Journal of Chromatography, 617 (1993) 51–57
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6885

Sperm cell heterogeneity revealed by centrifugal counter-current distribution in an aqueous two-phase system

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(First received February 15th, 1993; revised manuscript received April 14th, 1993)

ABSTRACT

In recent years, partitioning in aqueous two-phase systems has been developed as a mild separation procedure of high selectivity for studying cell surface properties. This work was undertaken to study the surface heterogeneity of bull and ram sperm cells by using centrifugal counter-current distribution in dextran–poly(ethylene glycol) two-phase systems. The revealed heterogeneity appears to be associated with different viability states of the fractionated cells. Various strands of evidence support this conclusion: (a) assessment of viability by fluorescence probes showed a different enrichment of live cells in the different fractions tested, dead spermatozoa showing enhanced affinity to the dextran-rich phase; (b) freezing or thermic shock of sperm cells, known methods of promoting loss of viability, provoked an increase in the number of cells with enhanced affinity to this phase; (c) the same effect was observed when sperm cells were obtained from ejaculates after longer periods of abstinence. The results presented here strongly suggest that loss of semen viability results in a decreased hydrophobicity of the cell surface. Thus this may be suitable for detecting sperm surface changes and to separate spermatozoa populations enriched in some functional and/or structural surface features.

INTRODUCTION

In recent years, partitioning in aqueous two-phase systems formed from poly(ethylene glycol) (PEG) and some other polymers, such as dextran or Ficoll, have been developed as a mild, highly selective procedure for the separation of cells or subcellular particles based on differences in their surface charge or hydrophobicity [1–3]. In populations of homogeneous cells, this procedure has become very useful for analysing subtle cell surface heterogeneity. In these cases, repeated phase partitioning (or counter-current distribution, CCD) is generally used to improve the resolution of the separation process.

The general topic of sperm surface structure and properties has been thoroughly studied, because mammalian fertilization is dependent on a series of events involving surface changes of the sperm cell, such as capacitation or acrosomal reaction (for review see refs. 4–7). However, techniques that enable the study of these surface changes using whole cells are scarce. The availability of these techniques would allow further determination of the surface changes associated with functional states of the sperm cell.

In this respect, the heterogeneity of bovine sperm revealed by CCD in a dextran–PEG two-phase system has been recently described [8]. Furthermore, this separation method has also been used for assessing subtle surface changes of bull, boar and ram spermatozoa dependent on possible interactions of ejaculate plasma proteins with the sperm cells [9]. However, because long periods of time are necessary for the separation of the two rather viscous phases, a CCD run re-

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quires a few hours to achieve good resolution in separation [2,8–10]. Shorter separation procedures can be carried out by using new equipment in which centrifugation speeds up the phase separation (centrifugal counter-current distribution, CCCD). Using relatively large centrifugal fields (1000 *g*), a whole CCCD of 60 transfers can be achieved in a short time (no longer than 60 min), provided that cell sedimentation is avoided in the system.

We have recently shown that management of sedimentation in CCCD of sperm cells, in an aqueous two-phase system of dextran–PEG, can be achieved if Percoll is added to the system [11]. Therefore, we have undertaken a study of the surface heterogeneity of ejaculated sperm cells assessed by CCCD. Using this technique, cell death during the separation process could be greatly diminished, thus allowing a better assessment of the surface properties of the ejaculated sperm cells.

The results presented here describe the surface heterogeneity of bull and ram sperm cells, revealed by CCCD in two-phase systems, containing either Percoll or Ficoll. This heterogeneity appears to be somehow associated with different viability states of the fractionated cells, suggesting that loss of viability results in a decreased hydrophobicity of the cell surface.

EXPERIMENTAL

Chemicals

Percoll (colloidal PVP-coated silica, d = 1.13 g/ml) was obtained from Sigma (St. Louis, MO, USA). Dextran T500 (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, NY, USA). All other chemicals used were of analytical-reagent grade.

Two-phase system

Two different two-phase systems were employed. The Percoll-containing system consisted of 4.5% (w/v) dextran T500, 4.8% (w/v) PEG 6000, 13.6% (w/v) Percoll, 0.25 *M* sucrose, 0.1 m*M* ethylene glycol-bis(β-aminoethyl ether) (EGTA), 1 m*M* sodium phosphate (pH 7.5) and 10% (v/v) of "10 × buffer stock HEPES" [4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid] (50 m*M* glucose, 100 m*M* HEPES and 20 m*M* KOH). The Ficoll-containing system 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, 4 m*M* sodium phosphate (pH 7.5) and 10% (v/v) of "10 × buffer stock HEPES" (see above).

