Cey. J. Sci. (Bio. Sci.) Vol 24, No.1, 1995

HYPOOSMOTIC SPERM SWELLING TEST: REEVALUATION OF ITS POTENTIAL USE

***W.D. RATNASOORIYA and R.J. AITKEN**

* Department of Zoology, University of Colombo, Colombo 03, Sri Lanka, and MRC Reproductive Biology Unit, Centre for Reproductive Biology, 37, Chalmers Street, Edingbur EH3 9EW, U.K.

ABSTRACT

The aim of this study was to assess the predictive value of the hypoosmotic sperm swelling test. Good correlations were observed between the percentage of spermatozoa undergoing swelling and the percentage motility both in normal ($r = 0.68$; $p < 0.001$) and infertile patients ($r = 0.65$; $p < 0.001$). In contrast, sperm swelling did not significantly correlate either with sperm oocyte fusion (assessed by the zona-free hamster egg penetration test) or with the ability of the spermatozoa to penetrate human mid-cycle cervical mucus.

INTRODUCTION

The conventional semen analysis, which provides information on sperm number and morphology, is of limited value in the diagnosis of male fertility since it does not assess the functional competence of spermatozoa (Aitken *et al.* 1984a). Several techniques, such as measurement of ATP content (Comhaire *et al.* 1983), detection of reactive oxygen species (Aitken *et al.* 1987b), ability to penetrate bovine (Alexander *et al.* 1981) and human (Katz *et al.* 1980) cervical mucus and zona-free hamster oocytes (Yanagimachi *et al.* 1976) or human zona pellucidae (Overstreet *et al.* 1980) are available to analyse specific aspects of sperm function. However, most of these techniques are complex, costly and not suitable for routine application. Moreover, many laboratories do not possess the expertise or facilities to perform these tests. Thus, there is a clear need for the development of alternative techniques to evaluate the fertilizing ability of human spermatozoa, which are simple, inexpensive and can be performed routinely.

The hypoosmotic swelling test developed by Jayendran *et al.* (1984) appears to satisfy these criteria. It is claimed to measure the functional integrity of the sperm plasma membrane, detected as the ability of the spermatozoa to swell when immersed in hypoosmotic media. A high correlation ($r = 0.90$) between the percentage of spermatozoa that swelled under hypoosmotic conditions and their ability to penetrate denuded hamster oocytes was demonstrated using donor semen (Jeyendran *et al.* 1984). However, in a larger study employing 171 heterogeneous semen samples (Chan *et al.* 1985) no significant positive correlation between sperm swelling and zona-free hamster egg penetration was

observed. This discrepancy creates some doubts about the value of the swelling test in sperm function analysis. On the other hand, it is possible that the different assay conditions used by these authors resulted in the conflicting data. Several factors such as the composition of the medium, sperm concentration, motility, the number of oocytes or duration of capacitation time are known to alter the sensitivity and the outcome of the hamster oocyte penetration test (Aitken *et al.* 1984b; Aitken *et al.* 1987a).

Therefore, in the present study a reassessment has been made of the relationship between sperm swelling and the capacity for oocyte fusion using an alternate protocol, incorporating the divalent cation ionophore A23187. The use of this ionophore bypasses capacitation and synchronises the acrosome reaction hereby optimizing the sensitivity and reliability of the test (Aitken *et al.* 1987a). In addition, we have taken the opportunity to investigate the relationship between sperm swelling and other aspects of sperm function including the capacity for cervical mucus penetration.

MATERIALS AND METHODS

Semen samples : Semen samples were obtained from healthy donors by masturbation (sperm concentration 20 x 10^6 spermatozoa/ml, motility 40% motile spermatozoa, morphology 40% normal forms) as well as from men under investigation for infertility were used. The female partners of these patients were normal according to the criteria given by Aitken *et al.* (1984a) relating to their menstrual history, luteal phase progesterone levels and diagnostic laparoscopy.

Semen motility : The motility of each sample was objectively assessed under phase contrast microscopy using an eye-piece graticule and hand counter (World Health Organization, 1980).

