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Surface changes associated with ram sperm cryopreservation revealed by counter-current distribution in an aqueous two-phase system
Effect of different cryoprotectants

M. Ollero, T. Muiño Blanco, M.J. López-Pérez, J.A. Cebrian Pérez*

Departamento de Bioquímica y Biologia Molecular, Facultad de Veterinaria, C/Miguel Servet 177, 50013 Zaragoza, Spain

Abstract

Ram sperm was frozen in the presence of the most commonly used cryoprotectants. After thawing, the overall cell surface changes provoked by freezing were assessed by centrifugal counter-current distribution (CCCD). In addition, cell membrane integrity (viability) of all the treated sperm was estimated by fluorescent staining. Fresh and refrigerated sperm were used as controls. Our results show no improvement of the cooling-induced cell surface damage by freezing in the presence of bovine seminal plasma, proline, glycine-betaine and phosphatidylcholine. Better results were obtained with vitamin E and cholesterol. However, the best protective effects were found by employing seroalbumin and lactalbumin. Furthermore, freezing in the presence of bovine lactalbumin resulted in a good maintenance of the cellular viability and of the CCCD heterogeneity in respect to fresh cells.

Keywords: Centrifugal counter-current distribution; Aqueous two-phase systems; Partitioning; Cryopreservation; Cryoprotectants; Ram sperm

1. Introduction

Artificial insemination in rams or cattle requires one-tenth as many fresh sperm to achieve pregnancy rates matching those currently obtained with frozen–thawed semen [1,2]. This fact indicates that normal cryopreservation procedures result in loss of physiological competence in 80–90% of the original cells. This physiological competence involves a series of features on the cell membrane essentially acquired during the maturation process through epididymis transit [3,4]. However, the freezing–thawing process provokes an irreversible loss of compounds and mechanical disarrangement that dramatically changes the plasma and acrosome membranes of the cell; ram spermatozoa are very sensitive to this damage induced by the freezing process [2,5–8].

Partitioning in two-polymer aqueous phase systems is a highly sensitive method for the study and separation of cells, organelles and membranes on the basis of surface properties [9,10]. Thus, phase partitioning has been used already to study sperm cell heterogeneity [11–15]. Moreover, management of cell sedimentation in centrifugal counter-current distribution (CCCD) [16] has allowed this technique to be used as a valuable method to assess sperm cell

*Corresponding author.

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surface changes. More recently, it has been shown that sperm cell heterogeneity revealed by CCCD is associated with different viability states of the fractionated cells [14,17,18] and with surface changes associated with maturation of spermatozoa [7].

This work was undertaken in order to evaluate the effect of the most commonly used cryoprotectants on ram sperm cryopreservation. For this, cell surface preservation accomplished by different cryoprotectants was assessed by CCCD analysis and by estimation of the cell viability, a cellular condition directly associated with the membrane integrity and sperm quality [19].

2. Experimental

2.1. Chemicals

Dextran T-500 (M_r 500 000) and Ficoll 400 (M_r 400 000) were obtained from Pharmacia (Uppsala, Sweden). PEG (M_r 6000) was purchased from Serva Feinbiochemica (New York, USA). Percoll was obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used were of analytical reagent grade.

2.2. Two-phase system

The system used consisted of 5.5% (w/w) dextran T500, 2% (w/w) PEG 6000, 10.5% (w/w) Ficoll 400, 0.25 *M* sucrose, 0.1 m*M* EGTA, 7 m*M* sodium phosphate pH 7.5, 10% (v/v) of '10× buffer stock Hepes' (50 m*M* glucose, 100 m*M* Hepes, 20 m*M* KOH).

