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Antisperm antibodies modify plasma membrane functional integrity and inhibit osmosensitive calcium influx in human sperm

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BACKGROUND: The hypo-osmotic swelling (HOS) test evaluates the ability of the functional sperm plasma membrane to stretch following cell swelling when exposed to hypo-osmotic solutions. Sperm samples with low HOS scores show low fertilization and pregnancy rates during assisted reproductive techniques, though data are controversial. The aim of this study was to compare the results of the HOS test in a group of normozoospermic men with those in a group of subjects affected by autoimmune infertility due to the presence of antisperm antibodies (ASA) bound to the sperm surface. METHODS: Sperm from normozoospermic and from infertile subjects affected by autoimmune infertility were exposed to hypo-osmolar conditions to verify the effects on intracellular calcium concentrations and acrosome reaction. RESULTS: Sperm samples from infertile men with ASA showed HOS test scores that were significantly lower than those of normozoospermic subjects despite similar sperm viability percentages. Sperm with ASA bound to their plasma membrane showed a reduced rise in intracellular calcium concentrations and acrosome reaction after hypo-osmotic challenge with respect to sperm from normozoospermic subjects without ASA. CONCLUSIONS: Infertile subjects with ASA have a reduced sperm plasma membrane functional integrity that could explain, at least in part, the low fertilization and pregnancy rates observed in these subjects during assisted reproductive procedures. Evaluation for the presence of ASA in all sperm samples showing low HOS test scores in the presence of normal sperm viability percentages is suggested.

Key words: acrosome reaction/antisperm antibodies/calcium/human sperm/hypo-osmotic swelling test

Introduction

Jeyendran *et al.* (1984) introduced the hypo-osmotic swelling test (HOS) as a laboratory index of the functional integrity of the sperm plasma membrane. The HOS test measures the ability of the sperm plasma membrane to transport water when exposed to hypo-osmotic solutions, thus inducing cell swelling and plasma membrane stretching. If water transport does not occur, it can be assumed that the sperm plasma membrane is functionally inactive and that it cannot be functional during the fertilization process. This test differs from the viability stains that evaluate whether or not the sperm plasma membrane is physically altered due to sperm death. Sperm from normozoospermic fertile subjects have normal viability and HOS test percentages and usually these parameters show a strict correlation (Bahamondes *et al.*, 2001). Since sperm plasma membranes may be physically intact (live sperm) but functionally inactive, the HOS test gives additional information on the sperm functional status. In this regard the World Health Organization (WHO, 1999) has advised its use as an additional test in the routine analysis of semen.

Since its introduction, the results of the different published studies evaluating the clinical usefulness of the HOS test have shown that semen samples with low HOS test scores are associated with normal fertilization but low pregnancy rates during IVF techniques (Check *et al.*, 1995; Katsoff *et al.*, 2000) although controversial data have been reported so far (Check *et al.*, 1989, 1995, 2001b; Enginsu *et al.*, 1992; Abu-Musa *et al.*, 1993; Biljan *et al.*, 1996; Katsoff *et al.*, 2000).

Previous reports have described the association between the presence of sperm antibodies and reduced HOS test score (Kiefer *et al.*, 1996; Katsoff and Check, 1997; Wen *et al.*, 2000). As reported recently by Check's group, it is not clear if sperm from subjects affected by antisperm autoimmunity have some plasma membrane alteration leading to low HOS test scores or if the antisperm antibodies directly affect the sperm plasma membrane functionality (Jaraj *et al.*, 2000). The fact that antisperm antibodies can influence plasma membrane functional integrity (as evaluated by HOS test) has been further confirmed by a study reporting that long-term corticosteroid therapy for antisperm autoimmunity

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improves HOS test scores as well as sperm motility and pregnancy rates in a group of men affected by autoimmune infertility (Omu *et al.*, 1996).

In the present study, we evaluated the correlation between HOS test score and standard sperm parameters in a large group of infertile subjects with autoimmune infertility. The effects of antisperm antibodies bound to the sperm plasma membrane on the hypo-osmolarity-induced calcium influx and acrosome reaction were examined.

