andrologia 18 (5): 530–538 (1986) Received April 25, 1986

Department of Health and Human Services,
U.S. Public Health Service,
National Institute of Occupational Safety and Health,
Division of Biomedical and Behavior Science,
Experimental Toxicology Branch,
Cincinnati, Ohio
Institute of Reproductive Medicine and Infertility
Chicago, Illinois and
Rush University,
Rush-Presbyterian St. Lukes Medical Center
Chicago, Illinois/U.S.A.

Sperm Viability: A Comparison of Analytical Methods

S.M. SCHRADER, S.F. PLATEK, L.J.D. ZANEVELD,
M. PEREZ-PELAEZ and R.S. JEYENDRAN

Summary: Two methods of measuring human sperm viability, the stain exclusion assay and the hypoosmotic swelling (HOS) test, were evaluated. Human sperm were pretreated with 2.0% glutaraldehyde or 0.1% Triton X-100 and compared to untreated controls. Approximately one half of the sperm were found to be viable in the control samples and nearly all sperm were non-viable in the Triton X-100 treated samples by both the stain exclusion and HOS assays. After glutaraldehyde pretreatment, presumably inactivating the spermatozoa, the HOS test revealed that most sperm were not viable, while the stain exclusion test found no difference between glutaraldehyde pretreated sperm and control sperm. Investigations with scanning and transmission electron microscopy demonstrated that the HOS test caused the membrane of the sperm tail to swell and the tail fibers to coil several times within the swollen membrane. It is concluded that the stain exclusion assay merely measures the structural integrity of the sperm membrane, whereas the HOS test also provides an indication of the physiological integrity of the sperm membrane.

Spermatozoen-Lebensfähigkeit: Vergleich von analytischen Methoden

Zusammenfassung: Es werden zwei Meßmethoden für die Lebensfähigkeit menschlicher Spermatozoen ausgewertet: Die Färbe-Ausschluß-Methode und der hypoosmotische Schwellungs-Test (HOS). Menschliche Spermatozoen wurden mit 2,0% Glutaraldehyd oder mit 0,1% Triton X-100 vorbehandelt und gegen Kontrollen verglichen. Annähernd die Hälfte der Spermatozoen wurden in den Kontrollen lebensfähig gefunden; nahezu alle Spermatozoen aus der Triton X-100-Gruppe waren sowohl in der Färbe-Ausschluß- als auch in der HOS-Methode nicht lebensfähig. Nach Glutaraldehyd-Vorbehandlung, wodurch die Spermatozoen offensichtlich inaktiviert wurden, offenbarte der HOS-Test, daß die meisten Spermatozoen nicht lebensfähig waren, während der Färbe-Ausschluß-Test weder bei Glutaraldehyd- noch bei der Kontrolle eine Differenz zeigte. Untersuchun-

Key words: Spermatozoa, viability – vital stain, spermatozoa – hypoosmotic swelling, spermatozoa – viability, spermatozoa

gen mit dem Raster- und Elektronenmikroskop demonstrierten, daß der HOS-Test eine Schwellung der Membran der Spermatozoengeißel verursacht und die Geißelfibern sich einige Zeit in der geschwollenen Membran schlängeln. Es wird die Schlußfolgerung gezogen, daß der Färbe-Ausschluß-Test lediglich die strukturelle Integrität der Spermatozoenmembran mißt, während der HOS-Test außerdem eine Indikation der physiologischen Integrität der Spermatozoenmembran liefert.

Introduction

Viability of spermatozoa can be determined by a stain exclusion method, which was originally developed by Lasley et al. (1942) and later modified to evaluate human sperm (Eliasson and Treichl - 1971). The basis of this assay is that the living cell membrane acts as a barrier to stain penetration thus not allowing nuclear staining. Recently, a new viability assay (Jeyendran et al. - 1984) was introduced which evaluated sperm response to hypo-osmotic stress (hypoosmotic swelling test – HOS test). When a viable sperm is exposed to hypoosmotic conditions, the tail curls and bulges due to the influx of fluids. This change in tail morphology can be identified under phase contrast microscopy and quantified by determining the proportion of sperm that undergo this change in tail morphology. The correlation between the two methods, while significant, is surprisingly low with a correlation coefficient of r = 0.52 (Jeyendran et al. - 1984). Martin and Taylor (1982) reported that no significant correlation existed between the stain exclusion assay and fertilizing capacity as measured by sperm penetration into zona-free hamster eggs. By contrast, the correlation between the results of the HOS and zona-free hamster egg penetration is very high (r = 0.90; Jeyendran et al. - 1984). Since both assays purportedly measure membrane integrity of "living" sperm. the current study was undertaken to clarify the apparent difference between the two assays. Two chemicals which are known to affect the sperm membrane were used: glutaraldehyde, a spermatocide and fixative of cell membranes (Gould - 1980); and Triton X-100, a detergent which dissolves the outer sperm cell membrane (Wooding - 1973).