2.3. Freezing–thawing of spermatozoa

Immediately after collection of semen with an artificial vagina, cell viability (see below) was determined. Then semen was frozen based on the method of Fiser et al. [20]: sperm cells were incubated for 10 min at 30°C with the cryopreservative up to a final concentration in the diluent composed of 16.5 m*M* betaine, 16.5 m*M* proline, 3.2% bull seminal plasma, 6.8% bull plus ram seminal plasmas, 1.55% and 4.65% phosphatidylcholine, 3.1% cholesterol, 30 m*M* dithiothreitol, 3.2 m*M* vitamin E, 11 μM lactalbumin, 11 μM bovine serum albumin, and diluted to a spermatozoa concentration of 2.0×10^9 cells/ml with Fiser's 1 diluent. The diluted semen was cooled gradually (0.2°C/min) from 30°C to 5°C and then further diluted 2:1 with Fiser's 2 solution. This preparation (now containing 4.7–4.9% glycerol) was equilibrated at 5°C for 2 h before packaging it in 0.25-ml straws. The straws were frozen by placing them in a −70°C refrigerator for 10 min and they were then stored in a liquid nitrogen container.

All the cryoprotectants were used in solution except phosphatidylcholine and cholesterol, which were added as liposomes made by sonication of a diluted sample in a chloroform–methanol solution previously vacuum-dried.

Semen was thawed by introducing the straws into a water-bath at 37°C for 30 s and motility and viability were determined prior to CCCD studies.

2.4. Preparation of cell samples

The pellet of spermatozoa obtained after washing through 4 ml 35% saline Percoll and 2 ml 70% saline Percoll (5 min at 200 *g* and 15 min at 1200 *g*) was resuspended in 5 ml of saline medium and centrifuged for 5 min at 700 *g*. The pellet was resuspended with 5 ml of the two-phase system medium and centrifuged for 5 min at 700 *g*. The supernatant was removed and the pellet was resuspended in the two-phase system medium.

2.5. Centrifugal counter-current distribution

The counter-current distribution machine used and a detailed scheme of the process have already been described [21]. To carry out CCCD experiments, a two-phase system of the above composition was prepared and mixed. In each experiment, the volume ratio was calculated in 5-ml batches. Then, the volume of the system loaded in chambers 1–59 (59 transfers) or 1–29 and 31–59 (29 transfers) was that estimated to maintain the desired volume of the bottom phase (0.7 ml). The cells were loaded in chamber 0 (59 transfers) or in chambers 0 and 30 (29 transfers). Shaking and centrifugation time was 60 s. After the run, the solutions were transformed into a one phase-system by addition of one volume of a

dilution buffer (a polymer-free medium as used in the two-phase system). The fractions were then collected and the cells counted under a light microscope. All operations were carried out at 20°C. For each CCCD run, results are expressed as the percentage of the value of cells counted in each fraction in respect of the value obtained in the chamber containing the maximal amount of cells. In some fractions assessment of viability was carried out by fluorescent stain with carboxyfluorescein diacetate and propidium iodide, according to Harrison and Vickers [22].

3. Results

In order to investigate whether the CCCD technique could be useful in assessing the cell surface damage produced by the freezing–thawing process, we first studied the cell distribution and viability throughout CCCD profiles of fresh, refrigerated and frozen–thawed ram spermatozoa. The clear heterogeneity presented in the CCCD profile obtained with fresh ram spermatozoa (Fig. 1a) decreases in the refrigerated sperm cells when they are kept at 5°C for 15 min, after the first part of the 'Fiser method' [20] to freeze them (Fig. 1b); the percentage of viable cells is also diminished from about 60% (fresh) to 20% (refrigerated) of the total cells (Table 1). Moreover, frozen–thawed ram spermatozoa were distributed as one broad single peak, indicating a dramatic loss of heterogeneity, with no viable cells present in all of the profile (Fig. 1c)

In the case of samples still containing viable cells, i.e. fresh (Fig. 1a) and refrigerated sperm (Fig. 1b), populations on the left of the CCCD profile were enriched in dead and those on the right in live cells. These results further confirm previous reports showing an enhanced affinity of live cells to the upper PEG-rich phase [14,17].