Semen swelling : 100ul of liquified semen was mixed with 1.0ml of hypoosmotic medium (7.35g sodium citrate and 13.51g fructose, in 1000ml distilled water) and incubated at 37°C for 60 minutes. The proportion of spermatozoa with coiled tails was assessed under phase contrast microscopy according to the criteria described by Jeyendran *et al.* (1984).

Zona-free hamster oocyte assay : This was performed using the methodology of Aitken *et al.* (1984a). In outline, the spermatozoa were suspended at a concentration of 10 x 10^6/ml in BWW medium containing a 100 μM suspension of the Ca++, Mg++ salt of the divalent cation ionophore A23187 (Calbiochem, Bishops Stortford, UK). The spermatozoa were incubated under these conditions for 3h, after which they were centrifuged and resuspended at a concentration of 10 x 10^6/ml in normal medium BWW prior to the introduction of zona-free hamster oocytes. The incubations were continued for a further 3h, at which time the oocytes were examined for the presence of decondensing sperm heads with an attached or closely associated tail under phase contrast microscopy.

Penetration of cervical mucus : Cervical mucus from patients attending an Artificial Insemination Donor clinic was aspirated 24-34h after the initiation of the LH surge as described previously (Aitken *et al.* 1986). The mucus was drawn into capillary tubes, having a depth of 300 μm and a volume of 25 μl (Camlab, Cambridge, UK). One end of the capillary tube was sealed with cristoseal (Lancet, Sherwood Medical, USA) and were equilibrated for 20 min. at 37°C in a humidified chamber containing an atmosphere of 5% CO_2 and 95% air. The open end of each tube was introduced into a 200 μl sample of semen, inclined at an angle of 45° and incubated for 30min. in an atmosphere of 5% CO_2 and 95% air. Then the capillary tubes were removed, and rinsed in a mucolytic solution containing 0.1% 2-mercaptoethanol and 10% formalin in Dulbecco's phosphate-buffered saline (Flow Laboratories, UK) to remove residual spermatozoa on the outersurface of the tube and non-penetrating spermatozoa from cervical mucus interface. The mucus was then extruded into 425 μl of mucolytic solution and the concentration of the spermatozoa was determined using an improved Neubauer haemocytometer. the mean linear velocity of progression of spermatozoa in the semen sample was measured using time-exposure photomicrography (Aitken *et al.* 1982) and the success of sperm interaction with cervical mucus (PSC) was calculated as described by Katz *et al.* (1980).

RESULTS

Samples from 30 different normospermic individuals were assessed for percentage motility and sperm swelling ability in the hypoosmotic medium. The mean ± SEM of percentage motility and percentage swollen spermatozoa were 60.20 ± 1.85% (range 42-71%) and 78.20 ± 1.97% (range 54-94%) respectively. A significant ($p < 0.001$) positive correlation ($r = 0.68$) was evident between the sperm swelling test and sperm motility. The percentage motility and percentage swelling in the 27 patient semen were also comparable to that of normospermic men (Table 1). Moreover, as with

normospermic samples, a significant ($p < 0.001$) positive correlation ($r = 0.65$) was found between percentage swelling spermatozoa and percentage motility. The mean percentage of oocytes penetrated and average number of spermatozoa penetrating each oocyte are shown in Table 1. As expected, there was considerable variation in these parameters since the samples originated from patients undergoing assessment for infertility. A comparable variation in the percentage swollen spermatozoa was also seen. However, no significant correlation was found between the ability of spermatozoa to swell in the hypoosmotic medium and their ability to penetrate zona free hamster oocytes using this particular protocol.

The results of the cervical mucus penetration test and the percentage swelling of the spermatozoa in the 10 samples from supposedly infertile men are summarized in Table 2. No significant correlation was demonstrated between the percentage of spermatozoa that under went swelling in response to hypoosmotic conditions and the concentration of spermatozoa penetrating the cervical mucus in unit time ($r = 0.52$) or the percentage successful collisjons ($r = 0.29$).