Scanning and transmission electron microscopy were used to clarify whether this change in tail morphology is due to coiling of the tail fibers within the outer sperm membrane or due to coiling of the tail along with the outer membrane.

Materials and Methods

Semen specimens from 11 healthy men were obtained by masturbation after 3 to 5 days of sexual abstinence. After 30 minutes to allow complete liquefaction, each semen specimen was divided into 3 aliquots. One was kept as a control and the remaining aliquots were treated with equal volumes of either 2% glutaraldehyde or 0.1% Triton X-100 (Sigma Chemical Company, St. Louis) in 0.15 M phosphate buffer for 10 min. All three aliquots were centrifuged at 1000 g for 3 min and the supernatant solution was discarded. The sperm pellet was resuspended to the original aliquot volume with 0.15 M phosphate buffer (pH 7.4) and aliquots from each of these samples were tested by both viability assays.

Viability by stain exclusion was determined using a method modified from Eliasson and Treichl (1971), in which a drop of sperm suspension and a drop of 0.5% Eosin Y stain (Sigma Chemical Co., St. Louis) in 0.15 M phosphate buffer (pH 7.4) were mixed together. The number of unstained (viable) sperm appearing bluish-white and the number

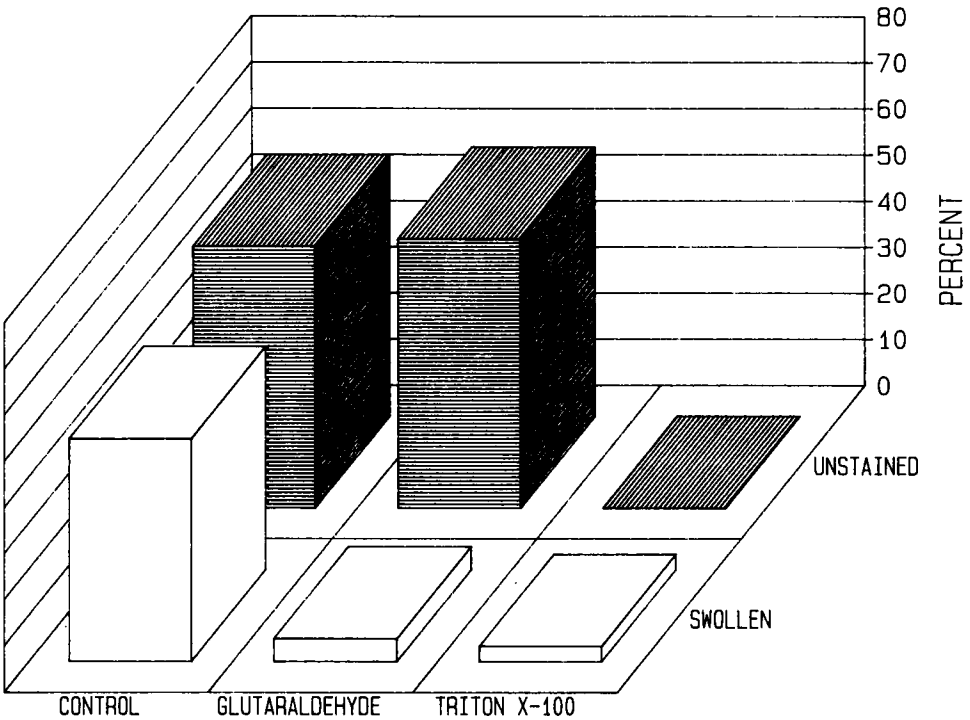


Fig. 1: The viability of sperm treated with Glutaraldehyde, Triton X-100 or controls assayed by the two methods. The hatched bars represent the percentage unstained ("alive") by the stain exclusion assay. The white bars represent the percentage swollen ("alive") by the HOS assay.

