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Reversion of thermic-shock effect on ram spermatozoa by adsorption of seminal plasma proteins revealed by partition in aqueous two-phase systems

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Abstract

Centrifugal counter-current distribution (CCCD) in a dextran, Ficoll, poly(ethylene glycol) two-phase system was used to study the effect of seminal plasma proteins on the partition behaviour of ram spermatozoa exposed to thermal shock. Ram spermatozoa freed from seminal plasma by a 'swim-up' procedure were submitted to thermal shock and fractionated by CCCD. Cell viability decreased from 68% to 18% after the treatment, showing a slight displacement of the cells from the right (where a higher enrichment of live cells is found) to the centre of the profile. A change of the distribution profile was shown in the presence of either ram or bull seminal plasma. Bull seminal plasma was able to move the profile to the right, whereas ram seminal plasma increased the proportion of cells with enhanced affinity for the lower dextran-rich phase. Plasma proteins isolated from both seminal plasmas moved the profile to the right. In addition, cell viability rose to 48% after the CCCD run in the presence of ram plasma proteins. This restoring effect was lost when ram plasma proteins were thermally denatured. Bovine serum albumin was not only unable to move the profile to the right but even promoted displacement of the profile to the left. This negative effect was also observed when proteins from bull seminal plasma were in the presence of protein-free ram seminal plasma. However, proteins isolated from ram seminal plasma still restored the profile in the presence of bull seminal plasma freed from proteins. The results presented here strongly suggest that seminal plasma proteins are adsorbed by a spermatozoal surface previously exposed to thermic shock. These proteins would exert a highly specific protective effect on ram spermatozoa. In addition, in the ram seminal plasma there must be some factor which avoids this adsorption.

Keywords: Partitioning; Spermatozoa; Ram seminal plasma; Proteins

1. Introduction

Counter-current distribution (CCD) in aqueous two-phase systems is a useful technique employed for the separation of cell populations based on differences in their surface properties [1,2]. The use of centrifugation to enhance the phase settling (centrifugal CCD; CCCD) allows the fractionation of samples in short periods, and it has already been used for the separation of sperm cell populations [3–5].

The sperm plasma membrane plays a central role in regulating spermatozoon functions. The exterior surface of the membrane may bind polypeptides secreted by the excurrent duct system, accessory glands, or lining epithelium of the female tract.

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Proteins from the epididymal [6,7] and seminal fluid [8,9] are adsorbed onto the sperm surface as they pass through the male reproductive tract. The end result of this remodelling is a functional mature sperm whose plasma membrane can participate in the complex set of membrane-mediated interactions with the egg during fertilization [10]. In addition, the need for some adsorbed proteins not only to acquire the fertilizing capacity but also to maintain cell viability has also been reported [11]. The presence of these components could modify the spermatozoon surface and, in consequence, different distribution profiles would be obtained with different spermatozoon functional states. Thus, surface changes of mammalian spermatozoa related to interactions of ejaculate plasma proteins with the sperm surface have been shown by using CCD [12]. Likewise, we have recently shown, by using CCCD, that the acquisition of adsorbed seminal plasma proteins by the sperm cell surface modifies the partition behaviour of bull spermatozoa, reproducing that of live cells [13].

Seminal plasma contains factors that may influence male fertility, as has been reported in different species. Comparisons of seminal plasma composition between males of different fertility have been made [14,15], as well as the isolation of factors from seminal plasma that facilitate or inhibit sperm capacitation or fertilization [16,17]. Detrimental [18–21] or beneficial [22] effects of seminal plasma on sperm motility and viability have also been described. Seminal plasma has also been reported to increase the resistance of boar spermatozoa to cold shock damage [23,24] or sperm survival after freezing–thawing [25,26]. These results may not provide accurate information and suggest the hypothesis that there are one or more components in seminal plasma that significantly alter the sperm function.

The following experiments examine the effect of homologous and heterologous seminal plasma proteins on the partition behaviour of ram spermatozoa exposed to thermal shock.

2. Material and methods

2.1. Chemicals

Dextran T-500 (M_r 500 000) and Ficoll 400 (M_r 400 000) were obtained from Pharmacia (Uppsala, Sweden). Poly(ethylene glycol) (M_r 6000) was purchased from Serva Feinbiochemica (New York, NY, USA). All other chemicals used were of analytical reagent grade.