New experiments were performed freezing ram sperm cells in the presence of the most commonly used cryoprotectants. Thus, proline, glycine-betaine, bovine seroalbumin, bovine lactalbumin, vitamin E, dithiothreitol, phosphatidylcholine and cholesterol were employed. All of them have previously been described as sperm cryopreservative agents in different freezing processes [20,23–29]. In all cases, ram

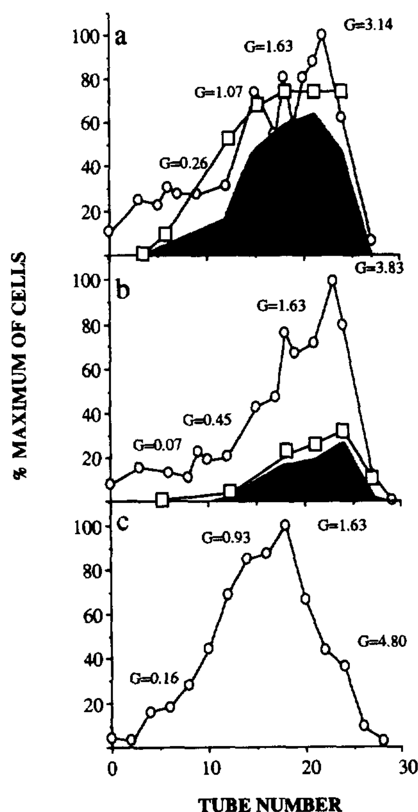

Fig. 1. Centrifugal counter-current distribution and cell viability of: (a) fresh, (b) refrigerated and (c) undiluted frozen ram spermatozoa. (○) Percentage of maximum of cells; dark area, distribution of total viable cells; (□) percentage of viable cells.

sperm was studied by CCCD of 29 transfers and the number of total and viable cells fractionated was then estimated. To better understand the results obtained, the percentage of viable cells was calculated in three different sectors of the profiles obtained, between 0 and 11, 12 and 19, and 20 and 29 chambers. The results thus obtained are represented in Table 1.

The profiles obtained for each cryoprotectant used were clearly different (Figs. 2–6). Thus, CCCD profiles obtained with ram sperm frozen in the presence of proline and glycine-betaine, previously described as good cryoprotectants [23], showed a high heterogeneity (Fig. 2) though the overall viability reached only about 12% and 18%, respectively (Table 1).

Benefits and detrimental effects have been attribu-

Table 1
Percentage of viable with respect to total cells obtained in the absence and presence of the cryoprotectant agents after centrifugal counter-current distribution

	A (0–11)	B (12–19)	C (20–29)	T (0–29)
Fresh	21	74	69	59,25
Refrigerated	0	20	26	18,97
Frozen-thawed	0	0	0	0
16.5 m*M* betaine	4	26	31	18,6
16.5 m*M* proline	1	14	26	11,79
3.2% bsp	0	12	34	9,78
6.8% brsp	9	16	35	18,25
1.5% ph.choline (1×)	0	5	20	5,62
4.6% ph.choline (3×)	2	9	24	9,12
3.1% cholesterol	4	6	4	5
30 m*M* dithiothreitol	0	0	4	1,93
3.2 m*M* vitamin E	19	20	41	27,3
Fiser's diluent	3	31	38	23,38
11 μM lactalbumin	13	29	48	32,31
11 μM serum albumin	7	24	34	22,33

A (0–11) chambers; B (12–19) chambers; C (20–29) chambers and T (0–29) chambers; bsp, bull seminal plasma; brsp, bull plus ram seminal plasmas.

ted to the presence of seminal plasma in the medium to freeze sperm [30–35]. This fact prompted us to study the cryoprotective effect of bovine and ovine plus bovine seminal plasma. Bovine plasma seems to partially avoid the freezing damage related to the sperm number of viable cells (10%) (Table 1), but a dramatic change in respect to control fresh cells (Fig. 1a) was observed in its CCCD profile (Fig. 3a). The

