BIOCHEMICAL ASPECTS OF RAM SPERM VIABILITY

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SUMMARY

A quantitative radiochemical assay has been used to measure Ca^{2+} fluxes in ejaculated ram spermatozoa. Intact motile sperm are largely impermeable and accumulate only low levels of Ca^{2+} (50-100 nmol $Ca^{2+}/10^9$ sperm). Exposure of the sperm to conditions which alter the plasmalemma integrity (*e.g.*, cold shock, hypotonic media) results in a rapid and large accumulation of Ca^{2+} (1200-1500 nmol $Ca^{2+}/10^9$ sperm). The assay has potential in defining suitable procedures for the cryopreservation of ram semen.

INTRODUCTION

The largest handicap to the development of effective procedures for the preservation of ram semen by deep freezing procedures is the absence of an *in vitro* assay to quantifate fertilizing ability (Linford *et al.*, 1976). The most common test used by the semen storage industry involves microscopic examination of sperm morphology and motility (Emmens and Blackshaw, 1956). However, many methods of assessing motility are quite subjective and incapable of detecting subtle variations in motility. Furthermore, it is now clear that sperm fertilizing ability is a complex phenomenon involving several metabolic parameters in addition to co-ordinated motility (Harrison, 1977).

Our laboratory has considered the metabolic factors essential for ram sperm viability, with particular emphasis on calcium (Ca^{2+}) metabolism. Ca^{2+} plays a vital role in regulating many biological functions (Carafoli and Crompton, 1978) and has been implicated in the control of motility and capacitation of spermatozoa (Hoskins *et al.*, 1978; Saling *et al.*, 1978). Within mature ram sperm, Ca^{2+} levels are regulated by a unique dual membrane system.

The plasma membrane has a $(Ca^{2+} + Mg^{2+})$-ATPase which pumps Ca^{2+} outwards (Bradley and Forrester, 1980), whereas the mitochondrial complex accumulates Ca^{2+} in an energy-dependent process (Bradley *et al.*, 1979) establishing a low Ca^{2+} concentration in the extramitochondrial space. This condition, characteristic of functional viable sperm, requires maintenance of membrane integrity. The results presented in this

paper demonstrate how the integrity of ram sperm membranes can be assessed using a quantitative radiochemical assay which measures intrasperm Ca^{2+} fluxes.

MATERIALS AND METHODS

Semen was collected by means of an artificial vagina from fertile Romney rams, and individual samples with high numbers of motile sperm were pooled. Sperm were separated by centrifugation (1100 *g* for 10 min) at room temperature and washed by centrifugation in sucrose buffer (0.25 mol/l sucrose, 5.0 mmol/l HEPES*-Tris, pH 7.4) containing 1.0 mmol/l EDTA† -Tris. The sperm were then washed once in sucrose buffer and finally resuspended in the same buffer at a cell concentration similar to that of the original semen. When required, semen and isolated sperm were cold-shocked by pipetting the sample down the inside of an ice-cold test tube and incubating at 0°C for 10 min. To hypotonically disrupt plasma membranes, isolated sperm were washed in 10 mmol/l potassium phosphate, pH 7.4, as previously described (Stewart and Forrester, 1979).

To measure Ca^{2+} accumulation, resuspended sperm (0.1 ml) were preincubated with 1.8 ml sucrose buffer, containing 0.5 mmol/l potassium phosphate, for 1 min at 37°C, and then 0.1 ml of sucrose buffer containing 5.0 mmol/l $CaCl_2$ and 10μCi/ml of $^{45}CaCl_2$ was added. Samples (0.1 ml) were removed at appropriate intervals and added to 0.16 ml of ice-cold quenching medium (sucrose buffer containing 2.0 mmol/l EGTA‡ -Tris). The quenched samples were then filtered through glass fibre filters (Whatman, GF/C) and washed four times with 1.0 ml of sucrose buffer at 0°C. The filters were dried and placed in vials containing 10 ml scintillation fluid (Butyl PBD, 6 g; toluene, 700 ml; triton X-100, 300 ml) and radioactivity determined by liquid scintillation counting. Results are expressed as nmol Ca^{2+} accumulated/10^9 sperm. Phase contrast microscopy was employed to assess motility, and cell numbers were determined using a Spencer haemocytometer. All chemicals used were of analytical grade. Filipin was a gift from Upjohn International Inc., Kalamazoo, Michigan.

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* HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid.

† EDTA, ethylenediamine-tetra acetic acid.

‡ EGTA, ethyleneglycol-bis-(β-amino-ethylether)-N,N'- tetra acetic acid.

