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Different functional states of ram spermatozoa analysed by partition in an aqueous two-phase system

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Abstract

The surface of spermatozoa plays a critical role in many stages involved in fertilisation. The plasma membrane undergoes important alterations in the male and female reproductive tract, which result in the ability of spermatozoa to fertilise eggs. One of these membrane modifications is sperm capacitation, a process by which sperm interacts with the zona pellucida receptors leading to the acrosome reaction. It has been proposed that the freezing process induces capacitation-like changes to spermatozoa, and that this premature capacitation could explain the reduction in longevity and fertilising capacity of cryopreserved mammalian spermatozoa. Our research focused on the relationship between membrane alterations occurring throughout freezing–thawing and the processes of capacitation and acrosome reaction. We used centrifugal countercurrent distribution (CCCD) analysis to compare the partition behaviour of ram spermatozoa that was either subjected to cold-shock or frozen–thawed with capacitated and acrosome reacted samples. In addition, the effect of the induced acrosome reaction on membrane integrity of ram spermatozoa was studied using biochemical markers and electron microscopy scanning. The CCCD analysis revealed important similarities between the surface characteristics of capacitated and cold-shocked sperm as well as between acrosome-reacted and frozen–thawed sperm. Cold-shocked and capacitated sperm showed an increased cell affinity for the lower dextran-rich phase as well as a decreased heterogeneity. Likewise, the induction of the acrosome reaction resulted in a loss of viability and an important decrease in cell surface heterogeneity compared to the untreated-control sample. Similar surface changes were found when semen samples were frozen with either Fiser or milk–yolk extender. These results confirm those obtained for membrane integrity by fluorescence markers. Thus, the high cell viability value found in the control sample (74.5%) was greatly decreased after cold-shock (22.2%), cryopreservation (26.38% Fiser medium, 24.8% milk–yolk medium) and acrosome reaction (6.6%), although it was preserved after inducing capacitation (46.7%). The study using electron microscopy scanning revealed dramatic structural alterations provoked by the induction of the acrosome reaction.

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1. Introduction

Sperm fertility depends upon the cell's ability to gain access to, interact with, and penetrate the oocyte. For this reason, sperm should be motile and

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retain membranes intact until encountering an oocyte. The plasma membrane, which assumes a key role in regulating spermatozoon functions, must undergo extensive remodelling in order to attain a functional mature sperm, whose plasma membrane can participate in the complex series of membrane-mediated interactions with the egg during fertilisation [1]. One of these membrane modifications is sperm capacitation, a process in which membrane destabilisation and cellular system activation reach the specific level so that interactions with the zona pellucida receptors lead to the acrosome reaction [2]. This process strikingly differs from other known exocytotic events in several ways: there are multiple fusion points between the outer acrosomal membrane and the overlying plasma membrane; exocytosis does not lead to a single fused membrane, but to vesiculation and actual membrane loss; and the acrosome reaction is a singular event, with no known membrane recycling taking place following exocytosis. The site where the point fusions first take place varies according to the species. This could reflect an intrinsic heterogeneity in the boundaries of the sites destined to fuse that determines the topology of this curious exocytotic process.

It has been suggested that the freezing process induces capacitation-like changes to spermatozoa [3–7]. Typical characteristics of an advanced stage of capacitation after thawing have been found in cryopreserved sperm [5,8–10], which present a decrease in survival ability [11]. Similarly, cold-shock and freezing–thawing, induce molecular changes in sperm coinciding with those caused by capacitation. Thus, both treatments were shown to lead to increasing protein tyrosine phosphorylation in ram spermatozoa [12]. Different behaviour of fresh and frozen–thawed spermatozoa when in contact with oviduct cells in vitro has also been reported [13]. This could be a consequence of the more advanced membrane state of the frozen spermatozoa upon thawing. It has been hypothesised that premature capacitation of frozen/thawed spermatozoa [14,15] may in some way be linked to sulfhydryl modifications in sperm proteins. The analogy between sperm membrane damage during cooling and the acrosome reaction has also been described, one being a disorganised version of the other [10].