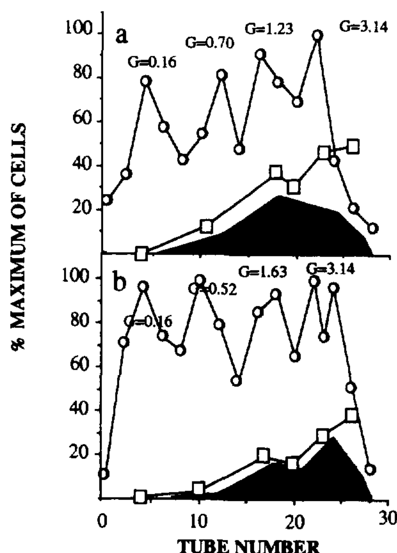

Fig. 2. Centrifugal counter-current distribution and cell viability of ram spermatozoa frozen in the presence of: (a) 16.5 m*M* glycine betaine, (b) 16.5 m*M* proline. (○) Percentage of maximum of cells; dark area; distribution of total viable cells; (□) percentage of viable cells.

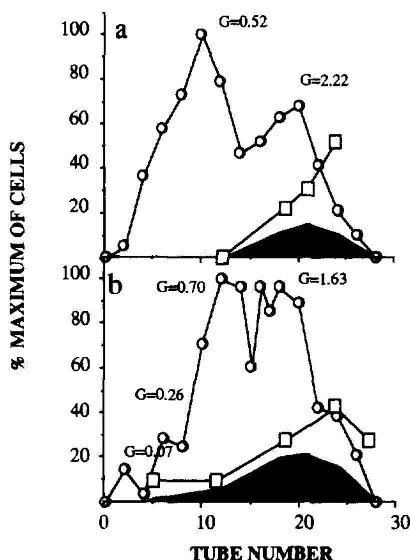

Fig. 3. Centrifugal counter-current distribution and cell viability of frozen ram spermatozoa in presence of: (a) 3.2% bull seminal plasma, (b) 6.8% of a mixture (50%) of bull and ram seminal plasmas. (○) Percentage of maximum of cells; dark area, distribution of total viable cells; (□) percentage of viable cells.

use of a mixture of both bovine and ovine plasma also induced detrimental effects on the spermatozoa CCCD profile (Fig. 3b).

One of the most important aspects related to the damage provoked by the freezing process is a lipid reorganisation with a consequent loss of lipids from the plasma membrane. Because of that, the use of different lipids, usually present in the cell membrane, has come to be used to cryopreserve cell structures. In our study, we also tested as cryoprotectants cholesterol and two different concentrations of phosphatidylcholine, added to the freezing medium as liposomes. Freezing in the presence of both concentrations of phosphatidylcholine (Fig. 4a,b) resulted in profiles similar to that obtained in the absence of cryoprotectants (Fig. 1c), whereas the overall viability also decreased to very low values (Table 1).

Different antioxidants have been also described as good cryoprotectants for bull sperm [25,36]. Dithiothreitol seems to preserve the initial sperm heterogeneity (Fig. 5a), though a clear loss of cell viability was also observed (Table 1). In the case of vitamin E, the overall cell viability (27.3%) was one of the highest obtained after the freezing–thawing process (Table 1), whereas its CCCD profile (Fig. 5b) was similar to that obtained in the case of refrigerated cells (Fig. 1b).

Different proteins have been used to protect sperm cell [37]. We have assessed bovine lactalbumin and seroalbumin as possible cryoprotectants. Cell viability estimated after freezing with both proteins was 32% and 22%, respectively (Table 1) clearly indicating a protective effect on the plasma membrane. Furthermore, the CCCD profiles show that freezing with bovine lactalbumin produced a maintenance of the heterogeneity observed in fresh cells (Fig. 6b) and a relatively high proportion of viable cells at the first sector (chambers 0–12) of the CCCD profiles (Table 1).