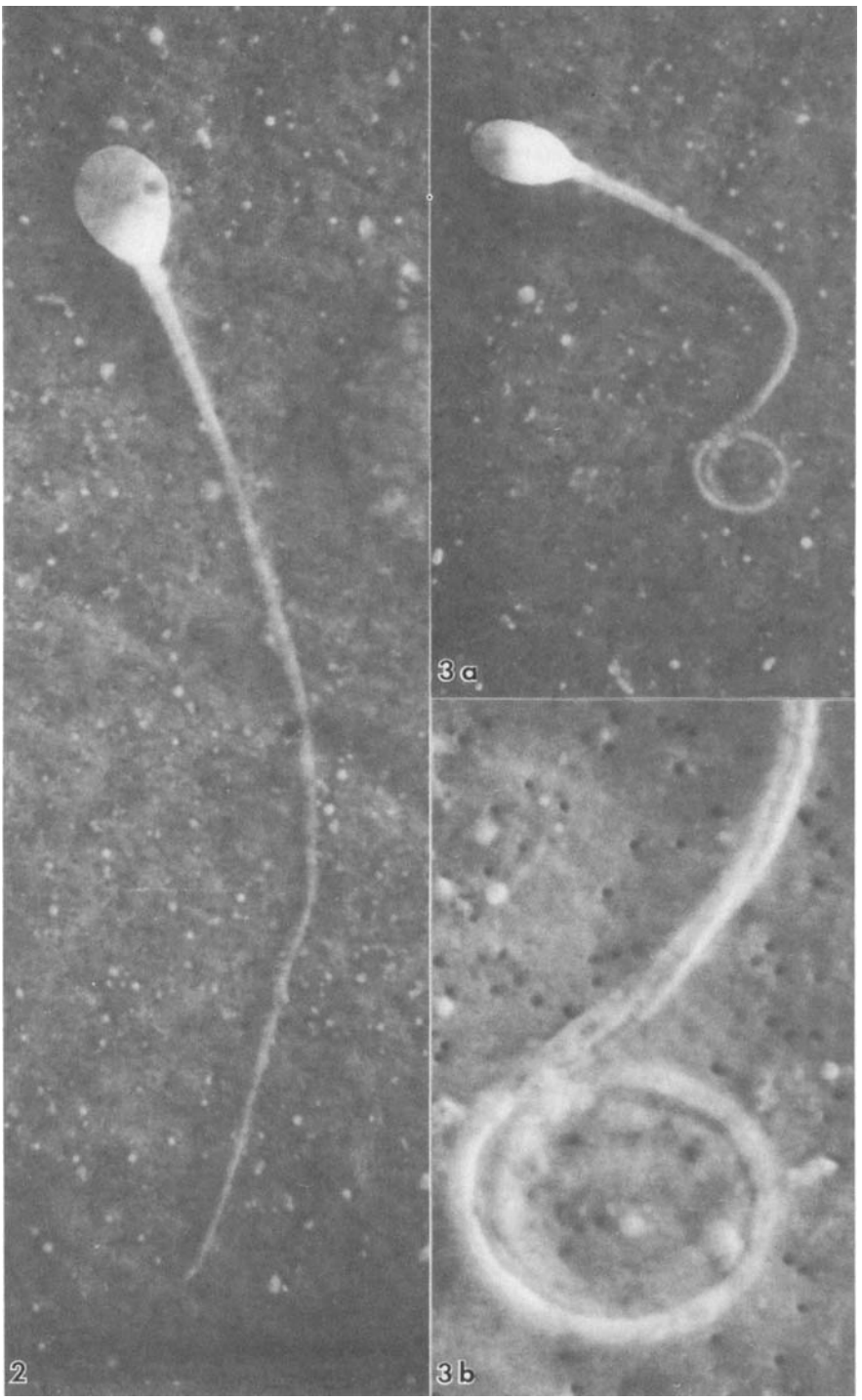
of stained (non-viable) appearing reddish-yellow to yellow were counted using phase contrast microscopy within five minutes after staining. The percent viable was calculated after evaluating a minimum of 200 sperm cells per sample.

