

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

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

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Introduction and Aims of the Thesis

In searching for reliable parameters of male fertility 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.

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-protamine replacement, acrosome development, cytoplasmic extrusion and sprouting of the sperm tail. In unison with cytoplasmic extrusion, there is also a remodeling of the sperm plasma membrane in late spermiogenesis, a step that facilitates formation of the zona pellucida and hyaluronic acid binding sites.

There are various nuclear and cytoplasmic markers that may reflect to the degree of arrested maturation. However, the major hallmark of arrested sperm maturity is the low expression of the 70 kilodalton chaperon protein HspA2, which in normal sperm development is expressed in two waves. First, in round spermatocytes, HspA2 as a part of the synaptonemal complex (SC) supports meiosis. 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, such as DNA repair enzyme, and proteins for sperm membrane remodeling. The HspA2 proved to be the main regulator of the production and transport of protamins also, and seems to have anti-apoptotic function as well.

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 indicates retarded nukleoprotein replacement, the immunostaining for apoptotic enzyme caspase-3 reflects to active apoptotic processes, and various approaches demonstrating DNA chain fragmentation, such as in situ nick translation (ISNT). 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.

In various studies of sperm with arrested maturity, similar relationships were established by objective biomarkers. In semen samples that have higher proportion of immature cells there was a relationship between arrested cytoplasmic development and frequency of disomy. There was similar relation between membrane immaturity and frequencies of chromosomal aneuploidies in samples of man with infertility.

We think that the correlation between different immaturity forms and between arrested maturity and meiotic nondisjunction can be explained by the hypothesis of central role of HspA2; its lower expression may disrupt the meiosis and can lead to abnormal maturation.

Though previous studies supported the theory on central role of HspA2, however the relationships among cellular and nuclear attributes of arrested sperm maturity and meiotic nondisjunction were examined in semen samples, but individual spermatozoon has not been assessed whether these relationships may be pertinent in intracellular level.

My concern in the thesis work was if low expression of HspA2 is causative in different forms of arrested maturation and meiotic nondisjunction by multiply probing of the same individual spermatozoa one may expect rather combination of different nuclear and cellular signs of arrested maturity and meiotic nondisjunction in affected cells than isolated abnormalities. In addition it we hypothesized that the common causative factor the low HspA2 resulted in similar degree of abnormalities.

In order to test the theoretical co-occurrence of different abnormalities related to low expression of HspA2 the technique of multiply, subsequent probing of the 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 spermatozoa in our experiments was commenced by evaluation of nucleoprotein replacement by AB and followed by a second probing like FISH, immuncytochemistry for CK, caspase-3 for apoptosis or ISNT. Localization-

relocalization of individual spermatozoa was facilitated by Metamorph™ digital imaging computer program that facilitated a detail morphologic assessment of cells in relation the biochemical immaturity.

In another study we studied the morphometric differences between haploid and disomic spermatozoa. We pursued this study since previous studies evaluating dimensional attributes of disomic spermatozoa were with conflicting evidences and formerly not native, decondensed spermatozoa were evaluated. 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.

There is no data whether the HspA2 related sperm immaturity forms and meiotic nondisjunction are related in the same individual spermatozoon. It is unclear whether the degree of HspA2 related abnormalities can be related in the same cell. There are conflicting data concerning morphometric differences between haploid vs. disomic spermatozoa. In my researches concerning to this thesis I have have studied the following issues.

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?

Materials and Methods

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 30-40x10⁶sperm/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 salineimidazol 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.

Aniline blue staining of sperm chromatin

Slides were stained by 5% AB solution acidified to approximately pH: 3.5 with acetic acid, The spermatozoa were evaluated at a magnification of 600x, and images of random microscopic fields were recorded. 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. 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).

Then slides were subjected to FISH probing. The DNA decondensation treatment of the destained spermatozoa was performed using DTT and lithium 3, 5-diiodosalicylic acid (LIS) then fixed in methanol: acetic acid (3:1).

