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Measurement of the Water Permeability of the Membranes of Boar, Ram, and Rabbit Spermatozoa Using Concentration-Dependent Self-Quenching of an Entrapped Fluorophore

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Published values for sperm membrane water permeability (L_p) obtained using a time-to-lysis methodology have produced anomalous results when used to model optimal cooling rates for cryopreservation of spermatozoa. As the lysis method is dependent on potentially questionable assumptions, we describe an alternative method for measuring sperm L_p. Spermatozoa were exposed to hypo- and hyperosmotic conditions using a stopped-flow apparatus and the time course of resulting volume changes was measured using concentration-dependent self-quenching of the entrapped fluorophore, carboxyfluorescein (CF). L_p was measured for boar, rabbit, and ram spermatozoa using a range of osmotic stresses (±50–100 mOsm). Values for exosmotic and endosmotic flow showed no evidence of rectification. Mean L_p values were 0.84 μm/min/atm (boar), 0.28 μm/min/atm (rabbit), and 2.79 μm/min/atm (ram). These values are lower than the lysis method estimates, with the ram value reduced by approximately two-thirds using the current methodology. The value for boar spermatozoa showed good agreement with published values obtained using an electronic cell-sizing technique. Substitution of the revised values for L_p into the model for optimal cooling rates brings the calculated optimal rate closer to the lower empirically observed value but does not fully account for the previously reported discrepancies.

A number of previously published reports have described the measurement of membrane water and cryoprotective agent (CPA) permeability, associated activation energies, and osmotically inactive water fraction for spermatozoa from a range of species (5, 9, 21). However, attempts to use these values to model cooling rates for sperm cryopreservation have resulted in theoretically calculated optimal rates which are orders of magnitude higher than empirically derived optimal rates (4, 13). The model used for these calculations predicts lethal intracellular ice formation and has been successfully applied to a number of cell types (12); so, its apparent failure with spermatozoa is of considerable interest. Either sperm cryoinjury is resulting from some other mechanism at rates slower than those calculated by the model to induce lethal intracellular ice formation or inaccuracies in the measured variables are sufficient to give an erroneous result even though the model itself is valid. Especially serious in this regard is the problem of measuring activation energy (E_a) at temperatures above 0°C and extrapolating to temperatures below 0°C measurements. A recent study using a differential scanning calorimeter technique has reported significantly different parameters for mouse spermatozoa when measurements were made at subzero temperatures compared with published suprazero values (6).

Spermatozoa are not straightforward subjects for biophysical measurement; their small size and irregular shape make surface area and volume measurement nontrivial (3, 11) and detection of osmotically driven volume changes even more difficult. An adaptation of an hypoosmotic cell lysis technique (21) has been used by a number of investigators to measure water permeability (L_p) and activation energy. However,

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this technique is technically laborious and involves two fundamental assumptions which may not be justified. First, only hypoosmotic cell swelling can be examined, whereas a cryopreservation freeze/thaw cycle involves dehydration of cells with exposure to an increasingly hyperosmotic environment that is reversed on thawing. Therefore, to use L_p values obtained from hypoosmotic lysis, there must be an assumption of no rectification of osmotic flow, i.e., that the permeability of the membrane to water is independent of the direction of water movement. Rectification is well documented in plant cells but remains a point of contention in animal cells. Toupin *et al.* (19) have described endosmotic flow as being some three times higher than exosmotic flow in human granulocytes, but other workers have failed to detect significant rectification in other systems (17).

The second problem with this method is that spermatozoa must be exposed to hypoosmotic conditions sufficient to swell cells beyond their critical volume in order to induce lysis. There are the necessary assumptions that the membrane permeability is constant and that the cells continue to behave as perfect osmometers under the extreme conditions near the lysis point. Good Boyle van't Hoff relationships have been demonstrated for spermatozoa from a number of species within limits (8) but not under the hypoosmotic conditions required to induce lysis. As cells swell and approach their critical volume it is possible that there may be other volume constraints, such as the presence of cytoskeletal elements (14), to be overcome before lysis occurs.