2.2. Two-phase system

The two-phase system used consisted of 5.5% (w/w) dextran T500, 2% (w/w) poly(ethylene 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, 10% (v/v) of '10× buffer stock Hepes' (50 m*M* glucose, 100 m*M* Hepes, 20 m*M* KOH).

2.3. Preparation of cell samples

All the experiments were carried out with fresh ram sperm. Semen was collected from three mature *Rasa aragonesa* rams by an artificial vagina. The rams were kept at the School of Veterinary Medicine under uniform nutritional conditions. Second ejaculates were pooled and used for each assay according to previous results [3]. For thermal shock, about 2×10^9 cells obtained after 'swim-up' procedure [27] were incubated for 5 min at 25°C, transferred to 5°C for 10 min, and then replaced at 25°C for a further 5 min. Then the 'swim-up' medium was eliminated by diluting twice with five volumes of a polymer-free medium and filtering through 5-μm pore size Millipore disks. A 0.5-ml aliquot of sample with about 1×10^8 cells was recovered and loaded in the CCCD sample chamber.

2.4. Obtention of seminal plasma and proteins

Seminal plasma was obtained by spinning 1 ml of semen at 12 000 *g* for 5 min in microfuge at 4°C. The supernatant was centrifuged again, and 400 μl of undiluted seminal plasma were removed and passed through a 0.22-μm filter. Phenylmethylsulphonyl fluoride was added (2 m*M*), and seminal plasma was kept at −20°C.

Protein-free seminal plasma was obtained by filtering the whole seminal plasma through MICROSEP microconcentrators of 3 kDa molecular mass cut-off (Filtron Technology, MA, USA), spin-

ning for 4 h at 3000 *g* at 4°C. To obtain seminal plasma proteins, the obtained sample concentrate was diluted with five volumes of the two-phase system medium and centrifuged again, and the seminal plasma proteins were recovered. Phenylmethylsulphonyl fluoride was added (2 m*M*) and stored at −20°C.

Thermally denatured proteins were obtained by incubating the sample for 10 min at 90°C.

2.5. Centrifugal counter-current distribution

The counter-current distribution machine used and a detailed scheme of the process have already been described [5]. 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 by batches of 5 ml. Then, the volume of the system loaded in all chambers was the one estimated to maintain the required volume of the bottom phase (0.7 ml). Cells were loaded in chambers 0 and 30, and 29 transfers were carried out. Shaking and centrifugation time was 60 s. After the run, the solutions were transformed into a one-phase system by the addition of one volume of a dilution buffer (polymer-free medium). The fractions were then collected and the cells counted under light microscope. All operations were carried out at 20°C. For each CCCD run, results are 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. As a consequence of the separation procedure, fractions located further to the left or right of the CCCD profiles will contain cells with higher affinity for the lower dextran- or upper PEG-rich phase, respectively. In some experiments assessment of viability was carried out by fluorescent stain with carboxyfluorescein diacetate and propidium iodide [28].

3. Results

We have previously shown that CCCD in a Ficoll–dextran–PEG two-phase system is a resolutive technique for revealing ram spermatozoa heterogeneity. Two different viability populations were isolated, live spermatozoa showing enhanced affinity for the upper PEG-rich phase (i.e. preferentially located in the right-hand fractions of the profile) [5].

In this work, the effect of thermal shock on the partition behaviour of a highly motile and viable ram spermatozoa population freed from seminal plasma by 'swim-up' procedure [27] was studied. Despite the decrease of cell viability from 68% to 18%, a slight modification of the CCCD profile was found. The profile of shocked cells (Fig. 1b) was more homogeneous than the control ones, showing a wide peak in the centre of the run (Fig. 1a). This change of profile would be reverted by seminal plasma if this fluid compensated the cold-shock damage. Fig. 2a shows a strong displacement to the right of the CCCD profile of shocked spermatozoa in the presence of whole bull seminal plasma (BSP), whereas ram seminal plasma (RSP) even increased the proportion of cells with enhanced affinity for the lower dextran-rich phase (Fig. 2b). An interesting observation is the significant displacement to the right of the treated cell profile, in the presence of bull seminal plasma, with respect to the untreated control cell profile (Fig. 1a), although cell viability was close to zero along all the profile.