Preparation of cell samples

Semen from rams and bulls was collected using an artificial vagina. Frozen bull sperm samples were packaged in 0.25-ml straws. The suspending medium was the one commercially used for routine cryopreservation of bovine sperm cells. For each experiment, cells were thawed at 35°C for 21 s. Fresh bull and ram sperm samples were diluted after collection in a saline medium [13]. Spermatozoa were washed and transferred to the top phase as previously described [9,11].

Thermal shock was carried out by incubating washed cells ($50 \cdot 10^6$ cells per ml) in the saline medium described by Shams-Borhan and Harrison [14] at 37°C for 30 min and cooling them rapidly to 20°C. The suspension was centrifuged at 2500 *g* in a microfuge, and the pellet was resuspended in saline medium and transferred to the top phase as described above.

Centrifugal counter-current distribution

The CCCD machine used was one constructed by us on the basis of that invented by Åkerlund [10]. 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, CCCD 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. Because no elution or pumping of any phase takes place, the overall process consists of a circular multistep transfer of 60 upper over 60 lower batch phases. Each transfer in this centrifugally enhanced CCCD 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. A detailed scheme of the device has already been reported by Åkerlund [10].

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 was that estimated to maintain the desired volume of the bottom phase (0.7 ml). Loading of cells was carried out in chamber 0. The shaking time was 60 s. The centrifugation time was different with each system: 15 s for the Percoll-containing system and 60 s for the Ficoll one. The number of transfers performed was 59. After the run, the solutions were transformed into one phase-system by the 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 light microscope. 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, the viability was assessed out by staining with fluorescent carboxyfluorescein diacetate and propidium iodide, according to Harrison and Vickers [13].

RESULTS

It has generally been assumed that centrifugal CCCD in aqueous two-phase systems cannot be employed for analysing cell populations, owing to sedimentation of large particles in the system caused by enhanced gravity. However, we have recently shown that addition of Percoll to a two-phase system avoids this sedimentation effect with frozen bull spermatozoa [11]. Fig. 1a shows the CCCD of fresh bull spermatozoa in a Percoll-containing dextran–PEG two-phase system. A major peak and a shoulder (with a minor peak) were clearly separated.

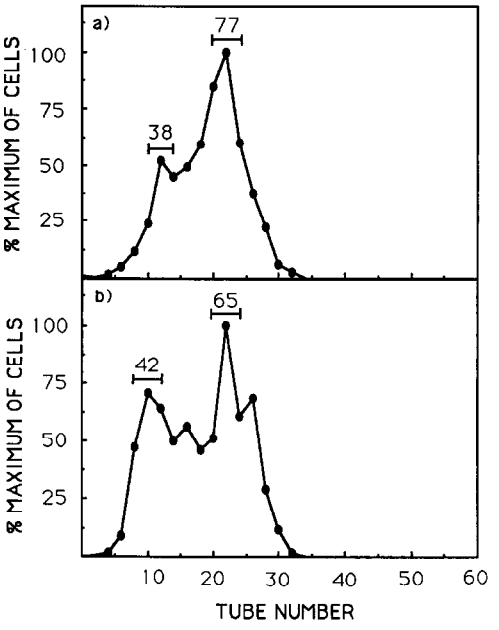

Fig. 1. Centrifugal counter-current distribution and viability of bull spermatozoa in a Percoll-containing two-phase system. Numbers on peaks show the percentage of live cells collected and mixed from marked chambers. (a) Fresh bull spermatozoa. (b) Frozen bull spermatozoa.

In order to discover which cell surface property is associated with this heterogeneity, we studied the viability of the cells in both populations. We found that the right peak (containing cells that have a greater affinity for the mobile upper PEG-rich phase) showed a partial enrichment in live cells with respect to that found in the left peak (with cells showing enhanced affinity to the stationary lower dextran-rich phase). New experiments were performed in which frozen bull spermatozoa were analysed (Fig. 1 b). The profile obtained was very similar to that previously obtained with fresh cells (Fig. 1a). Assessment of the cell viability also showed that the two separated cell populations had different enrichment in live cells. However, in the case of frozen cells the peak of the population enriched in dead cells was larger than that obtained from fresh spermatozoa.

