Ph.D. THESIS

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

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

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Debrecen, 2003

Introduction

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

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

Due to the elevated risk of transmission of genetic disorders to the offspring with ICSI, there is a need to eliminate spermatozoa with numerical chromosomal anomalies before assisted fertilization. To date, insufficient data are available on the efficacy of the widely utilized sperm preparation techniques (the *gradient centrifugation separation* based on the sperm density and the *swim-up method* based on the sperm motility) in decreasing the frequency of chromosomal abnormalities in the sperm used for assisted fertilization.

There is close correlation between the proportion of immature sperm characterized with cytoplasmic retention and frequency of chromosomal

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

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

Aims of the thesis

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

1. What is the incidence of numerical chromosome abnormalities in spermatozoa in men with low sperm count who represent a population of potential candidates for assited reproduction and ICSI and do the WHO definitions of spermiogram reflect the risk of genetic impairment? [*The Population Study: Determination of the incidence of the numerical chromosomal abnormalities (aneuploidy and diploidy) in infertile men with*

low sperm count and investigation of their relationship to the traditionally used WHO criteria of the spermiogram]

2. Can one eliminate sperm with aneuploidy/diploidy using the present sperm preparation techniques? [*The Gradient Centrifugation and Swim-up Studies: Examination and comparison of the efficiency of the traditional sperm preparation techniques (the gradient centrifugation and the swim-up method) in eliminating aneuploid and diploid sperm, and sperm with diminished maturity*.]
3. Can one visually select aneupolidy/diploidy-free sperm? [*The Morphology Study: Examination of the relationship of sperm morphology and numeric chromosomal abnormalities using objective computerized morphometry on the same sperm*.]
4. Can one select aneuploidy/diploidy-free individual sperm based on HA-binding? [*The HA-binding Study: Examination of the efficiency of selection of individual mature sperm with low frequencies of numerical chromosomal abnormalities by HA-binding*.]

Patient population and experimental design

The study population was composed of 44 men who presented for semen analysis at Sperm Physiology and IVF Laboratories of the Department of Obstetrics and Gynecology, Yale University School of Medicine. The sperm concentration and motility in the initial and prepared fractions were determined in a Makler chamber by computer assisted semen analysis. Samples of ten moderately oligospermic patients were examined with CK immunocytochemistry and FISH before and after gradient centrifugation, another ten moderately oligospermic patients before and after swim-up preparation. Furthermore, samples of twelve oligospermic patients before and after HA binding selection and gradient treated samples of twelve normospermic patients before and after HA selection. The FISH studies of the swim-up and HA experiments (68 samples

of 34 patients), cell scoring and data collection and analysis of the complete data-pool were performed by the author of this thesis.

With the use of gradient centrifugation in order to prepare the corresponding sperm fraction enriched in mature spermatozoa, an aliquot of the semen sample was centrifuged through 2 ml of an 80% single-phase Percoll gradient at 500xg for 20 minutes at room temperature.

During the swim-up preparation semen was diluted with HTF medium-0.5% bovine serum albumin and centrifuged at 400xg for 10 minutes in a flat bottom tube, which was then placed into a 36^{o}C incubator for 30 minutes. During the incubation, the motile sperm migrated into the supernatant, while the immotile and sluggish sperm and the particulate matter of semen remained at the bottom. After the incubation period, the top 0.5 mL of the supernatant, which is enriched in the motile sperm was withdrawn carefully.

During the course of the sperm HA-binding experiments we used HA of bacterial origin, which was permanently applied to plastic Petri dishes as 100-300 μm dots. The sperm progresses to the HA spots, and similarily to the zona binding pattern, the mature sperm attaches to the HA-spot. After incubation for 15 min, the HA-attached sperm were collected using an ICSI micropipette and placed onto a microscope slide.

Aliquots of the initial sperm suspension and prepared sperm fractions were then used for the FISH and CK-immunocytochemistry experiments.

