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Characterization of the lectin binding pattern in human spermatozoa after swim-up selection

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Summary. Capacitation is characterized by a hyperactivated pattern of sperm motility. The acquisition of highly motility is present in the early stages of capacitation. Sperm progressive motility is one of the most important parameters for determining the suitability of semen for processing. However, previous studies have shown that some sperm showing good motility have membrane damage. The aim of our study was to characterize the lectin staining pattern on the sperm plasma membrane of unselected and selected human sperm of normozoospermic donors. Sperm selection was performed by the swim-up technique. Fourteen samples from healthy consenting donors classified as normozoospermic according to the World Health Organization were used.

We observed changes in the distribution of the carbohydrate residues after the swim-up selection. With *Triticum vulgaris*, the most abundant pattern was dotted labeling all over the head plasma membrane in the unselected sperm. However, this lectin was distributed homogenously over the acrosomal region after selection. With *Arachis hypogaea*, the most abundant pattern in fresh sperm was a highly stained acrosomal region. In the highly motility sperm population, the most frequent pattern was dotted fluorescence on the acrosomal region and a highly stained equatorial segment. Meanwhile, with the *Aleuria aurantia* and *Canavalia ensiformis* lectins, the most representative patterns were the same before and after the swim-up selection. Our data indicate that modifications which occur in carbohydrate residues during swim-up selection could be important for the regulation of progressive motility and prepare the sperm for capacitation.

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Introduction

In vivo, after leaving the testis, mammalian spermatozoa from many species are morphologically differentiated but have not developed the capacity to fertilize a metaphase II-arrested egg. Human sperm undergo maturation and acquire the ability to fertilize an ovum during passage through the epididymis (Dacheux et al., 2009). After a period of time in culture or in the female reproductive tract, during capacitation, the sperm acquires hyperactivated motility, characterized by a high amplitude whip-like beating of the flagellum, asymmetrical flagellar bends, and a circular or erratic swimming trajectory (Yanagimachi, 1994). However, they are still incapable of fertilization. Fertilization capacity is gained after residence in the female tract for a finite period of time. The physiological changes within a sperm that gives it the ability to fertilize the ovum is called "capacitation". Capacitation was first described and defined separately by Chang (1951, 1955) and Austin (1951, 1952). The definition of this poorly understood phenomenon has been modified and narrowed down over the years. Today, it is known that spermatozoa from many species can be capacitated *in vitro* (Bavister, 2002). In humans, the IVF (*in vitro* fertilization) experiments of Edwards et al. (1969) demonstrated a need for sperm capacitation.

During the capacitation *in vivo* and *in vitro*, different molecular changes are produced (Yanagimachi, 1994; Visconti et al., 1995; Aitken et al., 1998; Baldi et al., 2000; Gadella et al., 2008; Krapf et al., 2010). These processes include the release of different membrane components and the reorganization of the sperm plasma

membrane (Topfer-Petersen et al., 1990; Myles et al., 1987; Cowan et al., 2001). The distribution of sugar residues on the sperm membrane of different mammalian species has been studied previously (reviewed in Benoff, 1997) indicating that carbohydrate heterogeneity was detected in the sperm population. Carbohydrate has been suggested to play a key role during gamete interaction in different species. The carbohydrate composition of the human gametes has been recently analyzed by glycomic analysis (Pang et al., 2007, 2012). Thus, it was recently reported that sialyl-Lewisx oligosaccaride present in the zona pellucida is an important carbohydrate ligand for human egg-sperm interaction (Pang et al., 2012).

The acquisition of motility and the hyperactivation of human sperm are the early stages of capacitation. The progressive motility of the sperm is one of the most important parameters for determining the suitability of semen for processing. However, previous studies have shown that some sperm showing good motility have membrane damage (Valcárcel et al., 1994). The functional activity of the sperm membrane has been shown to be a better predictor of fertilizing capacity than motility (Zaneveld et al., 1990). Some studies have indicated that a correlation exists between subtle changes in the sperm glycocalyx, and in the production of viable spermatozoa, which subsequently helps to determine fertility (Gabriel et al., 1994). Moreover, some changes in the sperm glycocalyx were found in infertile men (Purohit et al., 2008). Other authors used ConA for studying acrosomal status and egg penetration potential (Holden et al., 1990; Fierro et al., 1996; Runnebaum et al., 1995).