Although addition of Percoll to the dextran–PEG two-phase system prevents cell sedimenta-

tion and two cell populations are separated (refs. 11 and 12 and Fig. 1), the resolution of the system is limited because cells only were found in the first 30 chambers. This could account for overlapping of two putative populations of live and dead cells, thus causing the observed separation of two peaks with only limited differences in viable cell enrichment. However, increasing the phosphate concentration in the system (a way of enhancing affinity of cells to the upper PEG-rich phase, thus extending CCD profiles), caused abnormal phase separation, probably owing to changes in Percoll partitioning in the two phases. Therefore, we decided to examine a new two-phase system formed from Ficoll and dextran, in which part of the PEG concentration used in the former system was substituted by Ficoll. (Dextran and Ficoll are known to promote two-phase systems with enhanced viscosity that could also prevent cell sedimentation.) After testing different polymer and salt concentrations, we found that, by using a system containing 10.5% (w/w) Ficoll 400, 5.5% (w/w) dextran T500 and 2% (w/w) PEG 6000, bull spermatozoa could be fractionated among the 50 first chambers (Figs. 2 and 3).

Using this Ficoll-containing two-phase system, fresh bull spermatozoa were separated as an asymmetric peak (Fig. 2). Again, the percentage of live cells increased in the chambers farther to the right (Fig. 2). CCCD of frozen bull spermatozoa again resulted in the separation of a higher proportion of cells on the left part of the CCCD profile, where a higher enrichment of dead cells was found (Fig. 3a). In this last case, at least an obvious peak located at chambers 4–6 and a large heterogeneity in the first 25 chambers was visible (Fig. 3a).

To obtain further evidence for the different partition behaviour of dead and live spermatozoa, frozen sperm cells were submitted to thermic shock (Fig. 3b), a method of provoking loss of viability. CCCD of these treated cells revealed

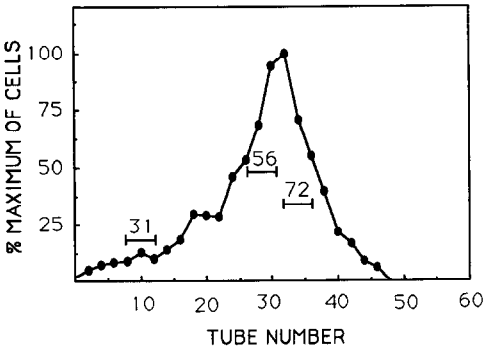

Fig. 2. Centrifugal counter-current distribution and viability of fresh bull spermatozoa in a Ficoll-containing two-phase system. Numbers along the profile show the percentage of live cells collected and mixed from marked chambers.

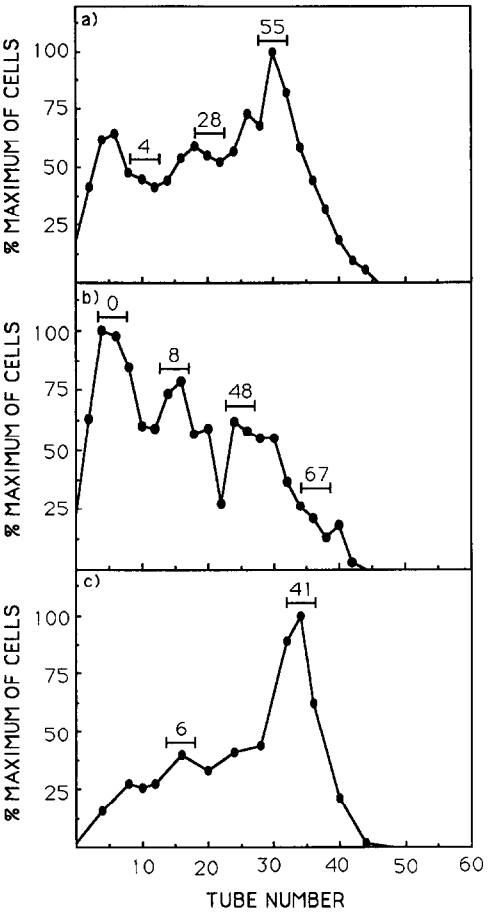

Fig. 3. Centrifugal counter-current distribution and viability of frozen bull spermatozoa in a Ficoll-containing two-phase system. Numbers along the profiles show the percentage of live cells collected and mixed from marked chambers. (a) Frozen bull spermatozoa. (b) Frozen bull spermatozoa after a thermal shock. (c) Frozen bull sperm (unwashed cells).

