SEMINAL PLASMA EFFECT ON RAM SPERMATOZOA STUDIED BY PARTITIONING IN AN AQUEOUS TWO-PHASE SYSTEM

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It has long been known that seminal plasma functions to transport and sustain motility of spermatozoa (19). Detrimental (7, 9, 10, 13) or beneficial (3) effects of seminal plasma on sperm motility and viability have been described. Likewise, seminal plasma contains factors that may influence male fertility. Comparisons of seminal plasma composition between males of different fertility have been made, (17, 18), as well as the isolation of factors from seminal plasma that facilitate or inhibit sperm capacitation or fertilization, (8, 16).

Since the adsorption of seminal plasma components could modify the spermatozoon surface, partition in aqueous two-phase systems is a useful technique to analyse seminal plasma interactions. This technique is based on the different cell surface affinity for inmiscible aqueous solutions of polymers, such as dextran and polyethylene glycol (PEG) (2). The upper phase is rich in PEG and is relatively more hydrophobic than the lower phase, which is rich in dextran. When cells are added to the system they partition between the interphase and the PEG-rich upper phase. The extent of partition is dependent on the cell surface properties.

With populations of homogeneous cells repeated phase partitioning (countercurrent distribution or CCD) is generally used to improved the resolution of the separation process. CCD is a chromatography process with one stationary (lower) phase and one mobile (upper) phase. The cell sample is partitioned in one system and the two phases are then, in a systematic way, brought into contact with fresh opposite phases. This separation method has already been used for studying mammalian sperm characteristics (5, 6, 14).

However, the long period of time necessary for phase separation may increase cell death during the run. Shorter separation procedures can be carried out by using new equipment in which centrifugation speeds up the phase separation process (CCCD, for centrifugal countercurrent distribution) (1). Thus, we have shown that CCCD in aqueous two-phase systems is a resolutive technique to reveal spermatozoa heterogeneity (20, 22, 23) and 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 (enhanced affinity for the upper PEG-rich phase) (11, 21).

The aim of this work was to characterize the effect of seminal plasma proteins on the distribution behaviour of fresh ram spermatozoa, as well as the effect of some seminal plasma fractions on ram sperm populations submitted to a freezing-thawing process.

Materials and Methods

Second ejaculates from 3 mature Rasa aragonesa rams were collected by an artificial vagina, pooled and used for each assay in order to eliminate individual differences, as described previously (20). About 1 x 10^8 cells obtained after "swim-up" procedure (12) were submitted to thermic shock as previously described (11). Sperm cell viability was assessed by fluorescent staining with carboxifluorescein diacetate and propidium iodide (15). The cells were then examined under a Nikon fluorescence microscope and the number of fluoresceine-positive (membrane-intact) spermatozoa and propidium iodide-positive (membrane-damaged) spermatozoa per 100 cells analysed was recorded. At least 200 cells were counted in duplicates for each sample.

Seminal plasma proteins and fractions were prepared as reported (11) and protein concentration was assessed by the Bradford's method (4).

A Ficoll-containing two-phase system was prepared as previously described (20, 23). The counter-current distribution machine used was one constructed by us on the basis of that invented by Akerlund (1). The apparatus contains 60 chambers arranged in a circle, allowing transfers of the upper (mobile) phases relative to the lower (stationary) phases. In this system, countercurrent distribution is performed during centrifugation by keeping the denser (bottom) phases in the outer half while the lighter (upper) phases are in the inner half part of each chamber. Since 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 countercurrent distribution includes the following: first, the phases are shaken at unit gravity to mix them thoroughly. The phases are then separated by centrifugation. After the phases have separated and while they are still rotating at full speed, the upper (inner) phases are transferred to the next chambers. After deceleration, a new cycle can be performed. To carry out CCCD experiments, a two-phase system of the above composition was prepared and mixed. In each experiment the volume of the system loaded in chambers 1-59 was the estimated amount to maintain the desired volume of the bottom phase (0.7 ml). Two cell samples (each about 1 x 10^8 cells) were loaded in chambers 0 and 30, and 29 transfers were carried out. So, two cell samples were analysed at the same time to allow direct comparison between them. Shaking and centrifugation time was 60 s. After the run, the solutions were transformed into one phase-system by 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.