On the other hand with the appearance of *in vitro* fertilization with uterine transfer of the embryos (IVF-ET), semen preparation techniques were developed to separate motile sperm that are morphological normal from the seminal plasma. The swim-up technique allows the selection of the higher motility spermatozoa (Boomsma et al., 2007). However, some authors have demonstrated that the use of this technique, as well as others, for isolating "functionally normal" can select spermatozoa with anomalies (at chromosomal, nuclear, organelle and/or membrane level) (Sakkas et al., 1996, 2000). Sperm preparation for assisted reproduction techniques should therefore minimize the risk that damaged spermatozoa could influence the outcome. A recent study has demonstrated that the swim-up technique was more effective than density gradient centrifugation for producing samples with lower nuclear vacuolization (Monqaut et al., 2011). With this process, the recovered spermatozoa had, on average, a longer telomerase size and a lower frequency of sperm with fragmented DNA (Santiso et al., 2010).

The aim of this study was to characterize the lectin staining pattern on the sperm plasma membrane of unselected and selected human sperm from normozoospermic donors. In this study, we demonstrated that a change in the lectin binding pattern was produced after swim-up selection.

Materials and methods

Semen samples

All activities involving human subjects were performed according to the Declaration of Helsinki.

Semen samples were obtained by masturbation from fourteen healthy consenting donors after 3 to 7 continuous days of sexual abstinence. The samples were received at the Biotechnology department laboratory and a basal semen analysis was conducted within one hour. At this point the semen sample was split into two aliquots, one fixed and used as the non-selected spermatozoa, and the other one was conducted by the swim-up procedure. All semen samples were classified as normozoospermic according to the World Health Organization (WHO, 2010).

Swim-up procedure

A highly motile sperm population was selected with the swim-up procedure using HAM F-10 medium (Invitrogen, Madrid, Spain). 1 ml of the semen sample was diluted 1:1 in the medium and the final volume was split into 4 conical tubes. After 10 minutes of centrifugation at 250 g, the supernatant was discarded. Then 250 μl of pre-warmed medium was carefully added to the pellet without mixing and incubated for 1 hour at 37ºC with 5% CO2 to allow motile sperm to migrate from the pellet to the medium. A 95% motile sperm population was selected recovering the supernatant.

Fresh and selected semen samples were washed twice in PBS and then fixed with a 2% Paraformaldehyde (Electron Microscopy Sciences, Hatfield PA, USA) solution in PBS for 15 minutes at 4ºC. Fixed sperm was washed twice in PBS and diluted to a final concentration of 1 million cells per milliliter.

Lectin labeling

Biotinilated WGA, PNA, ConA and AAA (Vector laboratories, Burlingame CA, USA) were used to label sperm cells. All the lectins were tested in the fourteen semen samples included in the study. The taxonomic names and specificity of lectins used in the present study are shown in Table 1.

Fixed unselected or selected sperm suspensions were placed on a round cover slip with a diameter of 10 mm (World Precision Instruments, Sarasota FL, USA) and then air-dried to enable the cells to attach to the surface. They were then rehydrated with PBS for 10 minutes and incubated with a blocking solution containing 2% of BSA (Sigma, Madrid, Spain) in PBS for 1 hour. After blocking, the cover slip was incubated with the lectin at a final concentration of 20 μg/ml for 1 hour at room temperature in a humid chamber. After being washed with PBS three times for 5 minutes each time, the cells were incubated with streptavidin-FITC (Sigma, Madrid, Spain) at a final concentration of 30 μg/ml for 1 hour at

room temperature in a humid chamber. The cover slip was washed three times in PBS for 5 minutes each time and then mounted with Vectashield H-1000 (Vector laboratories, Burlingame CA, USA) an anti-fading medium. Negative control experiments were performed omitting the lectin.

Statistical analysis

Quantification of staining patterns (P) was conducted by scoring 200 sperm for each cover slip using a Leica TCS SP2 (Leica Microsystems GmBH, Wetzlar, Germany) laser confocal microscope.