Directly labeled alpha satellite centromere-specific probes were used for FISH assay in case of chromosome X (Spectrum Green) and chromosome Y (Spectrum Orange). Chromosome 17 was hybridized by an indirectly, biotin labeled centromere-specific probe. To visualize the indirect biotin-labeled probe 17, slides were incubated with avidin-rhodamine (fluorescent red): avidine-FITC (fluorescent green,). Slides were washed 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

To decrease the subjectivity of the observations in scoring the chromosomal integrity, we used strict criterias. Nullisomies were not directly scored and were conservatively considered as equivalent to the incidence of disomies. 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: $25.0 \pm 6.2 \times 10^6$ sperm/ml, motility: $36.2 \pm 6.5 \%$).

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. 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; (ii) ISNT for detection of DNA chain breaks; and (iii) caspase-3 immunostaining to detect the apoptotic process in spermatozoa. 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.

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). The formaldehyde was removed by three washing steps with

PB-suc, then spermatozoa were exposed to a 3% bovine serum albumin (BSA) blocking solution in PB-such. After further washing, the spermatozoa were overlaid with a 1:1000 dilution of polyclonal anti-CK-Bantisenim. Following further PB-suc washes, the slides were treated with a biotinylated second antibody and were exposed to a Vector horseradish peroxidase/ABC kit according to the manufacturer's instructions. The avidin-biotin complex (ABC)-treated slides were further processed with diaminobenzidine and hydrogen peroxide. The developed brown color highlighted spermatozoa with various degrees of cytoplasmic retention. The specificity of the CK staining was established by using pre-immune serum in place of the first antibody, or by applying the second antibody only. In these experiments 1284 spermatozoa were studied (four men, concentration: $10.9 \pm 2.2 \times 10^6$ /ml, motility: $32.5 \pm 4.8\%$, 321 sperm/man, range: 209-610).

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. However, after the spermatozoa were exposed to the *We* BSA blocking solution, they were treated with a 1:300 dilution of active caspase-3 antibody. Further, the slides were processed with 1:1000 dilution of a biotinylated anti-rabbit second antibody. The brown color representing the caspase-3 content of spermatozoa was developed by the ABC method. The specificity of staining was established by applying the secondary antibody only. These studies were carried out in 2101 spermatozoa (4 men, sperm concentration: $11.9 \pm 1.4 \times 10^6$ sperm/ml, motility: $34.0 \pm 6.6\%$).

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 avidin in conjugated horseradish peroxidases. Thus, after the assay, the horseradish peroxidase-generated color in a spermatozoon is proportional to the extent of DNA breaks and repair. 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 mmol/l 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-HCl, 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. After further washing, the slides were developed in 3, 3-diaminobenzidine solution for 10-15 min. The slides were counterstained with Coomassie Blue in order to optimize visualization of the sperm contours. In these experiments 2446 spermatozoa were studied (5 men, sperm concentrations: $17.6 \pm 1.1 \times 10^6$ sperm/ml, motility: $38.4 \pm 5.9\%$, 489 sperm/ man; range; 276-602).

Evaluation of sperm morphology: Sperm smears were dried on glass slides and stained with Diff-Quik according to the manufacturer's instructions. The slides were scored by two investigators in a blinded manner according to the Tygerberg criteria. 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 $9.3 \pm 2.3 \times 10^6$ sperm/ml range (5-13); motility 39.7%-5.5% (range 30%-49%) and three normozoospermic men (sperm concentrations $46.5 \pm 21.0 \times 10^6$ 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.

In the second phase of the studies, sperm slides originating from 20 men (concentration $24 \pm 2.5 \times 10^6$ sperm/ml; motility $52.5\% \pm 2.5\%$) 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. 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..

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 $(4\pi \times \text{area})/\text{perimeter}^2$, where a value of 1 indicates a perfect circle; *Elliptical form factor*: calculated as long axis/short axis.

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

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.