It is widely accepted that partition in aqueous two-phase systems can be an efficient method to analyse external factors acting trough cell surface and plasma membrane components [16]. Surface changes in mammalian spermatozoa due to interactions with seminal plasma proteins have therefore been shown using countercurrent distribution (CCD) [17]. Likewise, using CCCD (centrifugal CCD), we have demonstrated that the acquisition of adsorbed seminal plasma proteins by the sperm cell surface modifies the partition behaviour of bull [18] and ram [19–22] spermatozoa. In addition, as a consequence of cryopreservation, we have also proven that frozen–thawed ram spermatozoa display a loss of heterogeneity, due to a decrease in cell surface hydrophobicity [23,24]. Sperm heterogeneity, the functional variability in response of spermatozoa, is known to be an inherent characteristic of sperm cells and to be highly involved in the fertilization success [1]. It can be explained through the coexistence of different sperm sub-populations within any typical mammalian ejaculate. The loss in heterogeneity is a proven effect of the freezing–thawing process [5], which drastically reduces the fertilising ability of semen. We have already shown that the induction of the acrosome reaction by ionophore A23187 accounted for an important decrease in cell heterogeneity with a significant displacement to the left of the CCCD profile [25]. These results prompted us to investigate the relationship between membrane alterations occurring during freezing–thawing and the processes of capacitation and acrosome reaction, in greater depth. In this study, CCCD analysis was performed to compare the partition behaviour of ram spermatozoa either subjected to cold-shock or having been frozen–thawed with those capacitated and acrosome reacted. In addition, the effect of the inducted acrosome reaction on membrane integrity of ram spermatozoa was analysed using biochemical markers and scanning electron microscopy.

2. Experimental

2.1. Reagents

Calcium ionophore A23187 (free acid), dimethyl sulphoxide (DMSO), chlortetracycline and chemicals used in cryopreservation were acquired from Sigma (St. Louis, MO, USA). Dextran T-500 (M_r 500 000)

and Ficoll 400 (M_r 400 000) were obtained from Pharmacia (Uppsala, Sweden). Polyethylene glycol (M_r 6000) was purchased from Serva Feinbiochemica (Heidelberg, Germany). All other chemicals used were of analytical reagent grade.

2.2. Semen collection

All the experiments were carried out with fresh ram spermatozoa. Semen was collected from six mature Rasa Aragonesa rams by an artificial vagina. The rams, which belonged to the National Association of Rasa Aragonesa Breeding (ANGRA), ranged from 2 to 4 years of age, were kept at the School of Veterinary Medicine under uniform nutritional conditions. Second ejaculates from three rams were pooled together and used for each assay in order to eliminate individual differences, as described previously [26].

2.3. Induction of capacitation

Sperm cells freed from seminal plasma by the dextran/swim-up [27] were incubated for 4 h at 39 °C in a humidified incubator with 5% CO_2 in air. The chlortetracycline (CTC) assay used was as previously reported [12] on the basis of the technique described by Ward and Storey [28].

2.4. Induction of the acrosome reaction

For the ionophore-induced acrosome reaction, calcium ionophore A23187 was dissolved in dimethyl sulphoxide (DMSO) and added to 20 µl of raw semen diluted 1:200 (2×10^7 cells/ml) with Hepes glucose buffer (149 m*M* NaCl, 2.5 m*M* KCl, 10 m*M* glucose, 20 m*M* Hepes and 3 m*M* $CaCl_2$. The pH was adjusted with NaOH to be 7.4 [29]). The final concentration of A23187 was 1 µ*M* and 0.3% DMSO. Control tubes had DMSO added but no ionophore, which was shown to be without effect (data not shown). The samples were incubated at 39 °C for 1 h, after which we assessed the acrosomal status and cell viability.

2.5. Cold-shock treatment

For cold-shock, aliquots of about 1×10^6 cells obtained after the swim-up process were immediately diluted up to 0.5 ml in Eppendorf tubes with the swim-up medium. After incubation for 5 min at 25 °C, the samples were transferred to 5 °C for 10 min, and then replaced at 25 °C for a further 5 min. All operations were carried out in a water bath.