After checking the adjustments of the percentage values to the normal curve, by using the Kolmogorov-Smirnov test, statistical differences between unselected and selected samples were tested for the different lectins using the Paired t-test. Differences were considered to be statistically significant at a 95% confidence level ($P<0.05$). The statistical analysis was performed using the SPSS software version 12.0 (SPSS Chicago IL, USA Inc.).

Results

Lectin labeling patterns in unselected and selected human sperm

We analyzed all the staining patterns observed with the different lectins and only considered those that were present in over 5% of the sperm in a sample, whether before or after selection. As a result of this pre-analysis, we detected seven patterns (Fig. 1a) that seemed to be consistent, namely: Pattern 1 (P1): highly stained acrosomal region; Pattern 2 (P2): dotted fluorescence at acrosomal region and highly stained equatorial segment; Pattern 3 (P3): dotted labeling all over the head plasma membrane; Pattern 4 (P4): dotted fluorescence at acrosomal region and the periphery of the post-acrosmal region; Pattern 5 (P5): faint labeling all over the head plasma membrane; Pattern 6 (P6): acrosomal and post-acrosomal region highly stained with no labeling in the equatorial segment; Pattern 7 (P7): acrosomal region with faint labeling and highly stained post-acrosomal region, with no labeling in the equatorial segment. Not all patterns were observed for all of the lectins (Fig. 1b).

Distribution of lectin in unselected sperm

AAA lectin shows patterns P2 (3.09%), P3 (19.5%) and P4 (77.33%). Patterns P1 (27.4%), P5 (12.98%), P6 (56.65%) and P7 (2.96%) were detected using ConA lectin. PNA shows patterns P1 (73.17%) and P2 (26.83%). The WGA lectin presents patterns P1 (24.42%), P3 (72.84%) and P5 (2.74%) (Table 2).

Characterization in the lectin staining patterns in selected human sperm

The distribution of lectin binding pattern was observed in human sperm after the swim-up selection (Table 2).

With AAA lectin the most frequent pattern after selection was P4 (71.77%), this pattern was also the most representative in fresh sperm. However, patterns P2 (7.86%) and P3 (12.10%) were significantly modified following the sperm selection process, increasing the frequency of the first and decreasing that of the second.

Significant differences were obtained with ConA, pattern P6 (72.9%) increased and P1 (9.18%) decreased after swim-up selection. No statistically significant differences in patterns P5 (9.44%) and P7 (8.48%) were obtained with the ConA lectin. With the PNA lectin, most of the higher motility sperm showed pattern P2 (52.47%), whilst pattern P1 (47.53%) diminished in selected cells. All the changes observed after the swim-up selection with the WGA lectin were significant. Patterns P1 (46.81%) and P5 (27.34%) increased in the selected spermatozoa. However, an important decrease in the percentage of the sperm showing pattern P3 after selection was observed.

Discussion

After the swim-up selection, the distribution in the lectin binding pattern in human sperm was different in our study. Although results could be interpreted as the mere removal of carbohydrates from fresh samples, the procedures for selecting the sperm cell have suggested that it is necessary to improve the probability of

Table 1. Taxonomic names and specificity of lectins used in the present study.

Taxonomic name	Abbreviation	Carbohydrate binding specificity	References
Aleuria aurantia	AAA	Complex-type N-linked oligosaccharides with a α1,6 fucosyl residue at the innermost GlcNAc. Terminal α1,2-linked Fuc residues.	Osawa and Tsuji, 1987; Yamashita et al., 1985.
Arachis hypogaea	PNA	Galα1,3GalNAc	Lotan et al., 1975
Canavalia ensiformis	Con A	Trimannosyl core of high mannose and bi-antennary complex type N-linked oligosaccharides	Bhattacharyya et al., 1987; Brewer and Bhattacharyya, 1986, 1988
Triticum vulgaris	WGA	Sialylated glycans, polylactosamine sequences	Bhavanandan et al., 1977; Debray et al., 1981; Gallagher et al., 1985

fertilization in IVF attempts (Van den Zwalmen et al., 1991). These techniques could also initiate the capacitation of human sperm (Tanphaichitr et al., 1990; Moreno-Fierros et al., 1992; Pasteur et al., 1992).

