head area, perimeter and short axis between disomic and haploid were with high significance (P=.001) the differences were in the mean values 32%, 13%, 21% respectively. But the difference in the length of tail was not significant. To check the dispersion of values of haploid and diploid cells our disomic population was not enough therefore we used values of 600 haploids vs. 178 disomic decondensed spermatozoa of a former study. In this second group differences of the mean concerning the head area and short axis were also significant between haploid and disomic cells (difference 10.6% P=0.02). However data of the dispersion were disappointing in relation to the possibility of safe selection in ICSI (**Figure 15.**). For instance, in the head area category it is clear that the majority (>60%) of the disomic sperm overlap in size with the haploid sperm. However, some disomic sperm are larger and others are smaller than haploid sperm. The latter group indicates the occurrence of disomies in microcephalic sperm. The right side of the curve suggests that approximately 20% of disomic spermatozoa show head sizes larger than haploid sperm. A similar difference in size distribution may also be detected in the other sperm shape attributes, for instance, in the shape factor or tail length. Conversely,

approximately 70% of the haploid and disomic sperm coincide in size ranges. The distribution of the nondecondensed haploid and disomic sperm follow the same patterns.

Our morphometric data indicates that disomic cells can not be separated by dimensional features. To increase the safety in ICSI one had better use biochemical selection method for instance HA binding in combination with the gradient centrifugation. By the use these methods one can reduce the chance of aneuploidy and other arrested maturity (Jakab *et al.*, 2005).

We have also examined the potential dimensional differences among spermatozoa with XX, XY, and YY disomies. Because of the chromosome size differences, one would expect an increase in the dimensions of the XX as compared to the XY and YY sperm. However, the YY disomic sperm were slightly larger compared to the other two types. This suggests that the size inconsistencies are related to the degree of decondensation and not to the absolute size of the chromosomal complement. In the 6,000 FISH images surveyed, the aggregate disomy frequency was 0.39% (0.15%, 0.19% and 0.08% for XX, XY and YY disomies, respectively) **Table 7**.

In summary, there are some differences in disomic versus haploid spermatozoa, but there is a 70% overlap between the dimensions of these sperm, and the remaining 30% of disomies that differ in dimensions from haploids consist of sperm that are both larger and smaller than haploids.

It was also of interest in the present study, in addition to the expected dimensional increase in the decondensed versus native spermatozoa, there were also slight but inconsistent differences in the degree of dimensional changes related to decondensation. This is likely related to the fact that in immature sperm, the remodeling of the plasma membrane during spermatogenesis is retarded and therefore the membrane is more pliable (Celik-Ozenci *et al.,* 2003 and Huszar, 1997). In immature sperm with disomies, other spermiogenetic processes, such as cytoplasmic extrusion and tail sprouting, are also arrested (Kovanci *et al*., 2001). As a result, head size is larger and the tail is abbreviated in sperm of diminished maturity (Gergely *et al*., 1999; Celik-Ozenci *et al*, 2004 and Cayli *et al.,* 2004). This finding was also confirmed in the present study: the ratio of tail length and long head axis is significantly shorter in disomic versus haploid spermatozoa in both the native and decondensed states (P=0.02 and .005, respectively).

Conclusions of the Thesis

1 Disomies occur mainly in spermatozoa with moderately arrested nucleoprotein replacement, the increase is 4-6 times higher comparing to mature cells (P<.001). There is also strong correlation between frequency of moderately immature cells and total disomy rate in particular semen samples (r=0.60, P=.002). These relationships support my theory that low expression of HspA2 disrupts meiotic disjunction and causes retarded spermiogenesis in the same cells. Surprisingly the spermatozoa with serious arrest in nucleoprotein replacement showed no FISH signal. The most likely that DNA integrity in these very immature cells is damaged by increased ROS activity of apoptotic processes and because of impaired DNA repair that is also consequence of immaturity. In the FISH process fragmented DNA releases from the sperm. Because of this one may surmise that the frequencies of disomies in semen samples with high proportion of immature cells are underestimated.

2 The attributes of arrested maturity of nuclear compartment such as DNA fragmentation, persistent histones, and arrested cytoplasmic development defects like surplus cytoplasm, apoptosis are related and affect mainly same individual spermatozoa. Moreover the degrees of co-occurring abnormalities are also related. The correlation between maturity attributes is highly significant (kappa=0.8). In addition cells with different degree of disrupted maturity had conforming degree in abnormal Tygerberg morphology also. These correlations are in line with our theory that low expression of HspA2 causes damage in various spermiogenetic processes in the same cells. However in low proportion abnormalities may occur in heterogenic fashion thus to test sperm immaturity with high accuracy double probing is advised.