In order to detect immature sperm with cytoplasmic retention, the sperm smears were exposed to a polyclonal anti-CK-B antiserum, then slide was processed with a biotinylated second antibody conjugated with horseradish peroxidase. The brown color representing the CK-content of spermatozoa was developed by the ABC (avidin-biotin complex) method. Spermatozoa were characterized as *mature* (no cytoplasmic retention) or *immature* (CK-staining in the head indicating cytoplasmic retention).

Before the FISH experiments, in order to render the sperm chromatin accessible to DNA probes, the slides were first treated with dithiothreitol (DTT, decondensation agent) then with lithium diiodosalicylate (LIS, swelling agent). The FISH studies were carried out using five chromosome probes (10, 11, 17, X and Y) labeled indirectly with fluorochromes (FITC and rhodamine). In each individuals, the initial and post preparation fractions were examined using both two-color and multicolor FISH. In order to detect the frequency of autosomal disomy and diploidy using chromosome 10 and 11 probes, two-color FISH was utilized (10-11 assay). Since three probe is necessary to study the frequencies of disomy and diploidy in the sex chromosomes, multicolor FISH was performed when chromosome X, Y and 17 were hybridized together (X-Y-17 assay). In the triple-probe FISH experiments, the chromosome 17 was combinatorially detected with both biotin-labeled and digoxigenin-labeled probes, so that its fluorescence profile would be the combination of two-colors. For each patient in the gradient centrifugation group one slide (triple probe FISH) of both the initial and the mature sperm fractions, in the swim-up group two slides (double-probe FISH and triple-probe FISH) of both the initial and the swim-up sperm fractions, in the HA binding group one slide (triple-probe FISH) of both the initial and the selected fractions were scored according to strict scoring criteria (with the exception of the HA-bound fraction, where the number of the available spermatozoa were limited). For the assessment of disomy and diploidy frequencies, ~4-10 000 spermatozoa were evaluated in each fraction (472 345 sperm nuclei in the 88 fractions of 44 subjects) with the exception of the HA-bound fraction, where the number of the available spermatozoa were limited. The overall hybridization efficiency in these experiments was >98%.

Morphologic analysis studies and objective computerized morphometry of individual spermatozoa were carried out in two phases: (A) In the first set of experiments, we investiged whether sperm maintain their shape after decondensation and denaturation. (B) In subsequent experiments directed to both FISH and morphometry analysis, we investigated whether sperm shape and

numerical chromosomal aberrations are related. Non-aneuploid, aneuploid, and diploid sperm were detected with FISH, classified, and images were captured and further analyzed with computerized morphometry.

Differences between the frequency of chromosomal abnormalities in different WHO spermatogram groups were characterized using Odds Ratio (OR) with the confidence interval of 95%. Differences in disomy and diploidy frequencies, as well as immature sperm rates were analyzed using the χ^2 analysis of contingency tables. Mann-Whitney Rank Sum Test were used to analyze the motility differences between the fractions. Correlations between the motility, the proportion of immature spermatozoa and aneuploidy frequencies were examined with Pearson correlation test. At the morhometry analysis paired t-tests were performed for sperm parameters before and after decondensation within each group. ANOVA tests were performed in order to test the similarities and differences between the sperm shape categories both before and after decondensation. Level of significance was selected as $p<0.05$.

Results and Discussion

1. The Population Study: Determination of the incidence of the numerical chromosomal abnormalities (aneuploidy and diploidy) in infertile men with low sperm count and investigation of their relationship to the traditionally used WHO criteria of the spermiogram