The lectins provided an excellent tool for studying glycoprotein redistributions and allowed us to observe variations in the sperm plasma membrane components. It has previously been observed through this methodology that modification of the lectin binding pattern was detected in mice sperm after capacitation (Baker et al., 2004).

Labeling with lectins could therefore be useful in selecting the sperm subpopulation with the highest fertilizing capacity. Some authors suggested that the spermatozoa affected by acrosomal pathologies could be removed from the ejaculate by selective PNA agglutination (Singer et al., 1986; Ravid et al., 1990). Others have observed WGA bound to spermatozoa with good motility (Lasalle and Testar, 1994). Pérez-Pé et al.

Table 2. Differences in the staining patterns between unselected and selected sperm.

Lectin	Pattern	Unselected sperm Mean ± SD	Selected Sperm Mean ± SD	Significance
AAA	P2	3.09±1.76	7.86±4.57	p≤0.01
	P3	19.57±6.47	12.10±7.08	p≤0.01
	P4	77.33±6.17	71.77±24.50	n.s*
ConA	P1	27.40±15.66	9.18±13.70	p≤0.01
	P5	12.98±7.71	9.44±7.26	n.s*
	P6	56.65±15.93	72.90±14.03	p≤0.05
	P7	2.96±3.55	8.48±13.52	n.s*
PNA	P1	73.17±17.33	47.53±30.21	p≤0.01
	P2	26.83±17.33	52.47±30.21	p≤0.01
WGA	P1	24.42±6.71	46.81±26.22	p≤0.01
	P3	72.84±6.77	25.84±19.82	p≤0.01
	P5	2.74±2.34	27.34±15.16	p≤0.01

*n.s.: Not significant.