Viability by HOS was determined as outlined by Jeyendran et al. (1984), where 0.1 mL of sperm suspension was mixed with 1.0 mL of hypoosmotic solution consisting of equal volumes of 150 mOsm sodium citrate and 150 mOsm fructose. The mixture was incubated for at least 30 min at 37° C and the percent with swollen (coiled) tails was assessed using phase contrast microscopy after counting at least 200 sperm per sample.

Statistical significance was determined using analysis of variance.

In order to determine if the sperm membrane was intact, transmission electron microscopy was used. Aliquots of each of the three treatments (control, glutaraldehyde, or Triton X-100) were fixed (Schrader et al. - 1981) in 3% glutaraldehyde in 0.2 M cacodylate buffer and washed 3 times in this buffer. The sperm were post-fixed in 1% osmium tetroxide in 0.2 M cacodylate buffer and washed 3 more times in the buffer. The samples were dehydrated in a water acetone series and embedded in Spurr's resin

Fig. 2: Scanning Electron Micrograph of an untreated human spermatozoa X3, 300.
Fig. 3: Scanning Electron Micrograph of a hypoosmotic stresses sperm.
3a – whole sperm X3, 300
3b – Enlargement of tail X11, 900.


2
3a
3b

(Spurr - 1969). Sections were cut on a Reichart OMB 3 ultramicrotome and stained with uranyl acetate and lead citrate for 10 min each. The stained sections were examined with a RCA 3F transmission electron microscope at an accelerating voltage of 100 kV.

For scanning electron microscopy, one mL of the suspension was deposited on a 25 mm Nucleopore membrane filter with a pore size of 2.0 micrometers and vacuum filtered. The cells were then rinsed with distilled water. The filter was air dried and affixed to a carbon planchet with colloidal graphite paint. The sample was then sputter coated with a gold/paladium conductive layer. The samples were viewed in a Hitachi S-570 scanning electron microscope at an accelerating voltage of 15 kV using secondary electron imaging.