Table 1. Mean values of % swelling, % motility and hamster egg penetration parameters of a group of infertile men (n = 27)

Measurement	Means ± SEM	Range
% Swelling	73.22 ± 3.41	15 - 88
% Motility	55.40 ± 3.73	15 - 88
% oocytes penetrated	23.06 ± 6.47	0 - 100
Spermatozoa/oocyte	0.597 ± 0.229	0 - 4.25

Table 2. Relationships between cervical mucus test and % swelling in a sample of 10 infertile patients

Measurement	Means ± SEM	Range
% swelling	70.90 ± 3.25	57 - 87
Concentration of spermatozoa in servical mucus (x 10^6)	191.76 ± 6.70	0.85 - 63.75
Percentage successful collisions	62.02 ±11.65	21.80 - 155.00

DISCUSSION

The present results clearly demonstrate that there is no significant correlation between the ability of spermatozoa to swell under hypoosmotic conditions and their ability to penetrate zona-free hamster oocytes. In this study semen from a variety of infertile men were used and the penetration assay was performed with the divalent cation ionophore A23187, which circumvents some of the shortcomings of this heterologous system (Aitken *et al.* 1984b). Such results indicate that the lack of correlation observed between sperm swelling and oocyte penetration was not due to suboptimal conditions used for carrying out the penetration assay. The results are in agreement with those obtained by Chan *et al.* (1984) who found no significant correlation between percentage swollen spermatozoa in hypoosmotic media and their ability to penetrate denuded hamster oocytes. In contrast, Jeyendran *et al.* (1984) showed a high correlation ($r = 0.90$) between sperm swelling and oocyte penetration. Differences in the composition of the patient populations may account for this discrepancy.

On the other hand, a significant correlation between sperm swelling and percentage motility was observed both in samples from donors ($r = 0.68$) and infertile patients ($r = 0.65$) in accord ($r = 0.61$) with the study of Jeyendran *et al.* (1984) and the weakly significant correlation ($r = 0.22$) recorded by Chen *et al.* (1985). These observations suggest that the hypoosmotic swelling test can measure some aspects of sperm plasma membrane function related to motility. If this argument is correct, it is perhaps surprising that no significant correlation was observed between sperm swelling and the outcome of the cervical mucus penetration test which is heavily dependent on motility. The lack of correlation may reflect the fact that penetration of cervical mucus depends not only on the presence of motility, but on particular pattern of sperm movement (Aitken *et al.* 1985), which is not reflected in the outcome of the hypoosmotic swelling test.

The hypoosmotic sperm swelling test is based on the tenet that if spermatozoa are immersed in a hypoosmotic medium, an influx of water should occur to re-establish an osmotic equilibrium. If

the spermatozoon has an intact plasma membrane, this inflow of water, causes the cell to swell, which is finally reflected as a curling of the sperm tail. Jeyendran *et al.* (1984) claimed that this response is an indication of the functional integrity of the sperm plasma membrane. However, in reality this test is capable only of measuring those physical properties of the plasma membrane (fluidity and structural integrity) required to accommodate the swelling process (Schrader *et al.* 1986). The plasma membrane of spermatozoon is a complex and dynamic structure involved in many sophisticated biological fusions such as the membrane fusion events involved in acrosome reaction or sperm oocyte interaction, the generation of flagellar wave form of sperm tail, regulation of fluxes of biologically important irons (Na+, K+ or Ca++) superoxide anion production (Aitken *et al.* 1987) which are inturn mediated through the activity of membrane bound enzymes, for example ATPase (Breitbart *et al.* 1985) and phospholipase (Llanos *et al.* 1982). In view of the sophisticated activities performed by the sperm plasma membrane, it is an exaggeration to claim that the functional integrity of the sperm membrane can be assessed merely by assessing its ability to swell under hypoosmotic conditions. Such functions depend on diverse array of membrane properties, only a fraction of which involve dependence on the physical properties of the plasma membrane reflected in the outcome of the hypoosmotic sperm swelling test.

REFERENCES

Alexander, N.J. (1981) Evaluation of male infertility with an *in vitro* cervical mucus penetration test. *Fertil. Steril.* **36** 201-208.

Aitken, R.J., Best, F.S.M., Richardson, D.W., Djahanbakhch, O. & Lees, M.M. (1982). The correlates of fertilizing capacity in normal fertile men. *Fertil. Steril.* **38**: 68 - 76.