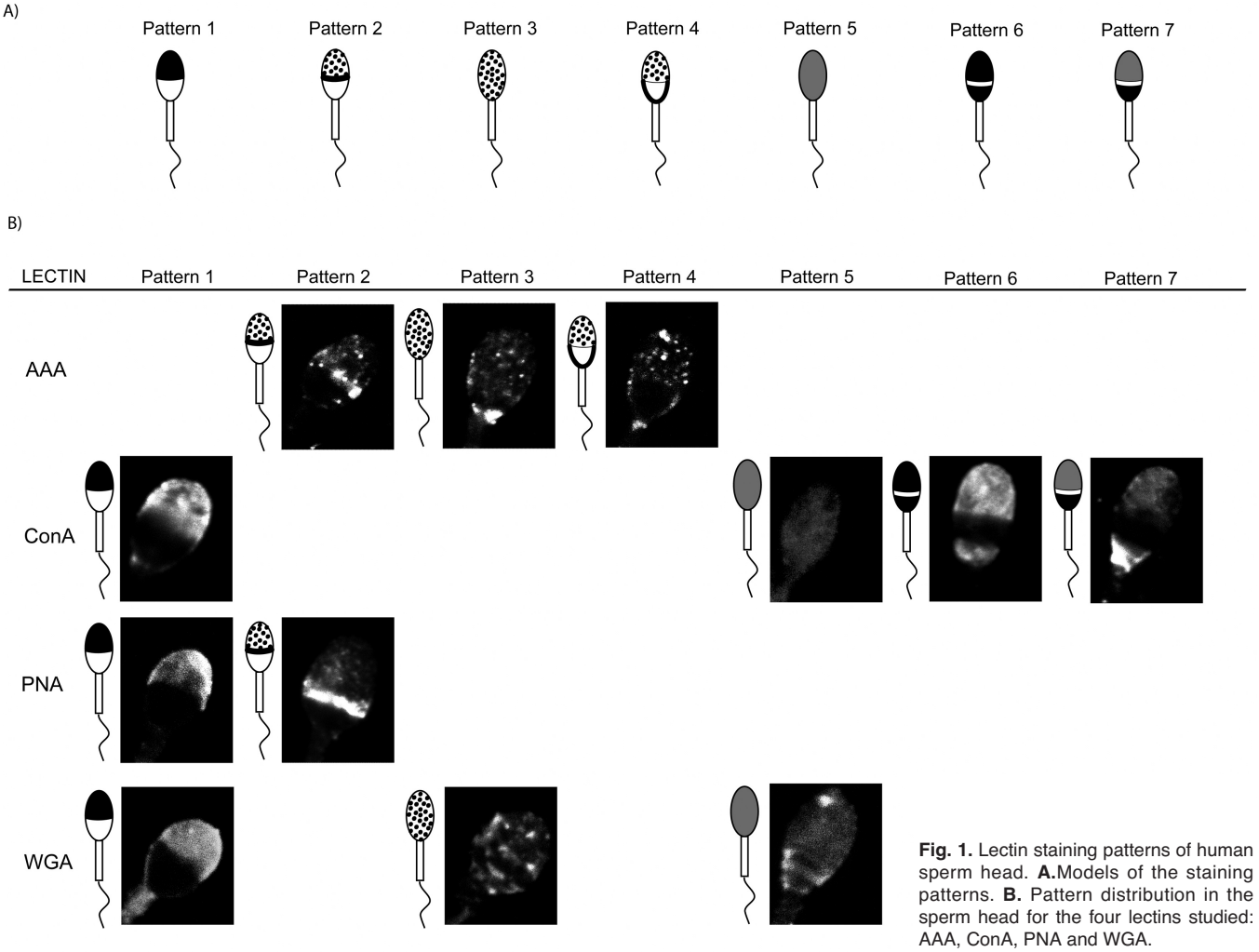

Fig. 1. Lectin staining patterns of human sperm head. **A.**Models of the staining patterns. **B.** Pattern distribution in the sperm head for the four lectins studied: AAA, ConA, PNA and WGA.

(1999) obtained highly-motile spermatozoa with preserved plasma membrane by sperm lectin agglutination combined with the swim-up technique in ram spermatozoa.

To our knowledge, a comparative study of the quantitative and qualitative distribution of lectin binding on unselected and on highly motile sperm of normozoospermic patients has not been performed. Some of the lectins used in this study showed a significant change in their binding pattern after the swim-up procedure.

Dotted staining was predominantly observed all over the head plasma membrane in both unselected and selected sperm using the AAA lectin. However, some sperm showed an equatorial band. The percentages of patterns P2 and P3 were approximately 20%, before and after selection. Due to the location of the labeling, this population of cells may correspond to spontaneous acrosome-reacted sperm. We observed that the most abundant pattern is the same before and after selection, which could mean that these carbohydrate residues do not change in those cells with higher motility. It is possible that the changes with AAA are produced during capacitation.

Jimenez et al. (2003) detected a significant increase of mannose residues in the acrosomal region of boar spermatozoa after capacitation. These authors did not report different patterns. However, we observed four patterns with ConA lectin. The spermatozoa with stained acrosomal and postacrosomal regions with no labeling in the equatorial segment (P6) were the most abundant subpopulation. This pattern increased after selection by swim-up, and remained the most representative. Cross and Overstreet (1987) studied the changes produced in the binding sites with ConA after 0 hours, 5 hours and 23 hours of incubation in a capacitating medium. They observed that about half of the anterior head of sperm labeled with this lectin was more heavily labeled than the posterior head, and that this pattern increased after 5 hours of incubation. These results were not consistent with our findings as the authors only described the patterns on the capacitated sperm, and they did not give a percentage of patterns. Moreover, experimental conditions used in both studies were different.

Purohit et al. (2008) found mannose residues on the acrosomal region, equatorial segment and post-acrosomal region of capacitated human sperm from normal and infertile subjects, but did not observe any labeling differences between the two types of subjects. This single pattern described by these authors corresponds to our pattern 5, and although they observed a moderate staining in the equatorial segment, we did not. By flow cytometric analysis, Maistre et al. (1996) studied the reactivity of ConA and found a very weak reactivity in fresh sperm; however, an increase was detected after percoll centrifugation. They only indicated that the large numbers of sperm were stained on the equatorial band.