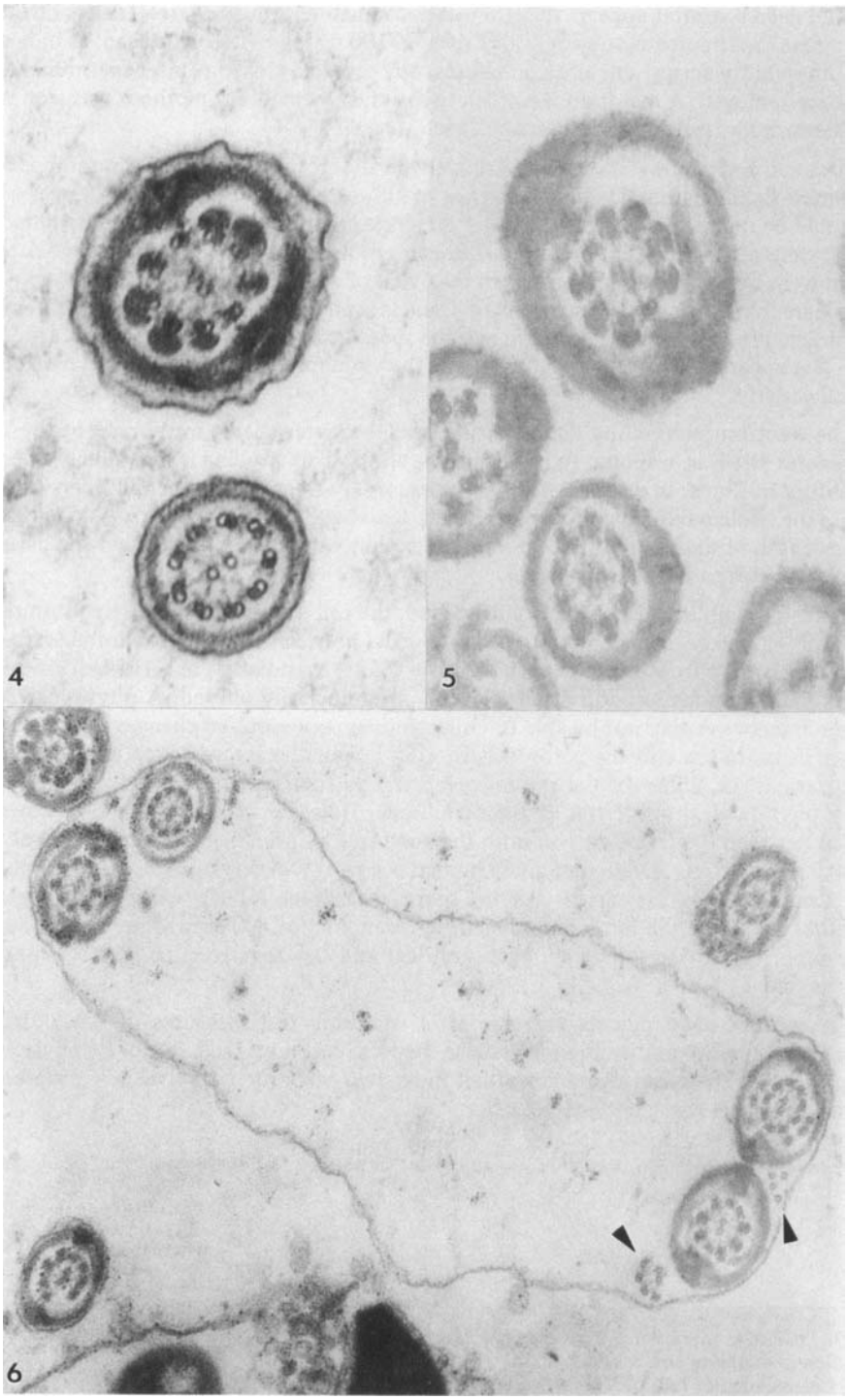
Results

Figure 1 illustrates the measurements of the two assays for each treatment. The mean (± standard deviation) percent alive found with the stain exclusion assay was 56.9 ± 9.7% for control, 58.5 ± 17.85 for the glutaraldehyde treated and 0% for Triton X-100 treated spermatozoa. The mean (± standard deviation) percent alive determined by the HOS test was 48.2 ± 11.2 for controls, 5.3 ± 1.9% for glutaraldehyde treated and 3.2 ± 1.8% for Triton X-100 treated. There were no significant differences found between the control samples and the glutaraldehyde treated samples using the stain exclusion assay; however, there was a difference ($p < 0.01$) between these treatments using the HOS assay. Both assays indicated a significant difference ($p < 0.01$) between the control samples and the Triton X-100 treated samples. There was no significant difference between the glutaraldehyde treated samples and the Triton treated samples using the HOS test while the difference was significant using the stain exclusion assay ($p < 0.01$). Scannning electron micrographs of normal (Fig. 2) and hypoosmotic swollen sperm (Fig. 3 a and b) demonstrate a noticeable shortening of the sperm tail. This shortening is often as much as a 3 to 4 fold reduction in the length of the swollen sperm tail compared to the normal sperm tail. Transmission electron microscopy revealed the status of the sperm membrane. Figure 4 is a cross section of a glutaraldehyde fixed sperm subjected to HOS. The sperm membrane is intact around each axoneme. Figure 5 is a cross section of sperm treated with Triton X-100 and then subjected to HOS. It is interesting to note the lack of a sperm membrane around the axoneme. Figure 6 is a cross section of the control sample subjected to HOS. The sperm membrane is swollen and surrounds 4 to 5 axonemes. Figure 7 (a and b) is a longitudinal section of a hypoosmotically stressed (HOS test) sperm. The outer sperm membrane appears to be intact and is continuous around the periphery of the coiled tail fibers. The cell membrane is seen only on the outside of the loop in Figure 7 and not along both sides of the tail structure as is seen in normal spermatozoa. The axoneme of the tail appears to disassemble under hypoosmotic stress (note arrows in Figure 6). Furthermore, the long microtubules can be seen free of the axoneme (note arrows in Figure 7b).

Discussion

We have investigated two methods of assessing sperm viability. While both of these methods are thought to be based on differences of the outer cell membrane between the

Fig. 4: Transmission Electron Micrograph – cross section of an untreated sperm tail X71, 000. ►
Fig. 5: Transmission Electron Micrograph – cross section of a Triton X-100 treated sperm tail X79, 000.
Fig. 6: Transmission Electron Micrograph – cross section of a hypoosmotic stressed sperm tail X35, 700.


4
5
6

live and dead sperm, it appears that they each evaluate different characteristics of the membrane. Spermatozoa subjected to Triton X-100 were identified as dead, as indicated by both viability assays, and electron microscopy revealed that the outer sperm membrane had been removed. A major difference between the two viability methods was seen when the sperm were treated with glutaraldehyde.