In order to further test the ability of plasma proteins to restore the CCCD profile after thermal shock, fractions of both seminal plasmas were assayed for their sperm viability-restoring effect or for their damaging effect. CCCD experiments were carried out in the presence of seminal plasma proteins isolated either from ram seminal plasma (RPP) or from bull seminal plasma (BPP). CCCD runs of 29 transfers were performed adding 90 μg of

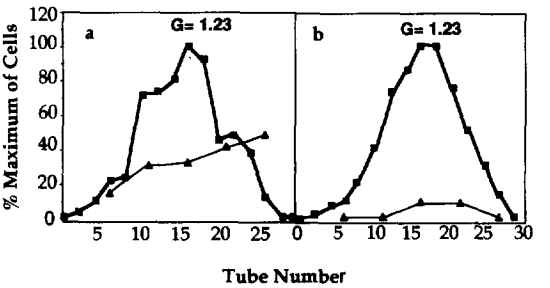

Fig. 1. Effect of thermal shock on the counter-current distribution of fresh ram spermatozoa. (a) Control cells; (b) cells submitted to thermal shock. (■) Percentage of maximum of cells; (▲) percentage of viable cells.

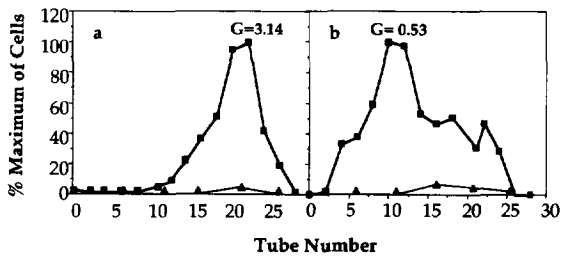

Fig. 2. Effect of the inclusion of 1% seminal plasma in the two-phase system on the counter-current distribution of fresh ram spermatozoa submitted to thermal shock. (a) Bull seminal plasma (BSP); (b) ram seminal plasma (RSP). (■) Percentage of maximum of cells; (▲) percentage of viable cells.

RPP or BPP to the indicated chambers. Fig. 3a and Fig. 3b show that both kinds of proteins were able to restore the profile obtaining only one peak at the right of the run. These results indicated that an important effect on the cell partitioning behaviour was induced by the presence of homologous and heterologous seminal plasma proteins, adsorption of which changes the cell partition properties, as previously suggested [12,13]. It is significant that cell viability after the CCCD run with RPP rose to 48% in chambers 19–21, whereas in the presence of BPP it was much lower.

In order to find out if this restoring effect was specific for seminal plasma proteins the effect of BSA and denatured plasma proteins was studied. BSA was not only unable to move the profile to the right but even promoted displacement of the profile to the left (Fig. 3c). Thermally denatured RPP were also unable to produce the profile restoring effect (Fig. 3d). However, when the thermal-shock treatment was performed adding RPP to the incubation medium, without therefore adding the proteins to the CCCD chambers, the same restored profile was obtained (Fig. 4).

Given the ability of both proteins (BPP and RPP) to restore the profile of treated cells despite the different effect of both seminal plasmas (BSP and RSP), some experiments were performed to find out if there was some factor in the ram seminal plasma capable of avoiding the protein adsorption. The effect of plasma proteins on partitioning of shocked spermatozoa, adding to the system 1% of protein-free heterologous seminal plasma was studied. The results are shown in Fig. 5. RPP caused a slight movement of the profile to the right, and so reverted, at least in part, the thermic-shock damage, when protein-free bull seminal plasma was added (Fig. 5a). However, no restoration of profile was achieved by BPP in the presence of protein-free ram seminal