Materials and methods

Semen sample analysis

We evaluated semen samples from 1228 subjects referred to our Centre for infertility. Of these, 116 infertile subjects showing antisperm antibodies bound on their sperm after semen analysis were recruited for the study. Semen was collected by masturbation after a 3 day period of sexual abstinence. Semen analysis was performed following WHO (1999) recommendations. After liquefaction, each semen sample was evaluated for pH, sperm number, motility, morphology, HOS test and viability (as evaluated by the red–eosin exclusion test). The presence of antisperm antibodies was detected using the Sperm Mar test for IgG family (FertiPro N.V., Belgium). Seminal parameters from 86 normozoospermic men without antisperm antibodies were considered as controls.

HOS test

The HOS test was performed as originally reported by Jeyendran *et al.* (1984): 0.1 ml of semen sample was mixed with 1.0 ml of a hypo-osmotic solution (150 mOsm) containing fructose and sodium citrate. After incubation for 30 min at 37°C, $\geq$100 sperm were analysed by phase-contrast microscopy, evaluating the modifications of the sperm tail to score swollen sperm, which were reported as a percentage of all sperm observed.

Sperm selection and incubation

When performing functional studies, we isolated sperm using density gradients (Isolate; Irvine Scientific, USA) following the manufacturer's instructions to obtain a population of highly motile sperm. All experiments were performed utilizing sperm isolated with this technique. Sperm isolated by this method from ASA positive subjects and fertile controls were collected and resuspended in standard saline containing (in mmol/l): 125 NaCl, 4.8 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 5.6 glucose, 25 $NaHCO_3$, 1.7 $CaCl_2$, 20 HEPES (pH 7.4, 37°C). Sperm concentration was adjusted to 15×10^6/ml.

To evaluate the effects of sperm exposure to hypo-osmotic medium on intracellular calcium concentration ($[Ca^{2+}]_i$) and acrosome reaction, sperm aliquots from seven infertile subjects, each showing 100% of sperm with ASA bound to their plasma membrane, were processed as described (Rossato *et al.*, 1996). As controls, we considered sperm samples from five normozoospermic fertile subjects that were treated as described for ASA positive sperm samples. To obtain a reduction of medium osmolarity, isolated motile sperm were diluted with distilled water to obtain a reduction of medium osmolarity of 30%.

$[Ca^{2+}]_i$ measurement

$[Ca^{2+}]_i$ was measured utilizing the fluorescent probe fura-2/AM as previously described (Rossato *et al.*, 1996): isolated sperm were incubated for 30 min at 37°C in the presence of fura-2/AM (2 mmol/l). After loading, sperm were washed by centrifugation at 800 g for 10 min, resuspended in standard saline and maintained at room temperature until used. $[Ca^{2+}]_i$ was measured before and after sperm medium dilution in distilled water as previously described (Rossato *et al.*, 1996).

$[Ca^{2+}]_i$ was measured using a Perkin Elmer LS50B fluorimeter equipped with a thermostatic and magnetically stirred cuvette holder and utilizing 1.0 ml sperm aliquots. The excitation wavelength was alternated between 350 and 380 nm and emission fluorescence was continuously monitored at 505 nm.

Acrosome reaction evaluation

Sperm aliquots were retrieved before and after reduction of external medium osmolarity by 30%. After fixation with formaldehyde the percentage of acrosome reacted sperm was assayed using an indirect fluorescence technique with fluorescein isothiocyanate-conjugated lectin from *Pisum sativum*, which selectively binds to the acrosomal matrix of acrosome-intact sperm. Non-fluorescent sperm were then scored as acrosome-reacted while fluorescent sperm were scored as acrosome intact. Two hundred sperm were scored in each sample to evaluate the percentage of acrosome-reacted sperm.

Statistical analysis

For statistical analysis the mean percentage of swollen and live sperm from controls and ASA positive subjects were compared using Student's *t*-test. A correlation analysis between HOS test scores and percentages of live sperm was performed by simple linear regression and determination of the correlation coefficients.

