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Revealing surface changes associated with maturation of ram spermatozoa by centrifugal counter-current distribution in an aqueous two-phase system

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Abstract

Centrifugal counter-current distribution (CCCD) in an aqueous two-phase system was used to detect changes associated with maturation of ejaculated ram spermatozoa. Spermatozoa obtained form three successive ejaculates of rams maintained in abstinence for one, two and three days were fractionated by CCCD. The results show that these ejaculates are relatively enriched in a cell population which presents a very high enhanced affinity to the lower dextran-rich phase. This cell population is not associated with loss of acrosomal integrity. In addition, it tends to disappear with longer abstinence periods, or after successive ejaculations at the same abstinence period, strongly suggesting that it is composed of immature cells. Therefore, phase partitioning can detect surface changes accompanying sperm maturation and offers a new possibility for sperm quality analysis.

1. Introduction

Thin-layer counter-current distribution (TLCCD) in aqueous two-phase systems is a useful technique for the separation of sperm cell populations based on differences in their surface properties [1–4]. However, because long periods of time are necessary for phase separation at unit gravity, TLCCD may increase cell death during the separation process, thus progressively modifying the cell partition behaviour. This experimental drawback can be avoided if phase separation during counter-current distribution is centrifugally enhanced, thus greatly shortening the time required for the cell separation process. Thus, although speeding up phase separation may result in reduced efficiency of cell separation [5,6], shortening of the process can be very advantageous for fractionating cells, such as spermatozoa, sensitive to environmental aggression. In this respect, we have recently shown that sperm cell heterogeneity revealed by centrifugal counter-current distribution (CCCD) is at least associated with different viability states of the fractionated cells [7], if cell sedimentation during the separation process is managed by using a Percoll- or a Ficoll-containing dextran–poly(ethylene glycol) (PEG) two-phase system [7,8].

On the other hand, partitioning in aqueous two-phase systems is a powerful method for tracing subtle surface changes that accompany *in vivo* processes. Thus, changes in the surface properties taking place during maturation or ageing of red blood cells and some tissue cells

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have been detected by using this method (for a review, see ref. 9). In the case of spermatozoa, maturation is a relevant physiological process that enables these cells to maintain viability and to acquire fertilizing capacity. In this respect, it is well known that important changes in the surface properties take place during maturation of mammalian sperm cells (for a review, see ref. 10) involving, among other effects, acquisition of new surface proteins [11] and changes in the exposed saccharide residues [12]. These surface changes could account for the different partitioning behaviour of spermatozoa depending on the maturation state of the cells. Thus, it has been already shown that surface changes during the maturation of rat spermatozoa can be detected by using partitioning in dextran–PEG two-phase systems [2,13].

In order to characterize further the biological basis underlying the sperm cell heterogeneity revealed by CCCD, we undertook a study of the partitioning behaviour of immature spermatozoa in such a system. For this, we used ram ejaculates obtained after short periods of abstinence, a known approach for increasing the number of immature ejaculated cells. The results obtained show that these ejaculates are relatively enriched in a cell population which presents a very high enhanced affinity to the lower dextran-rich phase. This cell population is not associated with loss of acrosomal integrity. In addition, it tends to disappear with longer abstinence periods, or after successive ejaculations at the same abstinence period, strongly suggesting that it is composed of immature cells.

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 and ionophore A23187 were obtained from Sigma (St. Louis, MO, USA). All other chemicals 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) and 10% (v/v) of "10 × buffer stock Hepes" {50 m*M* glucose, 100 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), 20 m*M* KOH}.

2.3. Preparation of cell samples

Semen from rams was collected using an artificial vagina and diluted to about $5 \cdot 10^8$ cells in a saline medium [14]. The pellet obtained after washing through 4 ml of 35% saline Percoll and 2 ml of 70% saline Percoll (5 min at 200 *g* and 15 min at 1200 *g*) was resuspended with 5 ml of saline medium and centrifuged for 5 min at 700 *g*. The pellet was resuspended with 5 ml of two-phase system medium [0.25 *M* sucrose, 0.1 m*M* EGTA, 7 m*M* sodium phosphate (pH 7.5) and 10% (v/v) of "10 × buffer stock Hepes") and centrifuged for 5 min at 700 *g*. The supernatant was removed and the pellet was resuspended with the two-phase system medium.

2.4. Centrifugal counter-current distribution

The counter-current distribution machine used and a detailed scheme of the process have already been described [7]. 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 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). The 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 such as that 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 with respect to the value obtained in the chamber containing the maximum amount of cells. In some fractions assessment of viability was carried out by fluorescent staining with carboxyfluorescein diacetate and propidium iodide, according to Harrison and Vickers [14].