We describe a stopped-flow technique for measuring the water permeability of spermatozoa utilizing a concentration-dependent self-quenching fluorophore to monitor cell volume changes. The technique is an adaptation of that described for membrane vesicles (1) and utilizes an entrapped fluorophore which undergoes changes in concentration as the cells shrink or swell; the concentration-dependent self-quenching properties of the fluorophore enable cell volume changes to be recorded as changes in fluorescence. This technique is able to measure permeability in response to relatively small hyper- and hypoosmotic differentials of the order of ± 50–100 mOsm and the use of stopped-flow allows volume changes to be monitored over short time courses of just a few seconds. Results are presented for boar, ram, and rabbit spermatozoa and the values obtained compared with published values obtained by the lysis method and by other techniques.

MATERIALS AND METHODS

Semen Collection

Semen was obtained by artificial vagina from New Zealand White rabbits and Friesland rams. Only ejaculates with a high percentage motility (>70%) were used. All ejaculates were used within 1 h of collection. Boar semen was collected by the gloved hand technique, diluted in BTS diluent (D glucose 205 mM, Tri-sodium citrate 20 mM, EDTA disodium salt 3.4 mM, potassium chloride 10 mM, sodium penicillin 1.7 mM; streptomycin sulfate 0.7 mM) and used at between 18–24 h post collection.

Sperm Membrane Integrity

Rapid mixing within the stopped-flow system is essential to ensure a completely mixed solution at as close as possible to time zero. However, rapid mixing may also result in mechanical damage to the spermatozoa. The speed of mixing is dependent on the design of the mixing chamber, which maximises turbulent flow, and on the pressure applied to the pneumatic drive syringes. Preliminary studies (data not shown) indicated that the ram spermatozoa were the most sensitive to damage during mixing. To determine the optimal mixing pressure, vital stains were used to assay for damage. Ram semen was diluted 1:2 with modified Hepes–Tyrode's solution (NaCl 146 mM, KCl 1 mM, NaH_2PO_4 0.42 mM, $MgCl_2$ 0.5 mM, Glucose 5.5 mM, Hepes 20 mM, pH 7.5, 300 mOsm), and carboxyfluorescein diacetate (5-CDFA; Sigma Chemical Co.; 1 mg/ml in DMSO) was added to a final concentration of 10 μg/ml. After incubation at room temperature for 30 min semen was washed through a two-step Percoll

gradient (35/70%) for 5 min at $200g_{max}$ followed by 15 min at $900g_{max}$. Pelleted spermatozoa were removed from the Percoll by washing with modified Hepes–Tyrode's solution ($500g_{max}$, 10 min) Spermatozoa were resuspended to final concentration of 75–100 $\times$ 10^6 ml^{-1}. Spermatozoa loaded with CFDA were mixed 1:1 with isosmotic Hepes–Tyrode's using a SF-61 single-mixing stopped-flow system (Hi-Tech Scientific, Salisbury, UK) using a range of mixing pressures between zero and 4 bar. After mixing, spermatozoa were recovered and propidium iodide (PI; Sigma Chemical Co.) was added to a final concentration of 5 μg/ml. Plasma membrane integrity was assessed using a flow cytometer (EPICS Profile Analyser; Coulter Electronics Ltd., Luton, UK) Cells retaining their CFDA staining were judged intact and cells having lost CFDA staining and acquiring PI-stained nuclei were scored as membrane lyzed (21). A minimum of 10^4 spermatozoa were scored for each sample.

Cell Loading

5-Carboxyfluorescein diacetate stock solution (100 mM in ME_2SO) was added to the diluted ejaculate to a final concentration of 1 mM and incubated for 60 min at 37°C. Excess CFDA was removed by centrifuging the cells through a two-step Percoll gradient as above. Spermatozoa were resuspended to final concentration of 75–100 $\times$ 10^6 ml^{-1}.