The presented CCCD profiles are representative of, at least, three different experiments. Partition 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 the CCCD experiments, cells from three consecutive chambers throughout the run were pooled and stained. Results are expressed as percentage of viable cells in each sample.

Results and Discussion

To analyse seminal plasma interactions a seminal plasma-free fresh ram spermatozoa population was obtained by a "swim-up" procedure (12). Fig. 1a shows the effect of thermic shock, a known method to cause loss of viability, on the partition behaviour of fresh ram spermatozoa. A notorious displacement of the CCCD profile to the right was observed when seminal plasma proteins were added (Fig. 1b). It is very significant that the percentage of viability after the CCCD run rose to 80 % in the right hand fractions. These results suggest that the profile restoring effect resulted from the acquisition of adsorbed seminal plasma proteins by the shocked spermatozoa surface. Moreover, seminal plasma proteins would exert a viability restoring effect binding regionally to specific areas of the thermic shocked spermatozoa plasma membrane. However, the protein adsorption appears to be prevented by some factor in the ram seminal plasma. Fig. 1c shows that the addition of the protein-free seminal plasma fraction avoided the viability restoring effect and promoted displacement to the left of thermic shocked spermatozoa profile.

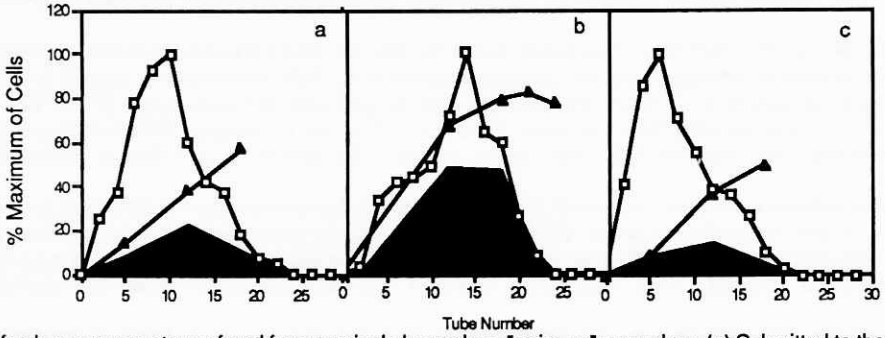

Fig. 1.- CCCD profile of fresh ram spermatozoa freed from seminal plasma by a "swim-up" procedure. (a) Submitted to thermal shock. (b) 900 µg of plasma proteins were added in the sample chamber and in the four previous chambers, and CCCD of 29 transfers was carried out. (c) The same as (b) adding to the system 1% of protein-free ram seminal plasma.. □ , % of maximun of cells. ▲ , % of viable cells. Dark area, distribution of total viable cells.

In order to find out if the addition of seminal plasma proteins could improve the cryopreservation protocols for ram semen, CCCD experiments were performed with ram semen samples diluted, frozen and thawed in the presence of >10 kDa seminal plasma proteins. The obtained results are summarized in Table 1. It is of note that viability after thawing as well as viability after CCCD run was clearly higher in the washed sample proving that protein adsorption is prevented by some factor in the ram seminal plasma. The isolation of these seminal plasma factors could help in the formulation of better cryopreservation protocols for ram semen.

Table 1. Percentages of cell distribution in the three sectors of CCCD (chambers 0-9, 10-19 and 20-29) corresponding to ram semen samples diluted, frozen and thawed in Fiser medium in the presence of 737 µl/ml >10 kDa seminal plasma proteins. C: cells in sector/ total cells; V: viable cells in sector/cells in sector; VT: viable cells in sector/total viable cells; TF: viability of fresh sample; TT: viability after thawing; TR: viability after CCCD run.

Treatment	additive	chambers 0-9			chambers 10-19			chambers 20-29			Total viability		
		C	V	VT	C	V	VT	C	V	VT	TF	TT	TR
Unwashed	>10 kDa rsp	45	1	7	48	6	55	7	32	38	72	11	5.6
Washed	>10 kDa rsp	32	3	6	60	21	73	8	46	21	71	22	17.1

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