Membrane fluidity predicts the outcome of cryopreservation of human spermatozoa

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Semen cryopreservation is an important procedure in the treatment of human infertility. However, the ability of spermatozoa to survive freeze/thaw processes varies between patients. Cryopreservation-induced stress may result in membrane injury with consequent loss of sperm motility and viability. We investigated the relationship between the physico-chemical state of the human sperm membranes and their tolerance to cryopreservation. Conventional characteristics of 20 semen samples were analysed before and after cryopreservation as well as their membrane fluidity assessed by measuring the fluorescence polarization anisotropy, which is inversely proportional to the fluidity. Correlation between fluidity and post-thaw recoveries of motile and viable spermatozoa were examined. Results showed that membrane anisotropy markedly varies between patients. In cryopreserved spermatozoa, anisotropy values were significantly higher than in fresh spermatozoa. Furthermore, recovery of motile and viable spermatozoa after freeze/thaw was strongly correlated with anisotropy of fresh spermatozoa (P < 0.05). The higher the membrane fluidity was before freezing, the better was the response of spermatozoa to cryopreservation. The results indicate that the freeze/thaw process results in a rigidifying effect on the sperm membrane and suggest that sperm adaptability to freeze/thaw-induced stress could be dependent on their initial membrane fluidity. The latter finding has practical implications for predicting the response of spermatozoa following freezing and thawing and for improving the recovery of viable spermatozoa.

Key words: cryoresistance/fluidity/fluorescence anisotropy/membrane/spermatozoa

Introduction

A considerable increase in the demand for semen cryopreservation has occurred over the last few years. In addition to donors’ spermatozoa, cryopreservation of ejaculated spermatozoa prior to radiation therapy, chemotherapy or other treatments liable to alter spermiogenesis is increasingly being practised (Lass et al., 1998; Naysmith et al., 1998). However, recovery of functionally intact spermatozoa from thawed samples is variable between patients (Centola et al., 1992; McLaughlin et al., 1992). Conventional sperm parameters have not allowed prediction of the behaviour of ejaculate to cryopreservation.

Structural and functional loss of plasma membrane integrity resulting from freezing and thawing have been well documented from ultrastructural studies (Barthelemy et al., 1990; De Leeuw et al., 1990; Holt et al., 1992). Membranes are destabilized by the passage to and from the storage temperature (thermal stress), the large volume changes associated with water and cryoprotectant movement and the exposure to high salt concentrations (osmotic stress). At a molecular level, changes in membrane organization such as modifications of specific lipid–protein interaction, phospholipid asymmetry and lipid composition are thought to be implicated in the loss of permeability (Mazur, 1984; Hinkovska-Galcheva et al., 1989; Parks and Graham, 1992). The reversibility of these changes could be dependent on membrane dynamics and the physical properties of the membranes has been assumed to be one of the pivotal factors in the resistance of spermatozoa to freezing (Watson and Morris, 1987; Quinn, 1989; Hammerstedt et al., 1990). However, this concept has never been directly tested for human spermatozoa. Determination of lipid fluidity has been shown to be a submacroscopic approach to the physical and dynamic state of the sperm membrane (Shinitzky and Yuli, 1982). To test the hypothesis that sperm membrane fluidity reflects the physiological status of the membrane and is relevant to sperm ability to be restored after freeze-induced stress, we studied sperm membrane fluidity on normal ejaculates before and after cryopreservation and examined whether the tolerance of human spermatozoa to the freezing/thawing process could be predicted from the fluidity assessment of fresh semen.

Materials and methods

Semen samples

Semen was provided by patients attending our laboratory of reproductive biology. After liquefaction at 37°C, sperm parameters were determined in accordance with World Health Organization guidelines (WHO, 1992) and surplus semen used for experimental analysis. A total of 20 semen samples was selected according to WHO criteria for sperm count > 20 x 10⁶ spermatozoa/ml, motility > 50%, viability > 75% and normal morphology > 30%.