Stopped-Flow Fluorescence Measurements

Stopped-flow measurements were performed using a SF-61 single-mixing, stopped-flow system (Hi-Tech Scientific). Aliquots of sperm suspension (150 μl) were mixed with equal volumes of modified Hepes–Tyrode's solution either with added distilled water to give various degrees of hypotonicity or with added sucrose to give various degrees of hypertonicity. Fluorescence was excited at 492 nm with an F/4 Czerny–Turner monochromator and detected using a 530 nm cut-off filter. Changes in fluorescence with time were recorded on a Pentium computer interfaced directly with the Hi-Tech SF-61 using a dedicated software package (Hi-Tech Scientific).

Time course data for fluorescence following mixing of ram spermatozoa with hypo- or hyperosmotic solutions was recorded over a 1-s period. Data for boar spermatozoa were collected over 4 s and for rabbit spermatozoa over 10 s. Each experiment used a single ejaculate from an individual animal (n = 6) exposed to a range of different osmotic stresses. Ten replicate curves were averaged to produce a mean curve for each osmotic treatment. The fluorescence data showed an initial time zero artifact of between 50 and 100 ms duration seen as a fluctuation usually in the form of an initial sharp rise followed by a greater fall in fluorescence. The reason for this artifact is unclear but a similar result has been described for fluorescence stopped-flow studies with red cell ghosts (18). To deal with this artifact, the first 75 ms of data were discarded, and a polynomial was fitted to a short section (100–500 ms) of the data to give a time zero fluorescence value.

Determination of Hydraulic Conductivity (L_p)

The cell volume vs time response is described by Equations 1 and 2 (7)

$$dVt/dt = -L_pA(C^e - C^i)RT \qquad [1]$$

$$C^i = C^i(t_0)\,[(V_{iso} - V_b)/(V_{(t)} - V_b)] \qquad [2]$$

where L_p is the hydraulic conductivity, A is the sperm surface area, V_{iso} and $V_{(t)}$ are the cell water at time zero and t, V_b is the osmotically inactive cell water volume, t is time, C^e is the extracellular concentration of nonpermeable solute, C^i is the intracellular concentration of nonpermeable solute computed assuming a Boyle van't Hoff relationship, R is the universal gas constant, and T is the absolute temperature. We show below that the cell volume is proportional to the cell fluorescence. L_p was determined by numerically integrating Equations [1] and [2] and least squares fitting to the data using the Powell variant of the Levenberg–Marquardt method (15), as implemented in the Scientist software package (MicroMath, Salt Lake City, UT, U.S.A.) (10).

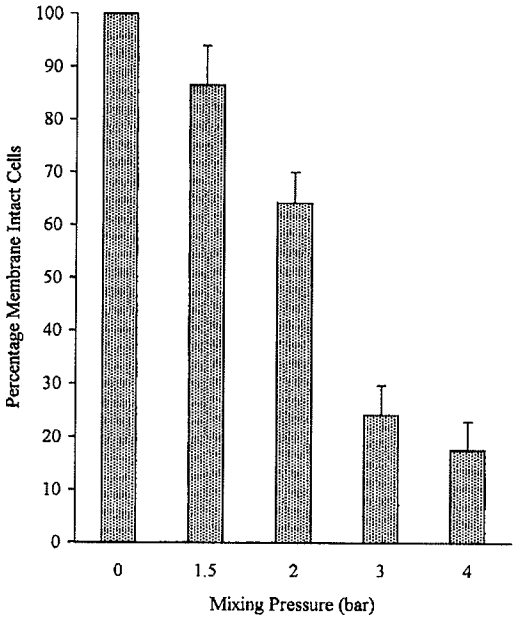

FIG. 1. Percentage (±SE, $n = 4$) of membrane-intact ram spermatozoa (control normalized to 100%) following stopped-flow mixing at increasing drive syringe mixing pressures.