Results

Among the 1228 subjects evaluated in the present study, 116 (9.4%) showed ASA bound to sperm plasma membrane. Standard semen parameters from normozoospermic control subjects and patients with ASA are reported in Table I and were similar in the two groups, apart from mean sperm motility which was slightly lower in patients with ASA although with no statistical significance. The mean percentage of HOS score in ASA positive sperm samples was 58.7 ± 12.1%, significantly lower than that observed in ASA negative sperm

Table I. Standard semen parameters and hypo-osmotic swelling (HOS) scores in patients with antisperm antibodies (ASA) and in control subjects

Subjects	Sperm concentration ($\times 10^6$/ml)	Sperm motility (%)	Sperm morphology (%)	HOS score (%)	Sperm viability (%)
Controls	60.4 ± 7.8	54.2 ± 7.2	53.3 ± 9.1	77.5 ± 5.9	79.9 ± 6.3
ASA positive	51.9 ± 8.9	41.2 ± 9.9	51.9 ± 8.2	58.7 ± 12.1[a]	78.8 ± 6.7

Values are mean ± SD.
[a]$P < 0.0001$ versus controls.

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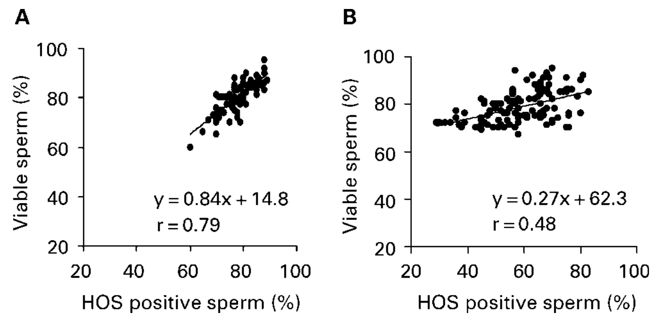

Figure 1. (**A**) Correlation between percentages of hypo-osmotic swelling (HOS) positive and viable sperm in normozoospermic subjects. (**B**) Correlation between percentages of HOS positive and viable sperm in infertile subjects with antisperm antibodies.

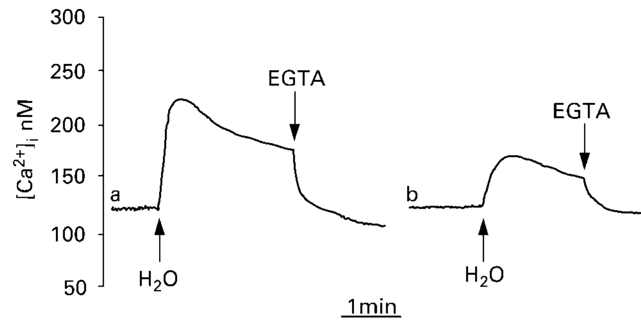

Figure 2. Effects of medium osmolarity reduction (–30%) on sperm $[Ca^{2+}]_i$ in isolated sperm from subjects without (trace **a**) and with (trace **b**) antisperm antibodies (ASA) bound on their plasma membrane (see Materials and methods for details). Traces represent the typical response observed in all samples evaluated from ASA positive and normozoospermic subjects. Where indicated, a proper amount of pre-warmed distilled water was added to reduce medium osmolarity by 30%. The calcium-chelating agent EGTA was added at the concentration of 2.0 mmol/l.

(77.5 ± 5.9%, $P < 0.0001$, Table I). On the contrary, mean percentages of live sperm, as evaluated with the eosin exclusion test, were very similar between ASA positive and negative sperm samples (78.8 ± 6.7 versus 79.9 ± 6.3 respectively, $P = 0.099$, Table I). Live sperm usually have a normally functional plasma membrane, thus HOS score and viability percentages should correlate (Bahamondes *et al.*, 2001) since a cell with a normally functional plasma membrane has to be alive. In fact, as reported in Figure 1A, HOS test and viability scores in normozoospermic subjects are closely related ($r = 0.79$; $P < 0.001$. On the contrary, when HOS test and viability scores in sperm from ASA positive subjects were analysed, a poor correlation was observed ($r = 0.48$; $P < 0.001$ Figure 1B). Among all subjects with ASA, there was a wide range of low HOS score, favouring the presence of other causes determining lower plasma membrane functionality other than direct effect of ASA. This seems confirmed by the observation that subjects with low ASA percentage also have a wide range of HOS score.