2.5. Acrosome reaction (AR)

The AR was induced by ionopore A23187. A stock solution of 1 m*M* in ethanol was prepared and added to a sperm suspension of $50 \cdot 10^6$ cells/ml in the calcium-enriched incubation medium described by Shams-Borhan and Harrison [15] to a final concentration of 1 μM. Spermatozoa were then incubated at 37°C for 30 min. Assessment of the acrosomal status was performed by the triple stain technique described by Garde *et al.* [16].

3. Results

We have previously shown that CCCD in a 10.5% (w/w) Ficoll 400-containing dextran–PEG two-phase system is a resolution technique for revealing ram spermatozoa heterogeneity [7]. In this system, assessment of viability by fluorescent probes showed a different enrichment of live cells in the different fractions tested, dead spermatozoa presenting enhanced affinity to the dextran-rich phase (*i.e.*, preferentially located in the left-hand fractions of the profile). Hence this two-phase system was also used in the experiments reported here undertaken to characterize the CCCD profiles of ram spermatozoa obtained after short periods of abstinence.

Washed spermatozoa obtained from three successive ejaculates of rams maintained in abstinence for one, two and three days were fractionated by 29-transfer CCCD. Representative profiles obtained (second ejaculates) are shown in Fig. 1. In agreement with previous results [7], the percentage of live cells increased in chambers further to the right. However, we had previously

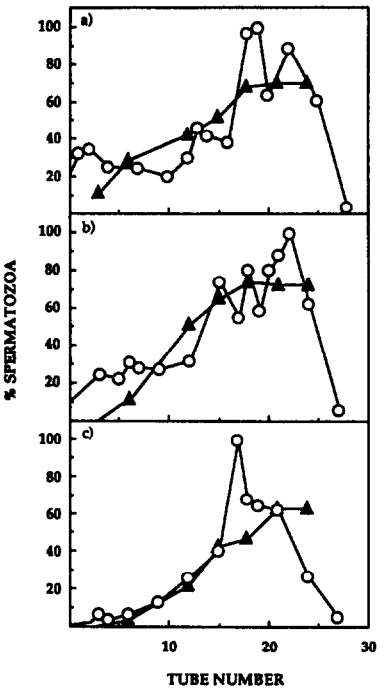

Fig. 1. Centrifugal counter-current distribution and viability of ram spermatozoa from second ejaculates after (a) 1, (b) 2 and (c) 3 days of abstinence. ○ = Percentage of the maximum of cells collected; ▲ = percentage of viable spermatozoa.

shown that loss of viability promoted by a prolonged (one month) period of abstinence accounted for a dramatic displacement to the left of CCCD profiles of ram spermatozoa [7]. In contrast, the appearance of an important number of cells fractionating in chambers 0–9 can be observed in Fig. 1. This dead cell-enriched population located in chambers 0–9 decreases as the time of abstinence increases, almost disappearing in ejaculated spermatozoa obtained after three days of abstinence (Fig. 1). This effect was large in the first and second ejaculates, whereas the percentage of cells found in these chambers was very similar in the third ejaculate obtained after any period of abstinence (Fig. 2). Thus, the number of cells found in the first nine chambers appeared to decrease as a consequence of two different effects: first, they decreased the longer the period of abstinence, and second, this cell population also tended to diminish as the cells were further submitted to maturation events

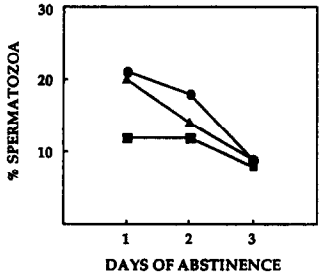

Fig. 2. Influence of the abstinence period on the percentage of cells found in chambers 0–9 with respect to the total number of cells counted in all the chambers (average of three experiments with different ejaculates) after centrifugal counter-current distribution of (●) first, (▲) second and (■) third ejaculates.

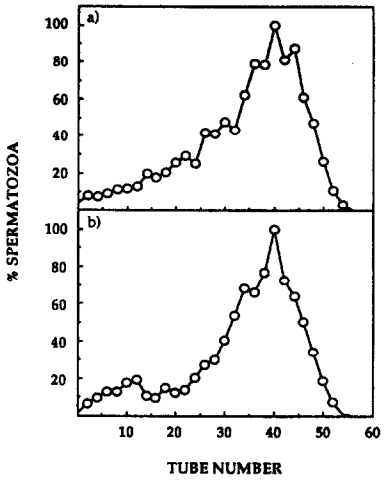

Fig. 3. Effect of the *in vitro* induction of acrosome reaction on the counter-current distribution of ram spermatozoa. Cells incubated in (a) the absence or (b) the presence of ionophore A23187. Data are expressed as a percentage of the maximum number of cells collected.

accomplished by successive ejaculations, as will be discussed later.