RESULTS

Sperm Membrane Integrity

Results for sperm survival after mixing at different pressures for four ejaculates from different rams are shown in Fig. 1. Results are expressed as the percentage of spermatozoa intact after mixing under control conditions for which the drive syringes were operated by hand with a slow push. Percentage survival (mean % ± SE, $n = 4$) were 86 ± 8; 64 ± 6; 24 ± 6, and 18 ± 5 for 1.5, 2, 3, and 4 bar, respectively.

A mixing pressure of 2 bar was adopted as the best compromise between effective mixing and acceptable cell survival. In the absence of any osmotically induced cell volume changes following isosmotic mixing of cells under the 2 bar pressure conditions there was no observable change in the fluorescent output from the cells.

Proportionality of Fluorescence and Cell Volume

The relationship between fluorescence and cell volume was investigated by plotting the final steady state fluorescence value of cells subjected to different osmotic stresses against calculated final volume, assuming that the cells behave as perfect osmometers (Boyle van't Hoff relationship). Within the osmotic range used (±100 mOsm) the relationship between fluorescence and volume was found to be linear for all three species investigated (Fig. 2). For convenience the data curves were inverted and normalized to unity $t = 0$ before analysis to determine L_p. Curves representing sperm volume changes with time under different osmotic conditions for each of the three species are shown in Fig. 3.

Water Permeability Measurement

Values for surface area and water volume were taken from published sources, 142 μm^2 and 15.5 μm^3, respectively, for ram spermatozoa (3), 156 μm^2 and 11.4 μm^3 for boar spermatozoa (9), and 139 μm^2 and 17.3 μm^3 for rabbit spermatozoa (5). The osmotically inactive fraction of the water volume was estimated as 20% and the curves were fitted with two adjustable parameters, L_p and a scaling factor to take account of the proportionality factor between fluorescence and volume. All measure-

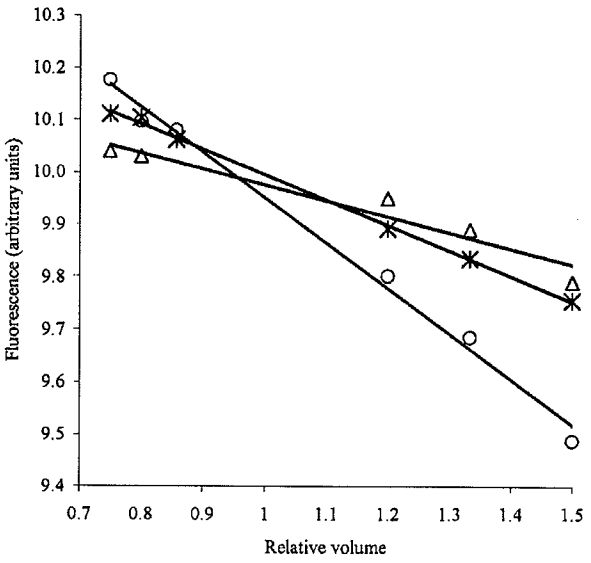

FIG. 2. Proportionality between sperm volume, calculated assuming a perfect Boyle van't Hoff relationship, and carboxyfluorescein fluorescence for boar (○) ($r^2 = 0.99$), rabbit (*) ($r^2 = 0.99$), and ram (△) ($r^2 = 0.94$); solid lines show least square fits to the data.