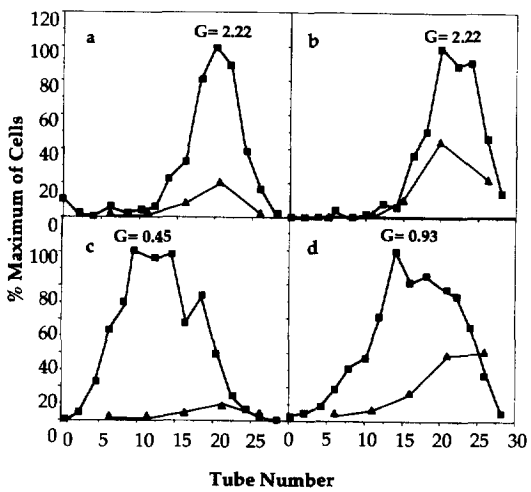

Fig. 3. Effect of the inclusion of proteins on the counter-current distribution of fresh ram spermatozoa submitted to thermal shock. (a) Bull plasma proteins; (b) ram plasma proteins; (c) BSA; (d) thermic denatured ram plasma proteins. A 90-μg quantity of proteins was added in the sample chambers (0 and 30) and in the four previous chambers, and CCCD of 29 transfers was carried out. (■) Percentage of maximum of cells; (▲) percentage of viable cells.

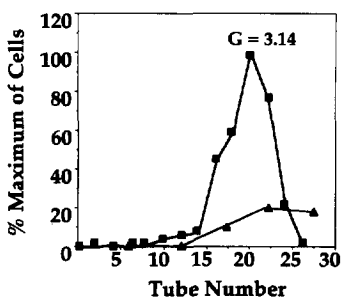

Fig. 4. CCCD profile of ram spermatozoa submitted to thermal shock with ram plasma proteins in the incubation medium. 2×10^9 cells were incubated with 450 μg of ram plasma proteins, and then filtered through 5-μm pore size Millipore disks by diluting twice with five volumes of a polymer-free medium. About 1×10^8 cells were loaded in the CCCD sample chamber. (■) Percentage of maximum of cells; (▲) percentage of viable cells.

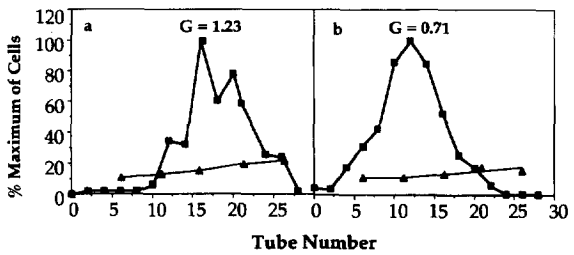

Fig. 5. Effect of plasma proteins on the counter-current distribution of thermally shocked ram spermatozoa adding to the system 1% of protein-free heterologous seminal plasma. (a) Ram plasma proteins and protein-free bull seminal plasma; (b) bull plasma proteins and protein-free ram seminal plasma. A 90-μg quantity of seminal plasma proteins was added in the sample chambers (0 and 30) and in the four previous chambers. CCCD of 29 transfers was carried out. (■) Percentage of maximum of cells; (▲) percentage of viable cells.

plasma (Fig. 5b), suggesting that ram seminal plasma contains a factor which interferes with the viability-stimulating effect of plasma proteins.

4. Discussion

Several studies have provided direct evidence that specific components of seminal plasma are adsorbed to the surface of ejaculated sperm [6–9,29–31]. In addition, adsorbed elements are responsible for maintaining the membrane cell stability against environmental aggression [32,33]. In the light of these results, and given the capacity of CCCD in an aqueous two-phase system to analyse sperm surface changes accounting for loss of viability [5] and different states of maturity [3], the adsorption of seminal plasma proteins to the ram spermatozoa surface was studied by changes on their partition behaviour. Our results show that ram sperm cells submitted to thermal shock, a method of inducing loss of viability, have different partition behaviour when RSP or BSP was added to the system.