Cryopreservation

As previously described (Grizard et al., 1999), semen was diluted with an equal volume of cryoprotective medium (glycerol 14%, v/v; sodium chloride 30 mmol/l; glycine 133 mmol/l; sodium citrate 40 mmol/l; glucose 69 mmol/l; antibiotics; pH = 7.4; osmolality = 430 mosm). Addition of the medium was carried out gradually (1 ml/min)
with care to avoid osmotic shock to spermatozoa. Samples were maintained for 15 min at room temperature for equilibration and sealed in 0.25 ml straws. Straws were frozen in a Minicool LC40 (Air Liquide Santé, France) following a standard freezing protocol (from 20°C to −4°C at a rate of 5°C/min, from −4°C to −30°C at 10°C/min and from −30°C to −140°C at 20°C/min). Straws were then transferred to liquid nitrogen for storage. Straws were thawed at 37°C for 5 min for estimation of sperm concentration, motility and viability.

Assessment of sperm viability and motility
The following analyses were performed on both fresh and frozen–thawed spermatozoa from each patient. Motility was assessed at 37°C and scored under light microscopy. Spermatozoa were classified as progressive, non-progressive or immotile spermatozoa. Viability was assessed using eosin–nigrosin test. Unstained (intact) and red coloured (with damaged membranes) spermatozoa were counted using nigrosin as a counterstain. Sperm viability was defined as the percentage of intact cells. The cryosurvival rates regarding the proportion of motile or viable spermatozoa that survived freezing were calculated from the respective formulae:

% Motility recovery = % post-thaw total motility × 100/ % fresh total motility
% Viability recovery = % post-viability × 100/ % fresh viability.

Fluidity assessment
Aliquots of fresh spermatozoa (200–300 µl) or frozen/thawed material (250 µl) were washed in 5 ml Earle’s medium supplemented with 0.3% BSA (bovine serum albumin) and centrifuged 10 min at 1800 g. The washed spermatozoa were suspended at a concentration of 1×10⁶ spermatozoa/ml in 3 ml of PBS (phosphate buffered saline) with 1,6-diphenyl-1,3,5-hexatriene (DPH, 10⁻³ mol/l prepared from a DPH stock solution of 2×10⁻³ mmol/l in tetrahydrofuran) (Giraud et al., 1999). The suspension was incubated for 30 min at room temperature. The molar ratio of DPH to phospholipid was lower than 1:2000 in order to minimize probe–probe interaction and probe–induced perturbation of the lipid bilayer. The molar ratio of DPH to phospholipid was calculated from the known content of phospholipids in human spermatozoa. Sperm suspensions containing no DPH (blanks) were similarly assessed to check light scattering. The sample was excited with vertically polarized light (365 nm) and emission (430 nm) was measured through polarizer both parallel and perpendicular to the excitation polarizer. The parallel (I//) and perpendicular (I⊥) fluorescence intensities were recorded at 37°C after careful temperature equilibration. The anisotropy r is calculated:

\[ r_f = (I// - I⊥)/I// + 2 I⊥ \]

The anisotropy refers to the rotational motion of the DPH distributed throughout the hydrophobic core of the lipid bilayers and is inversely proportional to the membrane fluidity; therefore, the lower the r is, the more fluid is the membrane.

Statistical analysis
Results are presented as individual data or mean ± SEM. Wilcoxon rank test was used to compare the fresh and post-thaw values. Relationships between anisotropy and sperm parameters or cryosurvival rates were analysed using Spearman’s rank correlation analysis. Results were considered significant when P < 0.05.

Results
Human spermatozoa with normal sperm characteristics were employed in this study (Table I). We assessed the fluorescence anisotropy r of washed spermatozoa labelled with DPH; r of spermatozoa from 20 patients was measured at 37°C using polarization fluorescence with DPH as a probe, before and after cryopreservation. (●) = individual values.

<table>
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<tr>
<th>Table 1. Sperm parameters and fluorescence anisotropy of fresh spermatozoa. Correlation coefficient between anisotropy and sperm features</th>
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<td>Human sperm features</td>
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<td>Concentration (×10⁶ ml)</td>
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<td>Progressive motility (%)</td>
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<td>Total motility (%)</td>
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<td>Viability (%)</td>
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<td>Morphologically normal (%)</td>
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<td>Anisotropy (r_f)</td>
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Figure 1. Rigidifying effect of cryopreservation processes on sperm membrane. Fluorescence anisotropy r of spermatozoa from 20 patients was measured at 37°C using polarization fluorescence with DPH as a probe, before and after cryopreservation. (●) = individual values.