Neat semen of 32 patients with low sperm concentrations were examined throughout these studies described above. Mean sperm concentration was $18.2\pm1.5x10^6$ sperm/mL semen (range 8.0-45.5, all data Mean±SEM, range), the mean motility 49.4±1.65% (30.0-69.2). A total of 200,696 sperm in the 32 initial semen were scored with a mean of 6272 (range 5030-7340) in each men. The

samples were grouped according to the WHO definitions of the spermatogram. In the 32 men involved in these studies the frequencies of sperm with 17 and sex chromosome disomies in the neat semen were 0.16% and 0.36%. Based on the detected disomy and diploidy frequencies, the estimated mean frequency of numerical chromosome abnormalities in these men was 8.3% with considerable inter-individual variations, which characterizes the risk of choosing a single spermatozoon for ICSI from a non-selected sperm population of an infertile patient with low sperm count. Our data represent a slightly elevated risk of aneuploidy in decreased fertility with the sperm concentration less than $50x10^6$ sperm/mL. It is also clearly shown by our data that the WHO definitions, such as the oligozoospermy below the sperm count of $20x10^6$ sperm/mL do not characterize the risk of elevated aneuploidy frequency. Although the sex chromosome disomy frequency alone was found to be elevated in the oligospermic men, there was not any correlation between the sperm concentration and the frequency of any numerical chromosome abnormalities in the examined sperm count range. The most frequent disomy was the disomy XY, and a more than two-fold increase of the disomy XY was detected in oligozoospermic men (0.17% vs. 0.08%). This finding is consistent with the observation that there is an increase in sex chromosome aberrations in ICSI children with a major contribution of Klinefelter`s syndrome (XXY). With respect to the poliploidies, we observed significant increase in diploidy frequency in samples with reduced motility, especially with oligoasthenozoospermy (0.96%). This finding supports the relationship between the diploids and meiotic defects, and it also suggests a reduced motility of sperm with extra chromosome set.

We made some novel observation with regard to the origin of diploid spermatozoa. Defective chromosome separation of either meiosis I. (M1) or meiosis II. (M2) can be responsible for the production of diploid spermatozoa. In sperm with higher than average diploidy rates, the dominance of either the M1 diploids or M2 diploids can always be observed. This suggests, that in the majority of the cases with moderately elevated frequencies of diploid

spermatozoa, chromosomal non-separation during meiosis I. and defective nuclear cleavage during meiosis II occur separately. However, in most cases of decreased sperm concentration and increased frequencies of diploids the failure of the meiosis II. is responsible for the formation of sperm with extra chromosome set, since frequency of XX and YY diploids of M2 origin are elevated. Consequently, the failure of the nuclear cleavage during meiosis II. will result not only in increased number of diploid spermatozoa, but also in decreased sperm count.

2. The Gradient Centrifugation and Swim-up Studies: Examination and comparison of the efficiency of the traditional sperm preparation techniques (the gradient centrifugation and the swim-up method) in eliminating aneuploid and diploid sperm, and sperm with diminished maturity.

Our data indicate that both the gradient centrifugation and the swim-up step eliminates sperm with disomies, diploidies, and sperm with diminished maturity with an overall significant reduction at the level of $P<0.001$. However, the results were not consistent. In the gradient centrifugation study all the ten patients showed a decline in disomy frequencies, and only two in diploidy frequencies in the Percoll pellet vs. semen sperm fractions. In the ten patients in the swim-up study there were seven that reached significant declines in proportions of immature sperm, and only one in the proportion of disomic sperm. Regarding diploidies, six of the ten men reached a significant reduction in the swim-up fraction, and this pattern was similar whether we considered data from three-color or two-color FISH. Also, the difference between the the gradient centrifugation and swim-up study approach was evident considering the clearance rates for disomic sperm of 3.2-fold and 1.5-1.4-fold (the three- and two-color FISH), respectively. It is of further interest that diploid sperm show much higher rates of clearance with swim-up (2.7 fold) as compared to those for

disomic sperm (1.5-1.4 fold). We gave strong evidences, that the gradient centrifugation effectively decreases the frequency of immature and aneuploid sperm, while the swim-up preparation is able to reduce significantly the diploid sperm due to their defective motility. There is a correlation between the rate of immature sperm and aneuploidy frequency, but no relationship seems to exist among sperm motility and aneuploidy.

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

3. The Morphology Study: Examination of the relationship of sperm morphology and numeric chromosomal abnormalities using objective computerized morphometry on the same sperm.