2.6. Freezing–thawing

Milk-galactose (MG) extender is based on the Söderquist et al. medium [30], and consisted of reconstituted non-fatty milk powder (11% w/v) heated at 95 °C for 10 min and then cooled to room temperature. Fraction I was prepared by adding egg yolk (5% v/v) and antibiotics (0.03 g penicillin and 0.04 g streptomycin/100 ml) to the reconstituted milk. Fraction II was prepared by adding egg yolk (5% v/v) and glycerol (14% v/v) to extender A along with 224 m*M* galactose and antibiotics, as described above.

Fiser extender (F) consisted of two parts: F 1 (3.25% Tris, 9.3% fructose, 1.7% citric acid, 25% egg yolk, 2% glycerol) and F 2 (0.68% sodium citrate, 0.15% TES, 0.36% glycine, 10.18% lactose, 1.18% raffinose, 0.5% fructose, 3.95% dextran (150 000–200 000 MW); 12% of the obtained solution was replaced by the same volume of glycerol) [31].

The freezing procedure was based on the method described by Fiser et al. [31]. Semen was diluted with the fraction I from each extender after collection. Samples were then placed in a thermocycler at 30 °C and the temperature was decreased to 5 °C during 1 h with an average cooling rate of 0.4 °C/min. Fraction 2 was then added and samples were maintained at 5 °C for an equilibration time of 2 h before freezing. The samples were packed in 0.25-ml plastic straws, kept at −70 °C for 5 min and stored at −196 °C in a liquid nitrogen container until thawing. In all cases sperm final concentration was 100 million/straw. The thawing process was carried out by keeping the straws in a bath at 60 °C for 5 s.

2.7. Evaluation of semen samples

Cell viability is defined here as both intact plasma and acrosomal membranes. It was assessed by fluorescent staining [32] with carboxifluorescein diacetate and propidium iodide (Sigma). The cells were then examined under a Nikon Labophot-2

fluorescence microscope with a B-2A filter at 400× magnification. The numbers of fluorescein-positive (plasma membrane-intact) spermatozoa, propidium iodide-positive (plasma membrane-damaged) spermatozoa and fluorescein-positive acrosome (acrosomal membrane-intact) spermatozoa per 100 cells were estimated and recorded. At least 200 cells were counted in duplicate for each sample.

Sperm concentration was calculated in duplicate using a Neubauer's chamber (Marienfeld, Germany).

2.8. Scanning electron microscopy

Cell suspensions (2×10^6) were fixed in Karnovsky fixative for 1 h at room temperature and centrifuged at 600 *g* for 1 min. The pellet was washed twice in 1.5% sucrose in 0.1 *M* phosphate buffer pH 7.4 for 45 min at room temperature. Samples were then placed on Isopore filters (0.2 μm diameter), dehydrated in ascending concentrations of ethanol twice for 5 min in each of 50, 70, 96, and 100% alcohol. Subsequently, samples were critical point dried (Jeol 50/60 Hz) and coated with gold–palladium (100–200 nm, 90 s, 20 mA). Specimens were observed and photographed using a Hitachi 4100 FE scanning electron microscope. In each instance, about 100 cells were observed and scored as membrane-intact or membrane-damaged spermatozoa.

2.9. Two-phase system

The two-phase system used consisted of 5.5% (w/w) dextran T500, 2% (w/w) polyethylene glycol 6000, 10.5% (w/w) Ficoll 400, 0.25 *M* sucrose, 0.1 m*M* EGTA, 4 m*M* sodium phosphate pH 7.5, 5 m*M* glucose, 10 m*M* Hepes, 2 m*M* KOH, final pH 7.5.