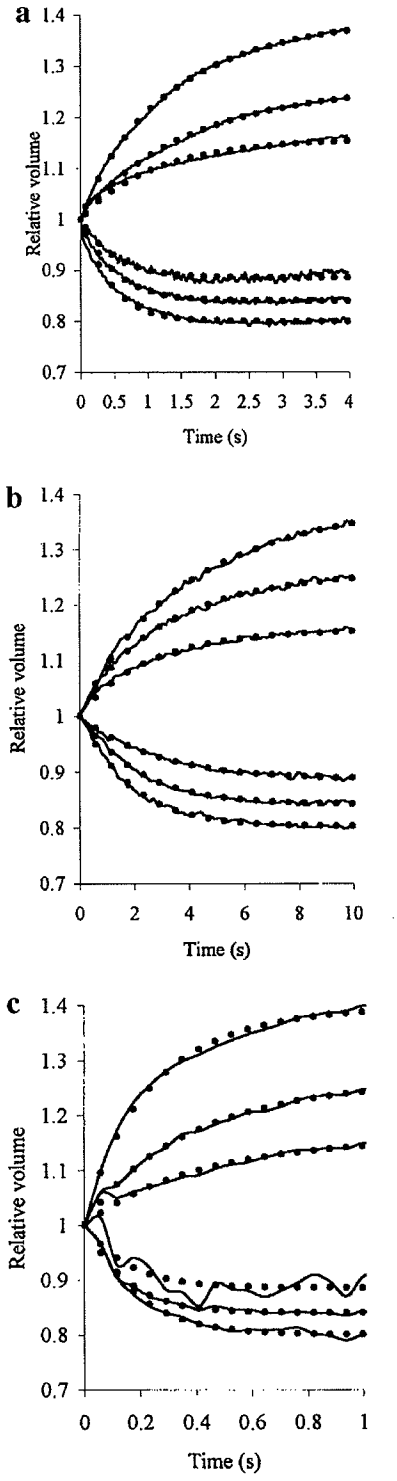

FIG. 3. Mean curves showing time course for sperm shrinkage at +50, +75, and +100 mOsm stresses and for sperm swelling at −50, −75, and −100 mOsm stresses (continuous curve), with fitted curves for L_p estimation (points) superimposed for (a) boar, (b) rabbit, and (c) ram spermatozoa.

ments were made at 25°C. Results for the three species are shown in Table 1 and ranged from a high L_p of 3.53 μm/min/atm for ram spermatozoa to a low L_p of 0.24 μm/min/atm for rabbit spermatozoa. Mean values were not significantly different for exosmotic and endosmotic flow for all three species and in each case the value for L_p was independent of the osmotic driving force. The ram spermatozoa showed the greatest degree of variation in L_p values. This is likely to be a reflection of their greater sensitivity to the handling procedures and particularly to the mixing stresses, compared with the boar and rabbit spermatozoa.

DISCUSSION

Stopped-flow methodologies have been used for measuring permeability in a variety of systems using light scattering to monitor volume changes with time (17, 20). However, the structural characteristics of spermatozoa (asymmetrical shape, large optically dense nucleus, small amount of cytoplasm) and the fact that they are motile cells make them unsuitable for light-scattering studies. Preliminary experiments (data not shown) failed to detect any consistent change in light scattering in response to osmotically induced sperm volume changes. In this study, a technique which is dependent upon the

TABLE 1
L_p Values for Boar, Rabbit, and Ram Spermatozoa Measured under a Range of Osmotic Stresses

Δ Osmolality (mOsm)	L_p (μm/min/atm)		
	Boar	Rabbit	Ram
+100	0.76	0.26	2.65
+75	0.83	0.25	3.44
+50	1.05	0.24	3.00
Mean (± SE) (exosmotic flow)	0.88 ± 0.09	0.25 ± 0.01	3.03 ± 0.23
−50	0.82	0.31	1.72
−75	0.72	0.29	2.40
−100	0.88	0.30	3.53
Mean (± SE) (endosmotic flow)	0.81 ± 0.05	0.30 ± 0.01	2.55 ± 0.53
Mean (± SE)	0.84 ± 0.05	0.28 ± 0.01	2.79 ± 0.28

Note. An isotonic value of 300 mOsm was assumed.