Table II. Effects of sperm exposure to hypo-osmotic medium on $[Ca^{2+}]_i$ and acrosome reaction percentages in antisperm antibodie (ASA) positive and normozoospermic control subjects

ASA positive	$[Ca^{2+}]_i$ (nmol)	Acrosome reaction (%)
1	55.6	8.3
2	60.5	13.6
3	58.8	11.2
4	63.6	7.9
5	60.3	8.4
6	42.1	6.8
7	80.2	7.5
Mean ± SD	60.2 ± 10.4[a]	9.1 ± 2.2[a]
Controls		
1	98.1	19.5
2	121.8	28.2
3	135.2	22.0
4	90.3	16.3
5	110.4	26.7
Mean ± SD	111.2 ± 16.1	22.5 ± 4.4

$[Ca^{2+}]_i$ values represent the increment of $[Ca^{2+}]_i$ above the basal levels; acrosome reaction percentages represent the absolute percentages of acrosome-reacted sperm observed after sperm incubation in hypo-osmotic medium.
[a]$P < 0.01$ versus controls.

Among subjects with ASA, 21 out of 116 (18.1%) showed ASA bound to 100% of their motile sperm. In seven of these subjects we evaluated the variation of $[Ca^{2+}]_i$ and acrosome reaction percentages induced by reduction of external medium osmolarity as previously described (Table II) (Rossato *et al.*, 1996). As reported in Figure 2, sperm from normozoospermic subjects exposed to hypo-osmotic medium show a rapid increase in $[Ca^{2+}]_i$ due to the opening of putative stretch-activated Ca^{2+} channels activated by plasma membrane distension after water influx within sperm cytoplasm and intracellular volume expansion (Rossato *et al.*, 1996). The hypo-osmotic challenge of sperm with ASA bound to their plasma membrane showed a significant reduction of $[Ca^{2+}]_i$ increase and acrosome reaction with respect to those observed in normozoospermic subjects (Table II, Figure 2).

In both groups of subjects the rise of $[Ca^{2+}]_i$ was due to an influx of calcium from the extracellular medium since it was completely blunted by the removal of calcium from the extracellular medium by the addition of the calcium chelator EGTA.

The location of antibody binding on sperm plasma membrane was mixed (head, middle piece and tail) and it was therefore not possible to correlate the effects of ASA location on changes in HOS test percentages, variation of $[Ca^{2+}]_i$ and acrosome reaction percentages induced by reduction of external medium osmolarity.

Discussion

Among the different laboratory procedures performed during semen analysis, the HOS test evaluates the ability of water to enter within sperm cytoplasm when exposed to hypo-osmotic solutions, thus inducing sperm swelling (Jeyendran *et al.*, 1984; WHO, 1999). Where this occurs, the sperm plasma membrane can be considered functionally active, thus suggesting the normal functionality of the plasma membrane of these swollen sperm. In contrast to the viability stains that measure whether the sperm plasma membrane is physically

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intact or disrupted, the HOS test is more specific, giving information on the functional integrity of the plasma membrane (Jeyendran *et al.*, 1984; WHO, 1999). On this basis it can be assumed that a dead sperm has a functionally inactive plasma membrane so that it does not swell when exposed to hypo-osmotic solution. On the contrary, a live sperm has a physically intact plasma membrane but one that could be functionally inactive, thus not swelling when exposed to hypo-osmotic solutions. The HOS test has become an important laboratory procedure during semen analysis for male infertility evaluation (Jeyendran *et al.*, 1984; WHO, 1999). Although some disagreements exist (Barratt *et al.*, 1989; Sjoblum and Coccia, 1989; Chan *et al.*, 1990; Avery *et al.*, 2000), it is accepted that sperm samples from fertile subjects have normal HOS test scores and that those from infertile men with low HOS test scores show low pregnancy rates during assisted reproductive techniques (Check *et al.*, 1995, 2002; Katsoff *et al.*, 2000). The main result of the present study is the demonstration that HOS test score in sperm samples from patients with autoimmune infertility is significantly lower than that observed in sperm from ASA negative normozoospermic subjects in the presence of normal and comparable sperm viability and motility. Interestingly, the mean HOS test score in sperm with ASA is very close to the threshold value of 50% that is considered critical for its association with male infertility or subfertility (Jeyendran *et al.*, 1984; Check *et al.*, 1989).