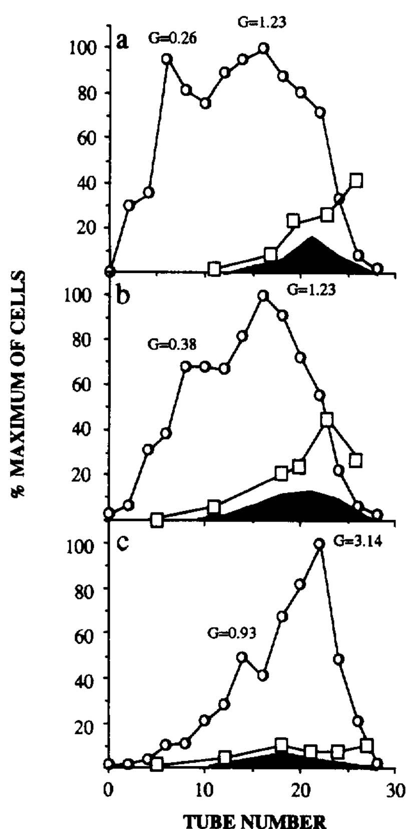

Fig. 4. Centrifugal counter-current distribution and cell viability of frozen ram spermatozoa previously incubated with: (a) 1.5% and (b) 4.6% of phosphatidylcholine liposomes, (c) 3% cholesterol liposomes. (○) Percentage of maximum of cells; dark area, distribution of total viable cells; (□) percentage of viable cells.

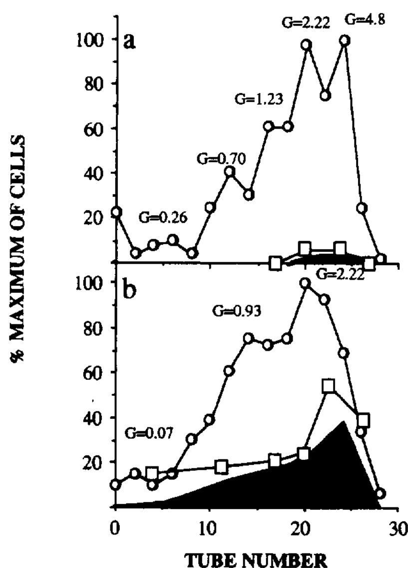

Fig. 5. Centrifugal counter-current distribution and cell viability of frozen ram spermatozoa in a medium containing antioxidant agents: (a) 30 m*M* dithiothreitol, (b) 3.2 m*M* vitamin E. (○) Percentage of maximum of cells; dark area, distribution of total viable cells; (□) percentage of viable cells.

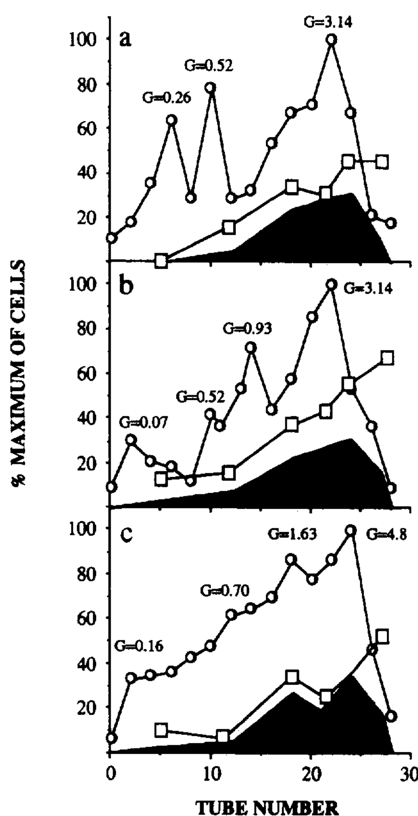

Fig. 6. Centrifugal counter-current distribution and cell viability of frozen ram spermatozoa in presence of: (a) Fiser's diluent, (b) 11 μM bovine lactalbumin, (c) 11 μM bovine seroalbumin. (○) Percentage of maximum of cells; dark area, distribution of total viable cells; (□) percentage of viable cells.