concentration-dependent self-quenching of an entrapped fluorophore has been used in place of light scattering. This technique has previously been used with a number of membrane vesicle systems (1, 20) and is dependent on loading high concentrations of fluorophore into the vesicles. As the vesicles shrink or swell in response to osmotic changes the concentration of entrapped fluorophore will rise or fall with a consequent change in fluorescent output (2, 16). Although it is relatively straightforward to load high concentrations of a nonpermeable fluorophore (≈10 mM) into a membrane vesicle which can be lysed and resealed (1), it is more difficult with a living spermatozoon. In this study the problem has been addressed using an ester form of the fluorophore carboxyfluorescein, which is membrane permeable but of low fluorescence until cleaved intracellularly by nonspecific esterases to give the impermeable fluorescent form. The concentration of the permeable form placed outside the cell is known but the extent to which it enters the cell and is converted to the entrapped fluorescent form cannot be directly determined. Therefore, the final intracellular fluorophore concentrations cannot be definitively stated. The external concentrations of CFDA used in this study with living cells are low compared with fluorophore concentrations used previously in other systems to obtain self-quenching. However, Chen *et al.* (1) have reported for fluorescein sulfonate that the fluorescence vs concentration relationship in vesicles is quite different from that in free solution with significant quenching observed at concentrations of <0.1 mM in vesicles compared with >10 mM in free solution. The basis for this amplification of the self-quenching effect is not fully understood but may involve a nonspecific quenching by intracellular membrane-bound proteins. The results presented here indicate that it is possible to produce both shrinkage and swelling kinetics data using this methodology.

Comparison of shrinking and swelling results for boar and ram spermatozoa show that there is no significant rectification effect. Mean L_p values of 0.88 and 0.81 μm/min/atm for boar and 3.03 and 2.55 μm/min/atm for ram were recorded for exosmotic and endosmotic flow, respectively. For the rabbit, the value for endosmotic flow (0.25 μm/min/atm) was only slightly lower than that for exosmotic flow (0.3 μm/min/atm), and although the mean values were significantly different at the $P = 0.01$ level, it seems probable that as for the ram and the boar there is no real rectification effect of any importance. In comparison Toupin *et al.* (19) found a threefold difference between exo- and endoplasmic flow for human granulocytes.

The overall mean value of L_p for boar spermatozoa, 0.84 μm/min/atm, is in reasonably good agreement with the published value of 1.03 μm/min/atm obtained using an electronic particle counter (9). The mean value for rabbit spermatozoa, 0.28 μm/min/atm, is somewhat lower than the published value of 0.63 μm/min/atm (5) obtained by the osmotic lysis method. Similarly, the mean ram sperm value, 2.79 μm/min/atm, is lower than the published lysis method value of 8.47 μm/min/atm (4). These comparisons with published values suggest a consistent overestimate of L_p with the lysis method. Such an overestimation does not appear to be present in the particle counter results for boar spermatozoa, for which measurement of L_p is not dependent on cell lysis and volume changes are monitored over a smaller osmotic range. Substitution of the new lower L_p value for ram spermatozoa into the model for prediction of optimal cooling rates brings the calculated optimal rate closer to the empirically observed value but is not sufficient on its own to account for the discrepancy between prediction and observation. Our previous contention that osmotic stresses occurring during cryopreservation cause additional damage to the sperm cell integrity over and above that related to the likelihood of intracellular ice formation remains valid.

Cryopreservation of spermatozoa is a subject of considerable interest for human infertility treatments, for improving the breeding performance in animals of agricultural importance, and for animal conservation. The causes of cryoinjury in such cells are not understood with any

certainty, but this study has confirmed that optimal cooling rates cannot be reliably estimated on the basis of water permeability alone. Unless the water permeability is considerably modified by the presence of cryoprotectant or its E_a changes significantly below 0°C, it is probable that other factors, such as that relating to osmotic stress, are also at work.

In summary, this study demonstrates that meaningful measurements of water permeability can be made by stopped-flow methodology in cells whose shape and size makes other methods imprecise. To the best of our knowledge this is the first time that living cells have been studied in this way. In sperm cells, there is little evidence that the direction of water flow, into or out of the cell, influences the estimate of L_p. Further studies are under way to examine the influence of cryoprotectants.

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