CCCD of treated cells (Fig. 1b) revealed a homogeneous peak in the centre of the run although cell viability had considerably decreased. Associated with this loss of viability a displacement of profile to the left, in relation to untreated control cells, would be expected according to our previous results [5]. However, in this case, the results obtained did not establish whether partition of cells somehow takes place on the basis of sperm viability. Some other cell surface heterogeneity could also underlie the CCCD fractionation, such as the acrosomal state already described [34] or thermotropic phase transitions in sperm membrane lipids due to thermal shock [26,35]. The effect on partition observed in the presence of ram or bull seminal plasma, which promoted a different distribution of shocked ram sperm cells without difference in cell viability (Fig. 2), would be in agreement with this interpretation. Thus, the effect of BSP on partition could be interpreted as an adsorption of proteins which increases the affinity of cells for the upper PEG-rich phase, reproducing the partition behaviour of live spermatozoa [5], although cell viability was close to zero along all the profile. Conversely, RSP promoted displacement to the left of the shocked spermatozoa profile without important differences in the cell viability value in all the assayed chambers. This effect could be interpreted as a change in hydrophobicity of the spermatozoa surface in the presence of seminal plasma proteins, since the low phosphate concentration in the two-phase system used here could result in a relatively charge-insensitive partition of cells [34,36]. In addition, a loss of membrane-bound protein following cold shock in spermatozoa has already been described [37], and the loss of adsorbed elements from the cell surface has been reported to modify the partition behaviour of bull spermatozoa [13].

This difference between the effect of both seminal plasmas would suggest that in the RSP there must be some factor which prevents the adsorption of proteins to the thermally shocked sperm surface, and, therefore, the displacement to the right of the CCCD profile. This would be in agreement with previous reports of Schmehl et al. [38], who found detrimental effects of ram seminal plasma on post-thaw spermatozoa survival.

The results obtained with proteins isolated from both seminal plasmas support the aforementioned suggestion. Thus, isolated proteins from either ram (RPP) or bull (BPP) seminal plasma promoted displacement to the right of the profiles (Fig. 3), cell viability increasing with RPP up to 48%. These results would indicate that adsorption of seminal plasma proteins to the shocked spermatozoa surface reverts, at least in part, the effect of thermal shock,

and that the protein adsorption is avoided by a negative factor in the ram seminal plasma. When RPP were thermally denatured, although the profile was displaced to the left, the fractions to the right exhibit high viability. Moreover, the protective effect of RPP in the incubation medium and the absence of any protective effect of BSA strongly suggest that the increase of viable cells resulted from the acquisition of adsorbed seminal plasma proteins. It also suggests that the protective effect of RPP is highly specific. Experiments with protein-free heterologous seminal plasma corroborate these results as RPP were still able to partially restore the profile when protein-free bull seminal plasma was added to the system (Fig. 5a). However, no restoration of profile was achieved by BPP when protein-free ram seminal plasma was added (Fig. 5b), proving that adsorption of plasma proteins is prevented by some factor of low molecular mass in the ram seminal plasma.

An interesting aspect of these results is the observation that although both kinds of plasma proteins promoted displacement of the profile to the right, the highest cell viability increase is obtained when isolated ram seminal plasma proteins are present. These results could be explained by a highly specific protective effect of ram seminal plasma proteins. As indicated by Courtens et al. [39], specific proteins bind regionally to specific areas of the ram sperm membrane, and this would be in agreement with previous observations about survival of ram spermatozoa [40]. The adsorption of these proteins to the shocked sperm membrane surface is prevented by some factor in the ram seminal plasma which avoids the displacement of the profile to the right, and, in consequence, leads to the loss of the protective effect. This opposite effect of ram seminal plasma on spermatozoa could be the explanation for the controversial results reported about seminal plasma effects on sperm cell characteristics [18,19,22,41,42].

The results reported here emphasize our previous observations that adsorption of seminal proteins reproduce the partition behaviour of live spermatozoa [13]. The study of the effects of seminal plasma constituents on spermatozoa may help in the formulation of better diluents for preserving spermatozoa during freezing, or storage at 4°C, room temperature, or even body temperature. Although use of bull semen is now routine and produces consistent results, this is not the case for the use of frozen ram semen which is inconsistent and usually produces unacceptably low fertility [43,44]. Cryopreservation techniques for frozen ram semen need to be improved to obtain a consistent and acceptable level of fertility. If the protective effect of ram seminal plasma proteins can be promoted at the time of insemination by changing the spermatozoa to a suitable medium, such a procedure may allow improvement of spermatozoa storage for extended periods and of cryopreservation methods for ram semen.

Experiments to characterise these surface and seminal plasma components are currently in progress.

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