As anticipated, cryopreservation resulted in a decrease of the percentages of total motility (from 59 ± 4 to 29 ± 9, P < 0.05) and viability (from 87 ± 4 to 45 ± 2, P < 0.05). The fluorescence anisotropy was higher for frozen/thawed aliquots of spermatozoa than for fresh spermatozoa from the same ejaculates (Figure 1). This rigidifying effect of cryopreservation was observed for each patient. The average anisotropy increased from 0.232 ± 0.015 for fresh spermatozoa to 0.295 ± 0.012 for freeze/thaw spermatozoa. This difference was statistically significant (P < 0.05).

Statistical analysis showed that neither progressive nor total post thaw motility was significantly correlated with post thaw anisotropy; however, a significant negative correlation was
found between post-thaw anisotropy and post-thaw viability ($l = -0.479, P < 0.05$). In a mixed system such as the different populations of spermatozoa present in individual ejaculates, the recorded anisotropy $r_f$ is given by $r_f = \Sigma f_i r_i$ where $f_i$ is the fraction of the total fluorescence intensity emitted from the system $i$ (Shinitzky and Barenholz, 1978). The average anisotropy recorded would therefore represent the sum of the anisotropy of the whole sperm population balanced by their relative proportions. Therefore, when the proportion of impaired spermatozoa increases, it significantly influences the recorded average anisotropy. This result indicated that a loss of membrane integrity was associated with increased membrane anisotropy.

To investigate whether the cryotolerance could be explained by the membrane anisotropy, we determined the correlation coefficient $l$ between fresh anisotropy and the cryosurvival rates. Both the proportion of motile spermatozoa and the proportion of viable spermatozoa recovered after thawing were significantly correlated with the anisotropy of fresh spermatozoa ($l = -0.455, P < 0.05$ and $l = -0.512, P < 0.05$ respectively) indicating that improved recovery of viable or motile spermatozoa was related to lower initial fluidity (Figure 2A and B).

**Discussion**

When spermatozoa are cooled to very low temperatures they could sustain irreversible injury. This cryodamage has been reduced by improvement in the processing protocols (slow cooling rates, use of cryoprotectant, etc.) (Royere et al., 1996; Gilmore et al., 2000). However, the decline in the number of motile and viable spermatozoa resulting from cryopreservation remains unpredictable from standard sperm parameters and varies markedly between patients. The present study showed that the membrane fluidity of human spermatozoa is decreased following cryopreservation and provides evidence that the response of spermatozoa to a standard freeze/thaw protocol could be predicted by the membrane fluidity of the fresh spermatozoa. We found that a higher recovery rate of motile or viable spermatozoa was associated with higher membrane fluidity.

The literature regarding the fluidity of mammalian sperm membrane is species specific and often indirectly assessed by lipid compositional data (reviewed by Ladha, 1998). Our observations indicate that variability of the average sperm fluidity existed between patients. Neither the sperm characteristics of fresh ejaculate nor those of thawed samples were significantly correlated with the sperm respective anisotropy except for the progressive motility of fresh spermatozoa and post-thaw viability. As far as the progressive motility is considered, our results agree well with others (Haidl and Opper, 1997), suggesting that membrane fluidity is implicated in the acquisition of progressive motility during epididymal maturation of human spermatozoa.

Cryopreservation processes result in a loss of membrane fluidity. The steady-state fluorescence polarization approach that we used provides information on the rotational motion of lipids in sperm membranous structures at the submacroscopic level. James et al. (1999) have also reported at the single molecule level that lipid diffusion in the membrane plane is reduced in thawed spermatozoa even when the cells survive freezing apparently undamaged. A possible explanation of the rigidifying effect could be the presence of glycerol molecules remaining within the lipid bilayer after washing. Glycerol is known to have a direct effect on the plasma membrane and to alter its fluidity by increasing the order of the fatty acid (Boggs and Rangaraj, 1985; Hammerstedt et al., 1990). Therefore, it is not clear whether the cryoprotectants per se are having a direct effect or whether the changes are caused by low temperature, or both.