2.10. Centrifugal counter-current distribution

We designed the counter-current distribution machine on the basis of that invented by Akerlund [33]. The apparatus contains 60 chambers arranged in a circle, which allows transfers of the upper (mobile) phases relative to the lower (stationary) phases. CCD is performed during centrifugation by keeping the denser (bottom) phases in the outer half while the lighter (upper) phases are in the inner half of each chamber. As no elution or pumping of any phase takes place, the overall process consists of a circular multistep transfer of 60 upper over 60 bottom batch phases. Each transfer in this centrifugal-enhanced CCD includes the following: first, the phases are shaken at unit gravity to mix them thoroughly and they are then separated by centrifugation (1000 *g*). After the phases have separated and while they are still rotating at full speed (1000 *g*), the upper (inner) phases are transferred to the next chambers. After deceleration, a new cycle can be performed. A detailed scheme of the device was already reported by Akerlund [33].

To carry out CCCD experiments, a two-phase system of the above composition was prepared and mixed. To avoid differences among experiments, batches of 400 g were assembled by weighing out stock solutions. When thoroughly mixed, the volume ratio was calculated by sampling batches of 5 ml, equilibrated at 20 °C, and the volume of the system loaded in chambers was the estimated amount to maintain the desired volume of the bottom phase (0.7 ml). Thus, three experiments were performed using the same system, in identical conditions, allowing direct comparison among six different samples.

Frozen and cold-shocked sperm samples were filtered twice throughout a 5-μm pore size Millipore disk (Millipore Corp., Bedford, MA, USA) mixing the sample with 20 volumes of the CCCD polymer free medium. Two samples of 0.5 g. each (approximately 1.2×10^8 cells) were mixed with the "loading system" which lacked 0.5 g. of medium to avoid alteration of the system composition, and the corresponding volume was loaded in chambers 0 and 30. Samples were analysed by performing 29 transfers (the whole process takes approximately 1 h). Thus, two different samples were analysed at the same time to allow direct comparison between samples. All experimentation was carried out at 20 °C. After the run, the content of each chamber was diluted with an equal volume of polymer-free medium to yield a single phase suspension which were collected and the cells counted under a light microscope.

Partition results were expressed as the percentage of cells counted in each fraction with respect to the value obtained in the chamber containing the maximal amount of cells. The partition of a certain fraction (CCCD peak) within a system is given by a

partition ratio, G, defined as ration between the amount of this fraction in mobile (upper) phase and in the stationary part (lower phase+interface). The G value is related to the position of the peak, $\hat{\imath}$, by the relation $G = \hat{\imath}/(n - \hat{\imath})$, where n is the total number of transfers [16]. For viability assessment, cells from three consecutive chambers throughout the run were pooled and stained as indicated above. Results are expressed as percentage of viable cells in each sample. As a consequence of the separation procedure, sperm cell populations with a marked affinity for the lower dextran-rich phase, (mainly due to a low hydrophobicity), partition in the left part of the profile. Sperm cells which partition almost equally in both phases distribute in the central sector. Finally, sperm populations with a high affinity for the upper PEG-rich phase (mainly due to a high hydrophobicity) partition in the right sector of the profile. The total number of viable cells recovered after the CCCD process was represented as a dark area which corresponds to the Σ_0^{29} % viable cells in each chamber respect to the number of cells in the chamber containing the maximum, i.e. in the largest sperm subpopulation.

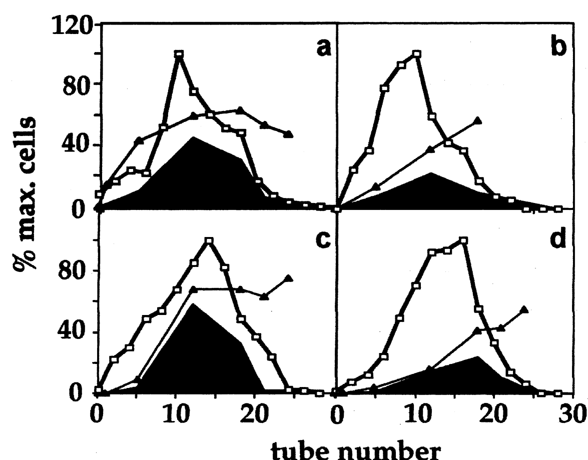

Fig. 1. CCCD representative profiles of ram sperm samples: (a) swim-up, (b) cold-shock, (c) capacitated-control, (d) capacitated. (□) Percentage of maximum of cells; (▲) percentage of viable cells. The shaded area represents the distribution of total viable cells.