Spermatozoa pretreated with glutaraldehyde did not absorb Eosin Y and appeared alive even though glutaraldehyde is known to kill mammalian cells rapidly (Dawes - 1980). It should be noted that glutaraldehyde treated spermatozoa washed and resuspended in buffer were not motile. The HOS test, however, indicated that the glutaraldehyde treated sperm were nonviable, as demonstrated by a lack of a functioning osmotic membrane. It appears that as long as the sperm membrane is intact, stain is excluded even if the membrane is physiologically inactive and the spermatozoa are nonviable. By contrast, the HOS test apparently measures the ability of the membrane to transfer fluid, i.e., a physiological activity.

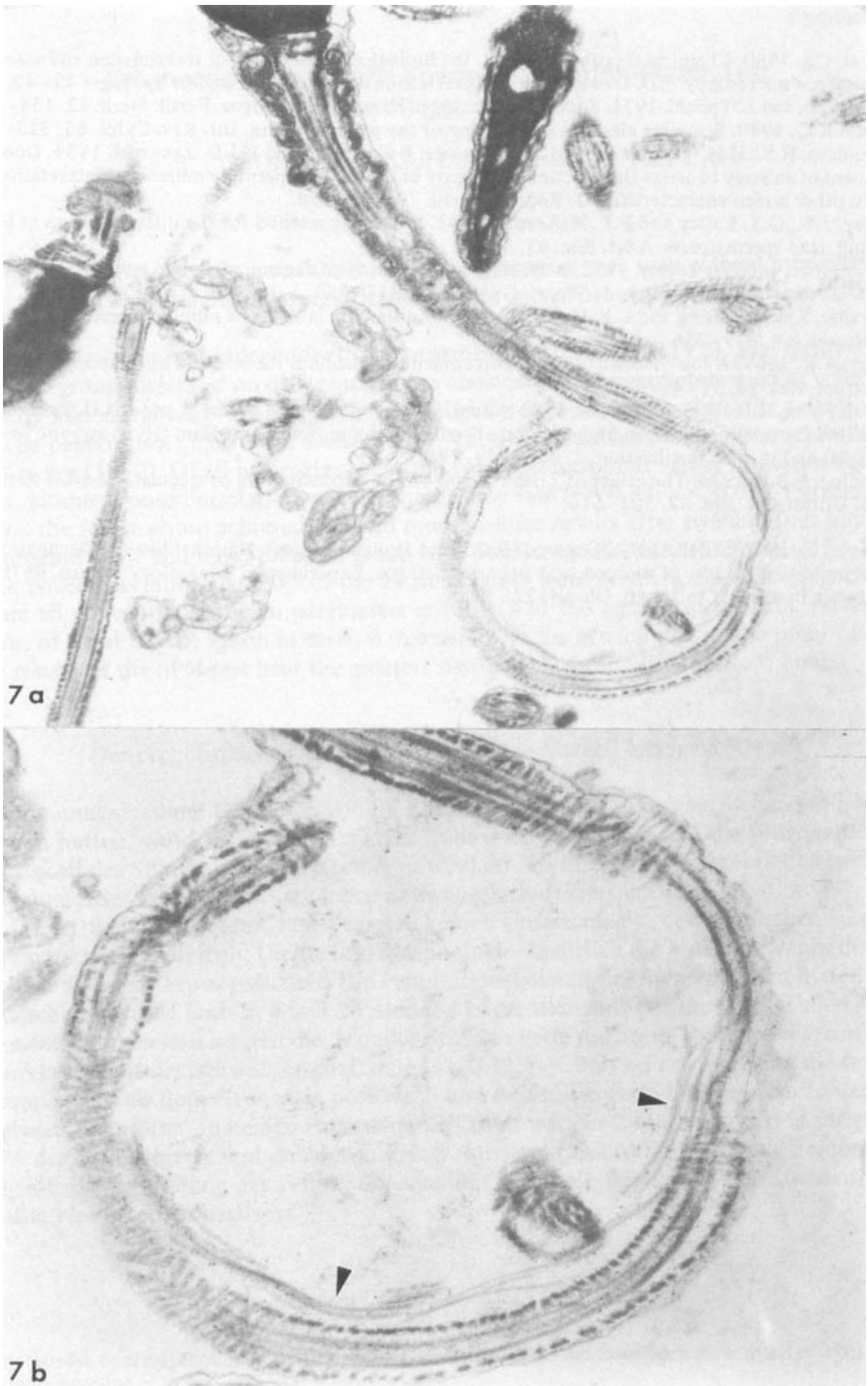
The electron microscopy data indicate that the outer sperm membrane of the tail bulges and swells in response to hypoosmotic stress. This swelling is most likely due to the influx of fluids. In response to changes caused by fluid influx, the tail fibers curl within the swollen outer membrane of the tail. It also appears that the 3 to 4 fold reduction in the length of the sperm is due to repeated coiling of the tail fibers within the plasma membrane as seen in Figures 6 and 7.