These results could suggest that surface changes promoted by death of hypermature (one month of abstinence) [7] or immature cells (one day of abstinence) (Figs. 1 and 2) could account for the very enhanced affinity to the dextran-rich phase. However, another possibility should be considered before drawing this conclusion. Cartwright *et al.* [2] recently described the low hydrophobicity of acrosome-reacted bovine spermatozoa located in left-hand fractions of a TLCCD in a dextran–PEG two-phase system. Further, we have also found that acrosome-reacted bovine spermatozoa show such low hydrophobicity when assayed by CCCD in the same two-phase system as used here (unpublished data). Therefore, the dead cell-enriched population that appears in chambers 0–9 after very short periods of abstinence (Figs. 1 and 2) could be composed of acrosome-reacted spermatozoa. This would imply that CCCD could be revealing the loss of acrosome by hypermature or immature cells rather than surface changes produced by loss of viability of these cells.

In order to find out whether the status of acrosome could significantly modify the CCCD behaviour of ram spermatozoa, acrosome reaction was induced *in vitro* by incubation in a calcium-rich medium in the presence of ionophore A23187 as described previously [15]. Incubation with the ionopore enhanced the number of acrosome-reacted cells (38%) with respect to controls (14%). However, the distribution profiles of treated and control ram spermatozoa did not show significant differences, even when assayed by 59-transfer CCCD (Fig. 3). These results strongly suggest that loss of acrosome does not appear to significantly affect the partitioning of ram spermatozoa, at least under the experimental conditions used here.

4. Discussion

Acquisition of adsorbed elements, such as proteins or lipids, by the sperm cell surface when spermatozoa pass through the epididymis and the vas deferens accounts for a masking effect of surface glycoproteins [17]. In addition, spermatozoa are stored in the cauda epididymis and vas deferens, when the last maturation events of sperm take place. Hence lately formed spermatozoa in short periods of abstinence could have lacked the masking effect of adsorbed elements, then presenting more saccharide residues exposed on the sperm cell surface. On the other hand, the low phosphate concentration used in the two-phase system used here could

result in a relatively charge-insensitive partitioning of cells. Therefore, the enhanced affinity to the dextran-rich phase shown by ram immature spermatozoa (chambers 0–9 in Figs. 1 and 2) could easily be interpreted as a low hydrophobicity of these immature cells which then would fractionate in the left-hand fractions of the CCCD.

These results agree with previous reports of Cartwright *et al.* [2] and Geada *et al.* [13], who found that rat spermatozoa obtained from the vas deferens (*i.e.*, more mature cells) presented a higher affinity to the PEG-rich upper phase than those obtained from the caput epididymis (*i.e.*, immature cells) when assayed either by batch experiments [13] or by TLCCD [2] in charge-insensitive or palmitate–PEG-containing two-phase systems.

On the other hand, adsorbed elements are responsible for maintaining the membrane cell stability against environmental aggression [18], thus somehow supporting viability of the cell. Consequently, immature cells could die more rapidly under experimental manipulations, further enhancing their affinity to the dextran-rich phase [7]. In addition, the dramatic enhancement of affinity to the dextran-rich phase observed in hypermature (dead) cells obtained after a very long period of abstinence (one month) [7] could be explained by the long exposure of the cells to proteolytic activity. Such proteolytic activity actually modifies sperm cell surface proteins during transit of the spermatozoa through the epididymis, as has already been described [19].

An interesting aspect of the results reported here was the observation that successive ejaculations diminish the percentage of cells fractionated in chambers 0–9. These results can be explained by cumulative maturation events undergone by preformed non-ejaculated spermatozoa (short times between ejaculations rule out the possibility of biogenesis of new cells). These cells, then kept at the cauda epididymus and/or vas deferens, would be further exposed to epididymal secretions and seminal plasma, enhancing maturation.

Experimental work in studies of the maturation of spermatozoa is usually performed by studying sperm cells isolated from different epididymal portions. However, to our knowledge, there are no methods available to discriminate populations enriched in ejaculated immature cells. The results presented here show that phase partitioning can be a useful technique for assessing such populations from ejaculates obtained after very short periods of abstinence.

In conclusion, these results emphasize the possibilities of using phase partitioning to detect surface changes accompanying cell maturation. Moreover, in the case of sperm cells, CCCD in an aqueous two-phase system can offer a new possibility for semen quality analysis, by which at least the integrity of the surface changes accounting for loss of viability [7] and maturity of cells (Figs. 1 and 2) can be assessed.

5. Acknowledgement

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