3 By morphometric data of haploid and disomic spermatozoa one can state, although there is statistical difference between haploid and disomic cells regarding the mean values of dimensions like head area, perimeter, short axis, however in the dispersion there is a substantial overlapping among sperm populations, and disomic sperm can be found among the microcephalic sperm population too. Because of the overlapping there is no chance in the practice to improve the safety of sperm selection by objective morphometry.

Summary

Biochemical markers are reliable indicators of sperm maturity and function. The major hallmark among biochemical markers is the 70 kDa heat shock protein, the HspA2. It proved to be very useful in assessment of sperm immaturity and fertilization potential. Expression of HspA2 is developmentally regulated, having a characteristic dual wave expression pattern. The first peak is in the round spermatocytes, the second a major peak is expressed in the elongating spermatids. Concerning the function of HspA2, it is part of the synaptonemal complex and supports the meiosis in round spermatocytes; abnormal expression therefore may cause meiosis defects, disomies. In the elongation phase HspA2 as chaperone protein facilitates assembly, folding and transport of different proteins among them protamins, DNA repair enzymes and proteins necessary for membrane remodeling. The low expression of HspA2 resulted in different forms of arrested nuclear and cytoplasmic maturities, like persistent histones, DNA chain brakes and surplus cytoplasm. Previous studies on HspA2 theory found correlation between different attributes of cellular immaturity in the semen samples. Other researches studied relationship between meiotic defects and various cellular immaturity forms in semen samples, and there was also strong correlation between them. These studies validate the HspA2 theory, but the intracellular relationship between the HspA2 related defects remained unclear.

My concern in this thesis was to find intracellular correlation between different HspA2 related defects such as nuclear and cellular immaturity attributes and meiotic nondisjunction. To test supposed co-occurrence of abnormalities double probing technique of individual spermatozoa has been developed. In the first experiment by consecutive AB and FISH probing we found that arrested nucleoprotein replacement and meiotic non disjunction are related within individual spermatozoa. This study validates our hypothesis on the central role of HspA2 in the spermatogenesis.

In another study AB staining was combined with CK immunostaining, ISNT, and caspase-3 immunocytochemistry. Data of this study proved that different attributes of arrested maturity in the nuclear and cellular compartment, persistent histones, surplus cytoplasm and DNA chain bakes are related in the same cell, moreover the degree of

abnormalities were also conforming (kappa=0.8). The apoptotic activity was also related to immaturity. These facts support the theory that HspA2 has central role in the spermatogenesis. However in low proportion there is heterogeneity in staining pattern. This is important for two reasons; first it proves that sperm after the defected maturation step may follow its changes in several pathways. Second the arrested spermiogenesis can not be examined correctly just by one biochemical probe only.

In the third study detailed morphometrical evaluation of the sperm head and tail was carried out in order to find differences between native, non decondensed spermatozoa with haploid and disomic set. Meanwhile there was significant difference in morphometric parameters such as head area, head area and the short axis between haploid and aneuploid cells by objective morphometry, disomic sperm heads were larger, but the by evaluation of distribution data were inconsistent, there was also a substantial (70%) overlapping in the of the values of these two populations, and disomic sperm heads were among the microcephalic spermatozoa also. Our data proves that the visual assessment in the selection of good chromosomal quality sperm using morphometric aspects is unreliable.

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List of author's publications in relation to the thesis

In-extenso Peer Review Publications

Óvári L., L. Sati L., Vigue L., Stronk J., Borsos A., Ward D.C., and Huszar G. (2009) Double probing individual human spermatozoa: aniline blue staining for surplus histones and fluorescence in situ hybridization for aneuploidies. *Fertil Steril*; reference number: **F and S7035R1**

Sati L, **Óvári L**, Bennett D, Simon SD, Demir R and Huszar G (2008) Double probing of human spermatozoa for persistent histones, surplus cytoplasm, apoptosis and DNA fragmentation. *Reprod Biomed Online*, **16**, 570-579.

Zavaczki Z., Celik-Ozenci C., **Óvári L**., Jakab A, Sati GL, Ward DC and Huszar G (2006) Dimensional assessment of X-bearing and Y-bearing haploid and disomic human sperm with the use of fluorescence in situ hybridization and objective morphometry. *Fertil Steril,* **85**,121-127.

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Peer Review Abstracts

L.G. Sati**, L. Óvári**, R. Demir, D.C. Ward, P. Bray-Ward, G. Huszar (2005): Persistent Histones in Immature Sperm are Associated with DNA Fragmentation and Affect Paternal Contribution of Sperm: A Study of Aniline Blue Staining, Fluorescence In Situ Hybridization (FISH) and DNA Nick Translation. American Society for Reproductive Medicine (ASRM) 60th Annual Meeting 2004. Abstract Book O-129, S 52**.**

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