Aitken, R.J., Best, F.S.M., Warner, P. & Templeton, A. (1984A). A prospective study on the relationship between semen quality and fertility in cases of unexplained infertility. *Int. J. Androl.* **5**: 297-303.

Aitken, R.J., Ross, A., Hargreave, T., Richardson, D.W. & Best, F.S.M. (1984b). Analysis of human sperm function following exposure to the ionophore A23187. Comparison of normospermic and oligospermic men. *J. Androl.* **5**: 321-329.

Aitken, R.J., Sutton, M., Warner, P. & Richardson, D.W. (1985). Relationship between the movement characteristics of human spermatozoa and their ability to penetrate cervical mucus and zona free hamster oocytes. *J. Reprod. Fert.* **73**: 441-449.

Aitken, R.J., Warner, P.E. & Reid, C. (1986). Factors influencing the success of sperm-cervical mucus interaction in patients exhibiting unexplained infertility. *J. Androl.* **7**: 3-10.

Aitken, R.J., Thatcher, S., Glasier, A.F., Clarkson, J.S., Wu, F.C.W. & Baird, D.T. (1987a). Relative ability of modified versions of the hamster oocyte penetration test, incorporating hyperosmotic medium or the ionophore A23187, to predict IVF outcome. *Human Reprod.* **2**: 227-231.

Aitken, R.J. & Clarkson, j.S. (1987b). Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa. *J. Reprod. Fert.* **81**: 459-469.

Breitbart, H., Rubinstein, S. & NAN-ARDEN, L. (1985). The role of calcium and Ca ATPase in maintaining motility in ram spermatozoa. *J. Bio. Chem.* **260**: 11548-11553.

Chan, S.Y.W., Fox, E.J., Chan, M.M.C., Tsoi, W-C., Wang, C., Tang, L.C.H., Tang, G.W.K. and Ho, P-C (1985). The relationship between the human sperm hypoosmotic swelling test, routine semen analysis, and the human sperm zona-free hamster ovum penetration assay. *Fertil. Steril.* **44**: 668-672.

Comhaire, F., Vermeulen, L., Ghedira, K., Mas, J., Irvine, S. & Callipotitis, G. (1983). Adenosine triphosphate in human semen: a quantitative estimate of fertilizing potential. *Fertil. Steril.* **40**: 500-504.

Jeyendran, R.S., Vander, Ven. H.H., Perez-peleaz, M., Crabo, B.G. & Zaneveld, L.J.D. (1984). Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. *J. Reprod. Fert.* **70**: 219-228.

Katz, D.F., Overstreet, J.W. & Hanson, F.W. (1980). A new quantitative test for sperm penetration into cervical mucus. *Fertil. Steril.* **33**: 179-186.

Llanos, M.M., Lui, C.W. & Meizel, S. (1982). Studies of phospholopase A2 related to the hamster sperm acrosome reaction. *J. Exp. Zool.* **221**: 107-113.

Overstreet, J.W., Yanagimachi, R., Katz, D.F., Hayashi, K. & Hanson, F.W. (1980). Penetration of human spermatozoa into the human zona pellucida and zona-free hamster egg: a study of fertile donors and infertile patients. *Fertil. Steril.* **33**: 534-542.

Shrader, S.M., Platek, S.F., Zaneveld, L.J.D., Perez-pelaez, M. & Jeyendran, R.S. (1986). Sperm viability: a comparison of analytical methods. *Andrologia*, **18**: 530-538.

World Health Organization: Laboratory Manual for the examination of human semen and semen-cervical mucus interaction (1980). Edited by M.A. Belsey, R. Eliasson, A.J. Gallegus, K.S. Moghissi, and C.A. Paulsen, Min. R. Prosad. Singapore: Press Concern.

Yanagimachi, R., Yanagimachi, H. & Rogers, B.J. (1976). The use of zona-free animal ova as a test for the assessment of the fertilizing ability of human spermatozoa. *Biol. Reprod.* **15**: 471-476.

ACKNOWLEDGEMENTS

Professor W.D. Ratnasooriya was supported by a Fellowship from the Association of Commonwealth Universities.