The increased anisotropy observed in frozen–thawed spermatozoa was related to the loss of post-thaw membrane integrity. The great range of quality in frozen–thawed spermatozoa allows a statistically significant correlation to be detected between the anisotropy and the percentage of viable spermatozoa. Alternatively, taking into account the additivity of the measured anisotropy, the lack of correlation in fresh spermatozoa may result from the large percentage of intact cells overwhelming the contributions from impaired cells.
The dye exclusion test that we used to determine the viability is related to the permeability of membrane, and our results suggest that loss of permeability induced by freeze/thaw is characterized by a decrease in the membrane fluidity. Moreover, Ladha et al. (1997) demonstrated that permeabilized ram spermatozoa presented a large immobile phase over the whole plasma membrane. This rigidification was irreversible. It is important to emphasize that membrane functions are dependent on an optimal fluidity. Change in the dynamic of the membrane would probably alter its barrier function.

The current study suggests that the adaptability of the membrane to damaging effects of the freeze/thaw process is markedly superior for spermatozoa with a high membrane fluidity. Attempts have been made to relate the membrane composition and its underlying physical structure to propensity for survival after a freeze/thaw cycle. Membrane lipid composition is commonly used to indicate change in membrane fluidity as the latter feature is modulated by the cholesterol level, degree of unsaturation of the phospholipid acyl chain, phospholipid composition and membrane protein. Greater resistance of mammalian spermatozoa to cold shock has been noted for species in which the cholesterol to phospholipid molar ratio and the degree of saturated fatty acids in the phospholipid fraction were high (Darin-Bennett and White, 1977; Watson and Morris, 1987). This comparison of species enabled the formation of two groups: the first one included bull, ram and boar spermatozoa and the second one included rabbit, dog, human spermatozoa, the spermatozoa of the former group being much more cryo-sensitive than the latter (White, 1993).

In terms of dynamics, a marked increase in the two chemical indices of fluidity, i.e. cholesterol/phospholipid and saturated/unsaturated molar ratios, indicates a significant decrease in fluidity of plasma membrane measured at physiological temperature. Therefore, it is conceivable that the first group of species mentioned above will have more fluid sperm membranes than the second one. Likewise, the study of Hinkovka et al. (1993) showed a greater rigidity of the rabbit spermatozoa compared to bull spermatozoa. However, this relationship is subject to controversy. It does not extend to rooster spermatozoa which are highly resistant to cold shock but have a lower sterol content and a greater overall fluidity compared to mammalian spermatozoa as demonstrated by Parks and Lynch (1992). Furthermore, in the current analysis we found a clear correlation between membrane fluidity and cryotolerance in human spermatozoa. Our results are in agreement with the general concept that cold tolerance is associated with membrane fluidity. This relationship was first demonstrated by Sinensky (1974) and then established for various organisms (Cossins and Raynard, 1987). The adaptation of cells to low environmental temperature results from a ‘fluidification’ of their plasma membrane. Steponkus and Lynch (1989) demonstrated that these membrane changes induced by cold acclimatization increased their cryostability. Moreover, as well as thermal stress affecting the lipid structure of spermatozoa, osmotic stress following cell dehydration is an important factor involved in the freeze/thaw-induced destabilization of sperm membranes (Gao et al., 1993; Curry and Watson, 1994). Rigidification of membrane has been shown to increase the fragility in response to osmotic stress, as demonstrated with erythrocytes (McGown et al., 1982).

In conclusion, our data provide further support for the view that sperm plasma membrane is a key organelle in controlling sperm cryosurvival. The capacity of human spermatozoa to withstand cryopreservation depends in part on their membrane fluidity. In-vitro manipulation of membrane fluidity may open novel approaches to cryopreservation processes. It will be interesting in future studies to investigate the effects of membrane-fluidizing agents on human sperm cryotolerance and fertilizing capacity.

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References


Sperm cryotolerance in relation to membrane fluidity

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