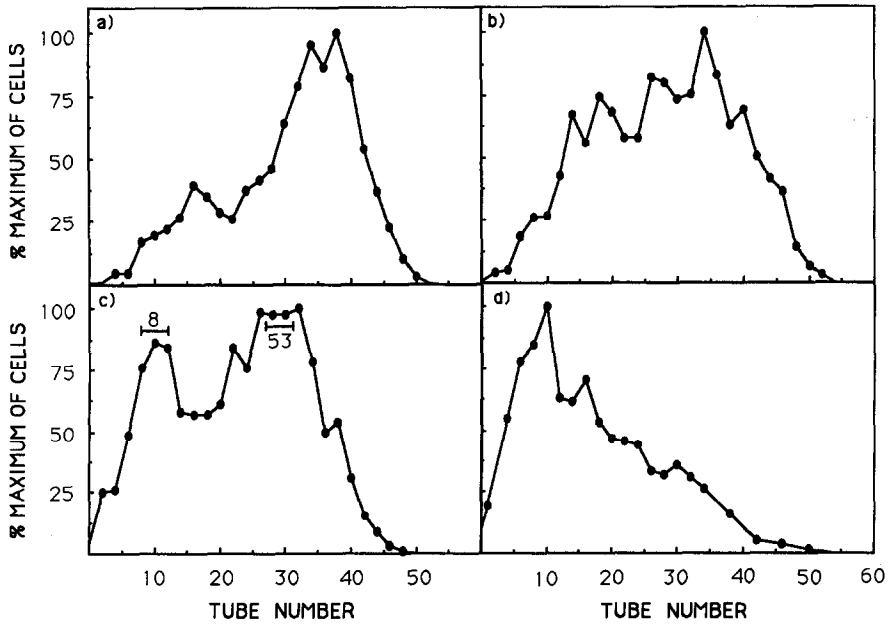

Fig. 4. Influence of the abstinence period on the centrifugal counter-current distribution of fresh ram spermatozoa in a Ficoll-containing two-phase system. Numbers on peaks show the percentage of live cells collected and mixed from marked chambers. (a) Two days; (b) six days; (c) twelve days; (d) one month.

(Fig. 3b) the existence of a majority of cells fractionating in the first 20 chambers and showing extremely low viability.

When CCCD of unwashed frozen cells (in the presence of the cryopreservative medium) was carried out (Fig. 3c), a profile similar to that found from fresh cells was obtained. However, the number of live cells present in the peak was smaller than that from fresh cells.

In order to determine if physiological changes of the viability could also affect the CCCD of sperm cells, some experiments were carried out with ram ejaculates obtained after different abstinence periods. Ram spermatozoa showed a similar CCCD profile to bull cells (Fig. 4c), the first chambers (8–12) of the set also containing a higher percentage of dead cells. As the abstinence period was longer, a higher proportion of cells was found in the first 20 fractions (Fig. 4). A dramatic change of the distribution was observed with cells after one month of abstinence. In this case, most of the cells were found in the first 20 fractions with a main peak located in chamber 8 (Fig. 4d).

DISCUSSION

Heterogeneity of bull spermatozoa was revealed by CCCD of 59 transfers using either a Percoll- (Fig. 1) or a Ficoll-containing (Figs. 2 and 3) two-phase system formed from dextran and PEG. However, the Ficoll-containing system proved to give better resolution than the Percoll one, because in the former system, cells were further fractionated: 45–50 chambers with cells in the Ficoll system compared with 30–35 in the Percoll system.

This higher resolution ability of the Ficoll system produced less sharp peaks, but offered the advantage of increased sensitivity to assess cell surface changes. In addition, ram spermatozoa also showed CCCD profiles similar to those found with bull cells when assayed in either a Ficoll- (Fig. 4) or a Percoll-containing system (data not shown).

The heterogeneity reported here appears to be somehow associated with different viability states of the fractionated cells. Some evidence supports

this conclusion. (a) Assessment of viability by fluorescent probes showed a different enrichment of live cells in the different fractions tested. Dead bull (Figs. 1–3) and ram (Fig. 4c) spermatozoa appear to show enhanced affinity for the lower dextran-rich phase (*i.e.* preferentially located in the left-hand fractions of the profile). (b) Frozen bull sperm, which are less enriched in live cells, are distributed with a higher proportion of cells on the left of profiles (Figs. 1 and 3). (c) Thermic shock dramatically moves distribution profiles to the left compared with those obtained from untreated cells (Fig. 3a and b). (d) Longer periods of abstinence again promoted displacement to left of ram spermatozoa profiles (Fig. 4).

The fact that viability figures close to 100% have never been found can be easily explained by the experimental observation that some cell death occurs after collecting fractions and during fluorescent staining for the viability assessment. In any case, it can be concluded from all the experiments presented here that viability increases as cells are further fractionated in chambers to the right, *i.e.* when they have greater affinity for the mobile PEG-rich phase.