3. Results

Initially, comparative CCCD analysis was carried out to determine whether the status of capacitated or acrosome-reacted sperm could reproduce the cell surface alterations due to the freezing–thawing process.

Under identical conditions CCCD yields highly reproducible results, as reported here and elsewhere [19,22,23,34]. The distribution profiles presented in this study are representative of four different experiments and reveal important similarities between the surface characteristics of capacitated and cold-shocked sperm (Fig. 1), and also between acrosome-reacted and frozen–thawed sperm (Fig. 2). The high sperm viability of the control sample (53.7%, Fig. 1a) was greatly reduced after cold-shock treatment (30.5%, Fig. 1b) also showing a significant loss of heterogeneity. Thus, a main viable cell-enriched population along with other smaller populations with lower viable cell content were found in the control sample whereas a main peak with two shoulders, corresponding to three subpopulations with extremely low viability, were located in the cold-shocked sample. The obtained G values of these subpopulations were 0.16 (chamber 4), 0.52 (chamber 10), 1.23 (chamber 16). Likewise, both characteristics, heterogeneity and viability, were significantly reduced after

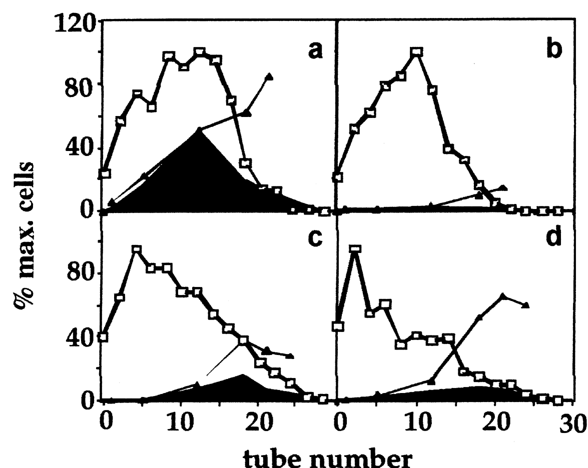

Fig. 2. CCCD representative profiles of ram sperm samples: (a) control containing DMSO, (b) acrosome-reacted, (c) frozen–thawed in Fiser extender, (d) frozen–thawed in milk–yolk extender. (□) Percentage of maximum of cells; (▲) percentage of viable cells. The shaded area represents the distribution of total viable cells.

inducing capacitation (Fig. 1d), revealing again three subpopulations with the following G values: 0.16 (chamber 4), 0.61 (chamber 11), 1.23 (chamber 16). Not only was the CCCD profile of the capacitated sample very similar to that of the cold-shocked sample (Fig. 1b), but also recovered sperm viability was very closed (27%, Fig. 1d). Incubation in the same medium under non-capacitating conditions did not however significantly affect sperm survival, since the viability of the sample maintained for 3 h. in the same medium at 15 °C remained well preserved (47.3%, Fig. 1c).

Similarly, a strong analogy was found between the partition behaviour of acrosome reacted and frozen–thawed sperm (Fig. 2). The induction of the acrosome reaction (Fig. 2b) caused a displacement of the profile to the left with a loss of viability and an important decrease in cell surface heterogeneity, compared to the untreated-control (Fig. 1a) and DMSO-control samples (Fig. 2a). Cell viability was almost zero along the sperm-reacted profile (Fig. 2b) whereas an important viable cell population still remained in the DMSO-control sample (40.7%, Fig. 2a). Similar surface changes were found when semen samples were frozen with either Fiser (Fig. 2c) or milk–yolk (Fig. 2d) extender. In both samples the sperm viability was very low, whereby the one containing milk–yolk demonstrated higher heterogeneity.