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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. 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 and discussion

Aniline blue and FISH study

In the 7 moderately oligozoospermic men, we have evaluated a total of 58,793 spermatozoa. Regarding nuclear maturity, the proportion of the light, intermediate and dark AB stained sperm (representing mature, intermediate maturity, and arrested maturity spermatozoa) were $73.1\pm 2.3\%$, $21.8\pm 2.1\%$ and $5.0\pm 0.5\%$, 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: $0.07\pm 0.01\%$, disomy Y: $0.09\pm 0.02\%$; disomy XY: $0.18\pm 0.03\%$, and disomy 17: $0.16\pm 0.03\%$. The frequencies of diploidy were $0.17\pm 0.04\%$.

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 $P<0.001$). 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 ($r=0.76$, $P<0.05$). There was no correlation between AB staining and diploidies.

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.

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. 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, 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, but not in case of disomy Y and disomy X..

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. 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. 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.

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: $25.0 \pm 6.2 \times 10^6$ / ml. motility: $36.2 \pm 6.5\%$).

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. There was good agreement between the two staining patterns with the AB and CK probes. 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 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 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 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 $0.5 \pm 0.3\%$ 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 $36.0 \pm 4.4\%$ (mean $\pm$ SEM), and in the individual men 34.4, 30.3, 30.4 and 48.9%, respectively, the mean incidence of intermediate-intermediate staining was $24.9 \pm 4.1\%$ (25.8, 15.8, 35.4 and 22.8%), whereas spermatozoa with dark-dark pattern were present in $21.1 \pm 5.9\%$ (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 $52.0 \pm 6.7\%$ (54.8, 66.4, 32.9, 55.2 and 50.8%), the mean intermediate-intermediate pattern was $19.7 \pm 1.9\%$ (20.6, 16.6, 25.9, 20.5 and 15.0%). The dark-dark pattern was $12.2 \pm 1.7\%$ (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 $49.4 \pm 6.7\%$ (44.5, 56.7, 33.2 and 63.3%) and the intermediate-intermediate stained spermatozoa were $23.3 \pm 3.5\%$ (30.3, 26.4, 20.9 and 14.4%), Further, the dark-dark spermatozoa were $12.7 \pm 5.5\%$ (11.3, 3.5, 28.3 and 7.6%).

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.

These data support our hypothesis that attributes of sperm immaturity in the cellular and nuclear compartment. That points to the validity of the theory that low level of HspA2 chaperone protein may disturb the protein synthesis and transport of those enzymes necessary for maturation processes, enzymes of cytoplasm extrusion, enzymes of membrane remodeling and protemin. The DNA repair enzyme synthesis is also defected, therefore DNA fragmentation may occur. The apoptosis is also activated in immature cells. Because of the same origine of these abnormalities, the low expression HspA2 level, the degrees of the abnormalities were conforming (84-87%). In our experiment we

found heterogeneity also between the the degree of abnormalities (14-18%). This phenomenon is in line to those observations that there is certain polymorphism in the maturation development. After the defected step in maturation process the spermatozoon may follow its changes in different pathway. This is an important knowledge for the reason that for the adequate estimation of sperm immaturity singel biochemical probe is not enough. However one may say that the AB staining is a simple and reliable staining metohod in evaluation arrested sperm maturity. The combination of sperm immaturity attributes in the same spermatozoa indicates that the medication of male infertility is with great limitation. The antioxydative therapy may be helpful in avoiding DNA fragmentation, but the co-occurring abnormalities may be persistent like reatarded histons.

Dimensions of Haploid vs. Disomic Spermatozoa

The comparison between haploid and disomic native nondecondensed spermatozoa is based on the 1,227 versus 24 spermatozoa.

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. 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.

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. 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%, .) 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).

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 my theory that low expression of HspA2 causes damage in various spermiogenetic processes in the same cells. However in low proportion abnormalities may occur in isolated 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 between the two, however in the dispersion there is a substantial overlapping sperm populations. Because of the overlapping there is no chance in the practice to improve the safety of sperm selection by objective sperm morphometry.

List of author's publications in relation to the thesis

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