Cartwright *et al.* [8] have also shown that bull sperm can be fractionated into two peaks by thin-layer counter-current distribution (TLCCD) in a two-phase system without centrifugation. In this case, the results obtained did not establish whether partition of cells takes place somehow on the basis of sperm viability. However, our results appear to be more conclusive in this respect. It must be taken into consideration that the long periods needed for TLCCD may increase for cell death, thus modifying progressively the cell partition behaviour during the separation process.

The phosphate concentration in the two-phase systems used in this work (1 or 4 m*M*) resulted in charge-insensitive partition [2,8]. Consequently, changes in partition behaviour are a consequence of modified hydrophobicity of the cell surface. Thus, the results obtained here strongly suggest that loss of viability results in a decreased surface hydrophobicity of dead or moribund cells, because they show an enhanced affinity for the lower dextran-rich (and therefore more hydrophilic) phase [2,3,8]. This change in the hydrophobicity could be due to partial rearrangement or the leakage of some plasma membrane components. De Leeuw *et al.* [15] have shown that cold treatment provokes a redistribution of membrane particles. However, these authors also reported that such redistribution was restored upon rewarming, as well as after freezing and thawing of bull spermatozoa [15]. However, frozen and thawed bull spermatozoa (Figs. 1 and 3) increased the proportion of cells with enhanced affinity for the lower dextran-rich phase. Therefore, these results would suggest that irreversible changes take place after freezing, perhaps owing to leakage of some membrane components.

In agreement with this interpretation could be the effect on partition observed in the presence of a cryopreservative medium, which promoted a more homogeneous distribution of sperm cells (Fig. 3c). It has been shown that a low-density fraction of the lipoprotein complex of egg yolk (a component of the cryopreservative medium) [16] is an afforded cryopreservative agent. Thus, the effect of the cryopreservative medium on partition could be interpreted as an exogenously supplied reimplantment of some lipoprotein component leaked from dead spermatozoa. However, it cannot be ruled out that the cryopreservative medium could directly modify the interface potential, thus affecting the cell partition behaviour.

Loss of viability brought about by freezing (Fig. 3a), thermic shock (Fig. 3b), or a long period of abstinence (Fig. 4d) resulted in fractionation of cells in the first 10 chambers and marked heterogeneity in the first 20–25 (this effect was not observed in the less resolving Percoll-containing two-phase system (Fig. 1b)). These observations would suggest that surface changes associated with sperm cell death are not due to a single effect. On the contrary, it appears that the longer or more pronounced the cell death-promoted effect, the more membrane damage is caused. The different of the damage would account for the observed dead cell heterogeneity described above.

Harrison *et al.* [9] have recently shown, in a precise study of the behaviour of spermatozoa

during TLCCCD, that partition varied with the number of spermatozoa in the sample. These authors suggested that PEG may exert a detergent-like effect on the sperm surface that is exacerbated in media free of protective proteins. However, we have found that CCCD behaviour of bull or ram spermatozoa does not depend on the number of cells fractionated (data not shown). This apparent discrepancy can be easily explained by the different duration of the two CCD techniques used (at least seven times longer in the thin-layer method). This produces substantially different exposure times to a putative negative effect of the phase system on cell membranes. Therefore, although it is known that speeding up phase separation can reduce the efficiency of cell partitioning [17], the shortening of time required for CCD can be advantageous for avoiding cell death, as occurs with sperm cells.

In conclusion, the evidence presented here strongly suggest that CCCD of spermatozoa discriminates cell on the basis of their different viability states. However, the large heterogeneity observed in bull and ram spermatozoa when fractionated by the Ficoll-containing system (Figs. 2 and 4) makes it reasonably likely that some other cell surface heterogeneity could also underlie the CCCD fractionation. Therefore, centrifugally enhanced CCD may be a promising technique for the separation of sperm cell populations enriched in some functional and/or structural surface features, such as capacitated cells or specific sex-chromosome-bearing populations. Alternatively, the use of *in vitro* models reproducing changes in partition detected after cryopreservation or under different physiological conditions could offer a new analytical approach to the study of sperm cell surface physiology.

ACKNOWLEDGEMENTS

This work was supported by Grant GAN 91-0950. We thank J. Folch (SIA, Zaragoza, Spain) and C. Rincón and F. Quintín (CENSIRA, Zaragoza, Spain) for help in obtaining semen samples. We thank Dr. M.V. Arruga (Facultad de Veterinaria, Zaragoza, Spain) for fluorescence microscopy facilities.

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