These results confirm those obtained for membrane integrity by fluorescent markers (Table 1). Thus, the high cell viability value found in the control sample (74.5%) was maintained in the capacitation-control sample (incubated for 3 h at 15 °C in the medium of capacitation, 64.5%) as well as in the acrosome reaction-control sample (DMSO-containing sample, 61.8%). Sperm viability was decreased significantly following cold-shock (22.2%), cryopreservation (26.38% Fiser medium, 24.8% milk–yolk medium) and acrosome reaction (6.6%), although it was preserved after inducing capacitation (46.7%).

Since the surface changes due to the acrosome reaction were more pronounced than those of capacitation, a scanning electron microscopy study was performed to go deeper towards the knowledge of the induced-acrosome reaction effect on sperm surface characteristics. Fig. 3 represents control and acrosome-reacted ram spermatozoa, and shows the dramatic structural alterations provoked by the induction of the acrosome reaction.

4. Discussion

Several methods have been used to investigate the morphological, biochemical, and functional changes of sperm cells, along with the subsequent reduction in fertilising ability caused by freezing/thawing processes. CCCD in aqueous two-phase systems is a useful technique to analyse the sperm surface changes accounting for loss of viability [20,23,35,36] and different functional states [25,37]. In the present work, we carried out a comparative study of surface properties of capacitated, acrosome reacted, cold-shocked and cryopreserved ram spermatozoa, based upon changes in partition behaviour.

Capacitation enables spermatozoa to undergo the acrosome reaction, once they bind to zona pellucida receptor of the oocyte as an initial step in the fertilization process. Reduced longevity and fertilising ability of cryopreserved mammalian spermatozoa can be explained by their early capacitated state [3–7]. The results of this investigation show that cold-shock induce membrane surface alterations similar to those caused by capacitation. An increased cell affinity for the lower dextran-rich phase as well as a decreased heterogeneity was found as a conse-

Table 1
Percentages of viable cells (CFDA/PI staining) of fresh (control sample), cold-shocked, capacitated (CA), CA control, frozen/thawed, acrosome-reacted (AR) and AR control (with DMSO) samples

Fresh ($n=14$)	Cold-shock ($n=14$)	CA control ($n=22$)	CA ($n=22$)	Freeze/thaw Fiser ($n=16$)	Freeze/thaw milk–yolk ($n=16$)	AR control ($n=14$)	AR ($n=14$)
74.50±7.65	22.22±5.29	64.53±9.55	46.70±12.13	26.38±6.38	24.84±8.06	61.82±12.23	6.68±3.36

Median values ±SD of the number of experiments indicated in brackets.

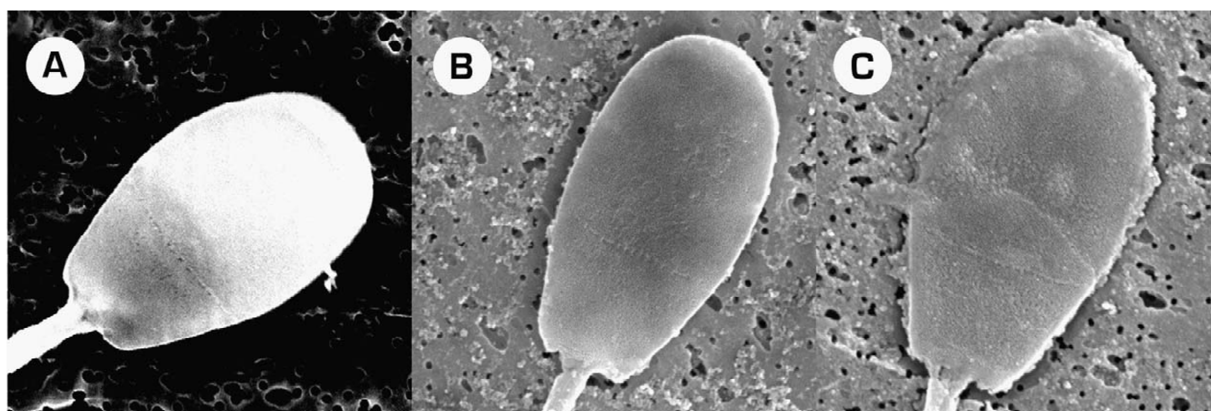

Fig. 3. Scanning electron micrographs of ram spermatozoa (A) obtained by swim-up; (B) control containing DMSO; (C) acrosome-reacted. Magnification ×13 000.