In our studies using objective morphometry and FISH on the same spermatozoa we addressed whether it is possible to predict the chromosomal status of a single sperm from its shape, thus allowing the ICSI embryologist to avoid selecting sperm with aneuploidies or diploidies by simply using their shape properties as a guide. Although several authors have reported the presence of sperm with abnormal morphology and increased frequency of aneuploidies in the same semen sample, the study of the common occurrence of these two factors within the same sperm is a novel finding of the present work. The prerequisite for the

morphometry study of the relationship between sperm morphology and disomies/diploidies in the same sperm was the confirmation that the original sperm shape remains conserved after the decondensation/denaturation steps, which are necessary for performing effective FISH on sperm. The study of the normal, intermediate, abnormal, and amorphus sperm categories, which in general represent an increasing degree of diminished maturity, indicated increases in aneuploidy frequencies in the four groups. Since we found that decondensation/denaturation tends to increase the cell size but does not affect cell shape, we were able to focus upon the question, using FISH, of whether sperm shape is associated with disomies/diploidies. The second phase of the morphology study revealed that among the 1073 selected, even the sperm with the most normal sperm population, which was classified as such by both visual evaluation or by objective morphometry, exhibited a 7% incidence of sperm with aneuploidies and diploidies. Both disomy and diploidy frequencies increased from the normal to the amorphus classes, and were highest among the amorphs, the most immature sperm population. However, the fact that sperm with chromosomal aberrations may occur among normal spermatozoa is now well established. Thus, selecting sperm for ICSI, based on shape properties alone, does not preclude the presence of chromosomal abnormalities, particularly disomies.

4. The HA-binding Study: Examination of the efficiency of selection of individual mature sperm with low frequencies of numerical chromosomal abnormalities by HA-binding.

Whether the HA-bound sperm were selected from the oligospermic or the normospermic groups, the frequency of aneuploidies were in the very low range of < 0.10%. Thus, HA selection eliminated sperm with disomy efficiently reaching a low level of frequency which is even less than that of characteristic for the normal men. Diploidy frequencies also declined in the HA fractions of both

groups, from 0.81% to 0.13% in the oligospermic group and from 0.58% to 0.10% in the normospermic group, representing a consistent 6.1-fold reduction for diploid sperm frequency regardless to the initial incidence.

The relationship between the proportion of immature sperm with cytoplasmic retention and frequency of chromosomal aneuploidies in men is based on the dual role of the HspA2 chaperone, which supports meiosis as a component of the synaptonemal complex. It was also found, that along with cytoplasmic extrusion and the initiation of HspA2 synthesis, a developmentally regulated plasma membrane remodeling also occurs, which facilitates the formation of the zona pellucida-binding and HA-binding sites. Immature sperm with retained cytoplasm, high CK content and low expression of HspA2 are apparently deficient in the zona-binding site. Mature sperm selectively attach and remain bound to solid state HA, similarly to the zona pellucida. The present data gave evidence that HA-selected mature sperm show low frequency of chromosomal aberrations comparable to that of sperm selected by the zona pellucida in conventional fertilization. HA is a normally occurring component of the female reproductive tract, such as the cervical mucus or the cumulus oophorus, to which sperm are regularly exposed, thus there should not be any ethical concerns. Fertilization with HA selected sperm, and potential attachment of a few molecules of HA does not appear to be different from natural fertilization. The 4-fold decline of sex chromosome disomies is consistent with the increase of chromosomal aberrations in ICSI children. In spite of the sample differences, the aneuploidy and diploidy rates in the HA-bound fraction declined to a narrow low 0.04-0.13% range, which comparable to that of sperm populations bound to the human hemizona. The similarly effective reduction in aneuploidy and diploidy frequencies to a very low level, based on selection by the sperm plasma membrane hyaluronic acid receptor, which is exclusively present only in mature sperm, gives a very promising perspective from the point of view of sperm selection for ICSI.

Conclusions of the Thesis

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

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

List of authors` publications in relation to the thesis

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