Human sperm is bound by the PNA lectin (Kallajoki and Suominen, 1984; Lee and Damjanov, 1985; Cross and Overstreet, 1987; Mortimer et al., 1987; Bains et al., 1992). Other authors have observed changes in the binding pattern with this lectin in mice (Baker et al., 2004). Purohit et al. (2008) observed that the capacitated spermatozoa were slightly stained with PNA at the acrosomal region, more intensely at the equatorial segment, and only slightly at the post-acrosomal region. They did not detect any differences between normal and infertile subjects in uncapacitated sperm. Our results were consistent with these authors in that some cells were PNA bound with high intensity to the sperm acrosomal region (P1), and others showed dotted fluorescence at the acrosomal region with a highly stained equatorial segment (P2), before and after selection. This pattern (P1) was described by Mortimer et al. (1987) who also showed an increase in this pattern after incubation for 20 hours. We observed that, in highly motile sperm population, the distribution of this lectin was more homogenous, and was restricted to the acrosomal region. Moreover, PNA has been used to evaluate the acrosome status (Patrat et al., 2004), as it is known that the lectin binds to the outer acrosome membrane (Cross et al., 1986). We still saw dotted labeling in the acrosomal region in P2, and results showed an equivalent proportion of sperm that changes from P1 to P2 after sperm selection, suggesting a reorganization of glycoconjugates that could be necessary in the early stages of acrosome reaction.

It has previously been reported that WGA binds to the whole plasma membrane of fresh human sperm (Cross and Overstreet, 1987; Bains et al., 1992; Fierro, 1996). In this study, we observed that the WGA lectin attached to the whole plasma membrane of the head forming clusters in fresh sperm. After selection by swim-up, WGA staining was restricted to the plasma membrane of the acrosomal region of the sperm. This lectin binding is also more homogeneous. Our results are consistent with Lassalle and Testar (1994) who observed a marked decrease in binding with WGA after incubation for 24 hours at 4ºC, suggesting that the release of sialic acid from the sperm plasma membrane could be one of the capacitation events necessary for unmasking certain sperm surface antigens implicated in zona pellucida recognition. Maistre et al. (1996) observed changes with this lectin after sperm selection with percoll gradient centrifugation, but these authors did not show the patterns of distribution. Also, other authors have suggested that sialic acid may be responsible for the net negative surface charge before and during capacitation, and that removal of sialic acid residues may be essential for sperm-egg interaction (Holt, 1980; Desantis et al., 2010; Ainsworth et al., 2011). In bovine sperm, Taitzoglou et al. (2007) demonstrated that capacitation is accompanied by a quantitative reduction in WGA binding sites. Gabriel et al. (1994) indicated a correlation between wheat germ agglutinin receptor and semen parameters, specifically, morphology. According to our findings the high motile

spermatozoa had the WGA receptors on the acrosomal region and these cells had normal morphology. This subpopulation of cells, which present this pattern for WGA, could be good quality spermatozoa.

For the WGA lectin, the most abundant pattern in unselected sperm is P3. However, this lectin is distributed homogenously over the acrosomal region (P1) after selection. With PNA, the most abundant pattern before selection was P1. Nevertheless, in highly motile sperm the most representative pattern was P2. Meanwhile, the most representative patterns with the lectins AAA and ConA in both unselected and selected sperm were P4 and P6, respectively. It is possible that the changes observed with these lectins correspond to a gradual remodelling of the sperm plasma membrane produced during the early stages of the capacitation process. A recent work has demonstrated that sialyl-Lewisx oligosaccaride is an important carbohydrate ligand for egg-sperm interaction (Pang et al., 2012), and it would be interesting to know if it changes during sperm selection and capacitation.

In conclusion, with this report we have demonstrated that after swim-up selection there are different subpopulations of spermatozoa characterized by a great diversity in carbohydrate distribution. This change in the carbohydrate composition or distribution is probably due to the extensive reorganization of proteins on the sperm head during early stages of capacitation which could be required for sperm to be able to undergo acrosome reaction and interact properly with the oocyte and successfully complete fertilization. In the future, we want to test whether these changes are correlated with other molecular parameters such as sperm DNA damage, apoptosis, nuclear vacuolization grade, sperm binding to the zona pellucida and fertilizing capacity of sperm. This information will probably contribute to the improvement of sperm preparation techniques for assisted reproduction.

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