quence of both treatments, although the main peak of sperm in cold-shocked sample (Fig. 1b) is in chamber 10, while in capacitated sample (Fig. 1d) is in chamber 16. A possible explanation could be that semen is very heterogeneous, with cells in different physiological states. Not all spermatozoa in a given sample will undergo capacitation at the same time. In addition, capacitation is a continuous process, starting and stopping at different moments depending on the cell and its degree of maturation. The in vitro induction of capacitation would affect the sperm in a given sample to different extents, since some are fully capacitated, others are partially capacitated, and others are starting capacitation. As a result, a wide range of surface changes is expected due to the induction of capacitation. Therefore, the exact position of the peak could change slightly, depending on the physiological state of the cells. However, the profile is always narrower and more homogeneous than the control one, which is similar to the results obtained after the cold-shock. In both cases the CCCD profile is made up of three hypothetical populations with closed G values, being therefore the similarity between both profiles very clear.

Since cold-shock has proven to be associated with thermotrophic phase transitions in sperm membrane lipids [38], our results support the hypothesis that morphological membrane surface changes that are consistent with a lipid phase transition contribute to sperm capacitation.

The acrosome reaction involves the occurrence of point fusions between the plasma membrane and the outer acrosomal membrane, followed by membrane vesiculation and extrusion of the acrosomal enzymatic content, and exposure of inner acrosomal membrane antigens [2]. The number of saccharide residues exposed to the phase system should change as a consequence of the induction of the acrosome reaction, thus accounting for a different CCCD distribution. As we have demonstrated, the important membrane alterations shown by the acrosome-reacted sperm are reflected in different partition behaviour, with a significant displacement to the left of the CCCD profile along with an important decrease in cell heterogeneity, in relation to control cells. Although the main population of the frozen–thawed sperm is more to the left than that in the acrosome-reacted sample, both treatments have similar effect on sperm surface characteristics. This difference could be because the effect of the acrosome reaction depends on the physiological state of the sperm, while the freezing process would strongly affect all cells in the sample. Thus, the induction of the acrosome reaction would cause a main population of cells with different degree of membrane damage. Some sperm would completely lose their acrosome, others partially lose it, and still others could have an acrosomal membrane which has started to fuse with the plasma membrane. As a result, impermeability to propidium iodide would be lost, together with an important decrease in surface heterogeneity. Moreover, the freezing medium con-

tains cryoprotectants which could affect cell partition. For this reason, the samples were filtered before CCCD analysis. The filtration process also affects sperm cells, promoting the displacement of the profile to the left [22], which could contribute to the observed difference in cell partition. However, in both cases about 65% of the cells were between chambers 0–10.

The low phosphate concentration in the two-phase system used in this study could result in a relatively charge-insensitive partition of cells [34,39]. Consequently, the different partition behaviour could be interpreted as a change in hydrophobicity of the spermatozoa surface induced by treatment. A higher affinity to the lower dextran-rich phase would indicate a decreased hydrophobicity of the treated sperm surface. The saccharide residues exposed to the medium as a consequence of capacitation or acrosome reaction would decrease the hydrophobicity of the sperm cells, as has already been described [1]. Likewise, we have already shown that loss of viability results in decreased surface hydrophobicity in dead or moribund sperm [36].

Our results have also shown that both cooling and freezing–thawing cause a displacement of the CCCD profile to the left and a loss of viability. As previously shown, sample dilution in Fiser's extender containing egg yolk and glycerol partially prevented these changes [24]. Sperm surface heterogeneity was however more effectively preserved when sample was diluted in milk–yolk. The higher heterogeneity found in the sample containing milk–yolk strongly suggests that the use of this extender for cryopreservation could result in a higher fertility rate. Furthermore, the use of CCCD in an aqueous two-phase system may be useful for analysing sperm subpopulations and for screening putative protective substances for their ability to maintain high heterogeneity and reduce damage caused by freezing.

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