Previous preliminary studies (Omu *et al.*, 1996, 1997; Jairaj *et al.*, 2000) have suggested that ASA may somewhat modify sperm plasma membrane integrity, leading to low HOS test score, but to date, no planned studies have confirmed these observations. The present data clearly demonstrate that sperm with ASA bound to their plasma membranes show low HOS test scores, and this non-specific alteration of the plasma membrane integrity may participate in the determination of infertility due to ASA. How ASA interfere with sperm plasma membrane functionality has been not elucidated yet but it is possible that ASA modify sperm plasma membrane permeability or fluidity, leading to low fertilizing potential. One possible explanation for low HOS test score in ASA positive sperm samples is that ASA modify water permeability. In this regard it has been demonstrated that water transport across cell plasma membranes utilizes specific water channels named aquaporins (Ishibashi *et al.*, 1997; Borgnia *et al.*, 1999; Calamita *et al.*, 2001). It is possible that ASA bound to sperm surface may non-specifically or specifically block these water channels, thus altering sperm water permeability. Furthermore, antibody cross-linking could prevent plasma membrane distensibility, thus reducing sperm swelling when exposed to hypo-osmotic medium. Further confirmation that ASA may alter sperm plasma membrane permeability derives from a recent study reporting that sperm from a subject who underwent vasectomy reversal show low HOS test scores that remain low also after 1 year from the vasectomy reversal (Wen *et al.*, 2000). It is well known that vasectomized subjects develop ASA (Gubin *et al.*, 1998) and thus it is possible that the low HOS test score reported by these authors in sperm from subjects after vasectomy reversal may be due to ASA bound to the sperm surface.

Jairaj *et al.* (2000) reported no significant reduction of HOS test score in sperm previously incubated with positive ASA serum. The different results obtained in that study compared to ours may be due to the fact that in our study ASA were present in semen and directly bound to sperm. Furthermore those authors incubated sperm with ASA positive serum for 60 min, and it cannot be excluded that longer incubation or higher ASA concentration may be necessary to induce sperm plasma membrane alteration. Our data suggest that subjects with ASA show a wide range of HOS scores irrespective of the percentage of ASA, thus favouring the association with some possible common aetiology other than a direct effect of ASA in determining the lower sperm plasma membrane functionality during incubation in hypo-osmotic medium. The nature of this phenomenon remains to be elucidated.

The role of sperm plasma membrane permeability to water in regulating important sperm functions has been shown in previous studies (Morisawa and Suzuki, 1980; Inoda and Morisawa, 1987) and we have demonstrated that human sperm exposure to hypo-osmotic medium activates an influx of water within sperm cytoplasm. This water influx induces a sperm volume increase and plasma membrane stretching, leading to the opening of osmosensitive calcium channels, calcium influx within sperm cytoplasm and activation of acrosome reaction (Rossato *et al.*, 1996). On the other hand, the osmosensitivity of sperm acrosome reaction in man has been reported previously (Bielfeld *et al.*, 1993) and the role of external osmolarity in the regulation of mammalian sperm functions is well known (Liu and Foote, 1998; Rossato *et al.*, 2002). The effects of ASA and of other still unknown mechanisms present in autoimmune infertility in reducing plasma membrane water permeability, as shown by reduced HOS test score, could induce also a reduction of sperm responsiveness to putative hypo-osmotic stimuli fundamental for sperm activation during the fertilization process, as suggested by the low osmolarity of female genital tract secretions with respect to that of semen (Polak and Daunter, 1984; Rossato *et al.*, 1996). Indeed this hypothesis was demonstrated to be true since sperm with ASA bound to their surface show a marked reduction of $[Ca^{2+}]_i$ rise and acrosome reaction percentage increase induced by sperm exposure to hypo-osmotic medium, as evidenced in sperm from normozoospermic subjects without ASA. These observations confirm that ASA alter sperm membrane functionality, impairing important transduction signalling pathways, leading ultimately to alteration of the sperm fertilizing ability.

The results of the present study, beyond the suggestion of performing the HOS test during routine semen analysis, could also have a practical aspect since, when a sperm sample shows a low HOS score associated with normal percentages of live sperm, ASA may be bound to those sperm. This may indicate evaluating the presence of ASA, a procedure that is not performed as a routine evaluation during semen sample analysis in a number of laboratories.

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Furthermore these observations could also have important clinical implications. When a semen sample shows a low HOS test score, it could be positive for ASA, and when performing an assisted reproduction technique, ICSI should be preferred to IVF or intrauterine insemination as recently suggested (Check *et al.*, 2001).

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