If a cell's membrane is physically disrupted, the cell will not swell in a hypoosmotic medium. Therefore, some correlation should exist between the stain exclusion test and the HOS test as our results have indicated ($r = 0.52$; Jeyendran et al. - 1984). However, a membrane may be structurally intact but physiologically altered. A physiologically altered membrane may not be able to undergo the many required changes that occur during capacitation and the acrosome reaction before the spermatozoa are capable of fertilization. An ability of the sperm membrane to transport fluid is only one of the many physiological properties of the membrane. However, based on the high correlations between the HOS test and both the zona-free hamster egg test (Jeyendran et al. - 1984) and in vitro fertilization of intact human oocytes (Van der Ven et al. - 1986), it appears that the fluid transport capacity of the sperm membrane reflects other physiological activities related to the fertilizing capacity of spermatozoa. No significant correlations were reported between the stain exclusion test and the zona free hamster egg assay (Martin and Taylor - 1982).

The present data indicate that the stain exclusion test measures the structural integrity of the sperm membrane, whereas the HOS test is an indicator of its physiological integrity. It is therefore concluded that these two viability tests evaluate different characteristics of the sperm membrane.

Acknowledgements: This research was supported in part by National Institute of Health grant PHS-HD 19555.

Fig. 7: Transmission Electron Micrograph of Longitudinal Section of Hypoosmotic Swollen Tail. ►
7a – Sperm midpiece and tail X12, 700.
7b – Enlargement of tail X26, 300.


7 a
7 b

References

Dawes, C.J. 1980. Chemical fixation for TEM. In: Biological techniques for transmission and scanning electron microscopy. ED. Dawes. Ladd Research Industries. Inc. Burlington Vt. pages 19–49.

Eliasson, E. and L. Treichl. 1971. Supravital staining of human spermatozoa. Fertil. Steril. 22, 134–137.

Gould, K.G. 1980. Scanning electron microscopy of the primate sperm. Int. Rev. Cytol. 63, 323–355.

Jeyendran, R.S., H.H. Van der Ven, M. Perez-Pelaez, B.G. Crabo, and L.J.D. Zaneveld. 1984. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. J. Reprod. Fertil. 70, 219–228.

Lasley, J.E., G.T. Easley and F.F. McKenzie. 1942. A staining method for the differentiation of live and dead spermatozoa. Anat. Rec. 82, 167.

Martin, R.H. and P.J. Taylor. 1982. Reliability and accuracy of the zona-free ova assay in the assessment of male fertility. Brit. J. Obstet. Gynaec. 89, 951–956.

Schrader, S.M., L.A. Eng and C.B. Metz. 1981. Immunoferritin labeling of rabbit spermatozoa. Gamete Research 4, 379–386.

Spurr, A.R. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. 26, 31–43.

Van der Ven, H.H., R.S. Jeyendran, S. Al-Hasani, M. Perez-Pelaez, K. Diedrich and L.J.D. Zaneveld. 1986. Correlation between human Sperm Swelling in hypoosmotic medium (Hypoosmotic swelling Test) and in vitro fertilization. J. Androl. 7, 196.

Wooding, F.B.P. 1973. The effect of Triton X-100 on the Ultrastructure of ejaculated bovine sperm. J. Ultrastruct. Res. 42, 502–516.

Address: Dr. S.M. SCHRADER, Dept. of Health and Human Services, National Inst. of Occupational Safety and Health, Div. of Biomed. and Behavior Science, Experimental Toxicology Branch, 4676 Columbia Parkway, Cincinnati, Ohio 45226 /USA.