The percentage of viable cells found in the three different sectors, (A: chambers 0–11; B: chambers 12–19; C: chambers 20–29), of the CCCD profiles is also shown in Table 1. The use of different cryoprotective agents significantly modifies the proportion of viable cells found in each sector. No viable cells were found on the further left fractions in most cases. A few viable cells were present only in fresh sperm and when freezing was carried out in the presence of vitamin E, lactalbumin and seroalbumin (Table 1). With some of the cryoprotectants used, the percentage of viable cells clearly increases even at the further right part of the profiles (Table 1). Interestingly, 50% of the cells distributed at this right part preserved their membrane integrity when they were frozen in the presence of lactalbumin.

4. Discussion

Since the discovery that glycerol is an effective cryoprotective agent for rooster sperm [38], semen from a broad range of animal species has been frozen and used successfully for artificial insemination. However, apart from cattle, frozen semen has not been usually employed for insemination in domestic animals, mainly because cryopreservation induces membrane and functional alterations during cooling and the usual protocols do not provided an acceptable level of fertility. Ram sperm is particularly affected by the freezing process. Its saturated/unsaturated fatty acids ratio differs considerably from that of other species such as man or rabbit [39] which tolerate the freezing–thawing process better.

CCCD of ram spermatozoa showed changes in the sperm distribution profile which depend on the treatment used. The sperm heterogeneity observed in fresh sperm CCCD is clearly affected by the cryopreservation process. Thus, the number of spermatozoa populations that can be observed in the CCCD profile of fresh cells is reduced by refrigeration and it is even lower after freezing (Fig. 1). These changes appear to be associated with dead cells and consequently with a partial or total loss of the initial cell membrane integrity (Table 1).

Our results indicate that improvement of cryopreservation is not achieved by adding either bovine or ovine seminal plasma to the freezing medium (Fig. 3). Likewise, the use of phosphatidylcholine liposomes (Fig. 4) do not improve the cell damage provoked by the freezing–thawing process. Moreover, although betaine and proline have been shown to enhance sperm motility after thawing [23], no positive effect in respect to control refrigerated cells was found when they were used as cryoprotectants (Fig. 2).

On the other hand, when vitamin E was present during the freezing process, the CCCD profile obtained showed an important loss of the original cell

heterogeneity. Cell affinity to the upper PEG-rich phase was increased, reproducing the partitioning behaviour of live spermatozoa [17]. The total amount of viable spermatozoa recovered (27%) was higher than that obtained with refrigerated sperm.

A similar effect was observed when cholesterol was used as cryoprotectant. In this case, spermatozoa showed an enhanced affinity to the upper PEG-rich phase (Fig. 4). However, cholesterol was not able to avoid cell damage as revealed by the low percentage of viable cells found (Table 1, Fig. 4). These results suggest that cholesterol could be taken up by the cell membrane as already described [25,40] thus modifying the cell partitioning behaviour but without any beneficial effect as far as protecting the cell surface from damage is concerned.

The best cryoprotective effect observed corresponded to use of lactalbumin and seroalbumin (Fig. 6). The capacity of these two proteins in binding to different lipids is well known. This capacity could somehow be involved in the maintenance of cell heterogeneity and viability (Table 1). This effect could account for the preservation of the cell surface against lipid disruption and, consequently, avoiding loss of membrane components. However, though both proteins were the best cryoprotectants tested in this work, significant differences were found between them, lactalbumin being the most effective in preserving cell viability (Table 1).

On the other hand, the CCCD spermatozoa distribution could be interpreted as a progressive transition from a population of impaired cell surface (which usually has a preferential affinity for the upper PEG-rich phase) to one of dead cells affected by dramatic surface changes (preferentially partitioning in the lower dextran-rich phase).

The results reported here show that CCCD is a useful method to evaluate the damage suffered by sperm during the freezing–thawing process. Two main considerations lead us to this conclusion. First, CCCD is probably a unique technique available to assess the overall surface state of a semen sample, a question of fundamental interest in estimating the fertilising capacity of a given sperm. Second, CCCD can be used for preparative purposes, making possible the isolation of specific spermatozoan populations enriched in a defined character.

Acknowledgments

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