RESULTS AND DISCUSSION

Previous reports from our laboratory have shown that the movement of Ca^{2+} across the mitochondrial membranes of hypotonically treated ram spermatozoa can be measured using the radioactive isotope $^{45}Ca^{2+}$ (van Eerten *et al.*, 1978; Stewart and Forrester, 1979). In the assay, nonspecific binding of Ca^{2+} to membranes is effectively eliminated by the inclusion of the cation chelating compound EGTA. The same procedure has now been modified to investigate Ca^{2+} flux systems within intact ram sperm. Using the polyene antibiotic filipin (Babcock *et al.*, 1975), we have found that the Ca^{2+} permeability of the sperm plasmalemma can be modified (van Eerten *et al.*, 1979; Bradley *et al.*, 1980). Motile intact sperm obtained by gentle fractionation of ram semen accumulate very little Ca^{2+} over the first 4 min, but on exposure to filipin (0.2 mmol/l) a rapid and large accumulation of Ca^{2+} (1200-1500 nmol $Ca^{2+}/10^9$ sperm) occurs over 4 to 8 min, Fig. 1). This influx of Ca^{2+} is accompanied by an immediate loss of motility. The Ca^{2+} uptake induced by filipin represents accumulation into the mitochondrial compartment of the ram sperm (van Eerten *et al.*, 1979; Bradley *et al.*, 1979). Variations in this Ca^{2+} uptake pattern are obtained when harsh physical

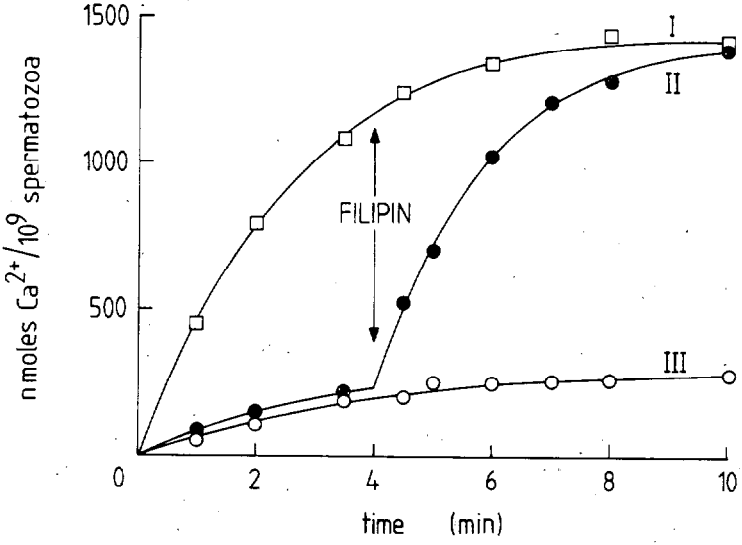

FIG. 1: *Calcium uptake in ram sperm. Sperm isolated at 4°C (line I); room temperature (line II); and room temperature following washing in hypotonic media (line III). Filipin added at 4 min to all samples.*

conditions are used during the sperm isolation procedure. Sperm washed at low temperatures (0 to 4°C) can rapidly accumulate Ca^{2+} into the mitochondrial compartment by a process which is unaffected by the subsequent addition of filipin (Fig. 1, line I). In comparison, sperm initially exposed to hypotonic buffer and then washed at 20°C have very little capacity to accumulate Ca^{2+} (Fig. 1, line III). The above experimental results can be interpreted as follows:

Line I: A high level of Ca^{2+} accumulation before exposure to plasmalemma disrupting agents indicates the sperm have a damaged plasmalemma, but with the mitochondria still metabolically intact and capable of active Ca^{2+} influx.

Line II: A low level of Ca^{2+} accumulation before plasmalemma disruption (*e.g.*, using filipin) followed by a high level of Ca^{2+} accumulation after plasmalemma disruption indicates that the sperm had intact membranes plus an integrated metabolism generating sufficient ATP to maintain the intrasperm/extracellular Ca^{2+} concentration gradient. These sperm are, in a metabolic sense, totally viable and should be capable of fertilization.

Line III: A low level of Ca^{2+} accumulation both before and after plasmalemma disruption indicates that the mitochondria of the sperm are metabolically damaged, incapable of synthesizing ATP and lacking an energy-linked Ca^{2+} transport system. These are effectively dead sperm.

We have now investigated the possibility of exploiting this procedure to measure the viability of sperm during some of the stages leading to the cryopreservation of ram semen. The outline of the experimental approach is shown in Fig. 2. A sample of ram semen was divided into the following five categories: fraction A was untreated; fraction B was obtained by subjecting the original semen to cold shock; fraction C was obtained by washing the cold-shocked semen at 20°C; fraction D was washed sperm; and fraction E the washed sperm subjected to cold shock. All five fractions were then assayed for Ca^{2+} accumulation, with 0.2 mmol/l filipin being added after 2½ min incubation in 0.25 mmol/l $^{45}CaCl_2$. The results are summarized in Table 1.

Untreated semen (fraction A) accumulated very little $^{45}Ca^{2+}$ until addition of filipin, which is consistent with the metabolic properties of viable sperm possessing membrane integrity. In comparison, cold-shocked semen (fraction B) showed an immediate,

TABLE 1: RELATIONSHIP BETWEEN MEMBRANE INTEGRITY, CALCIUM UPTAKE CAPACITY AND MOTILITY IN RAM SPERM

Fraction	*Membrane Integrity*[1]	*Ca^{2+} Uptake*[2] *(nmol $Ca^{2+}/10^9$ cells)*	*Motility (%)*
A	+	910	90
B	—	1000	2
C	—	190	2
D	+	1260	60
E	—	1280	1

[1] Demonstrated by change in Ca^{2+} uptake rate on addition of filipin.
[2] Ca^{2+} level attained after 10 min incubation.

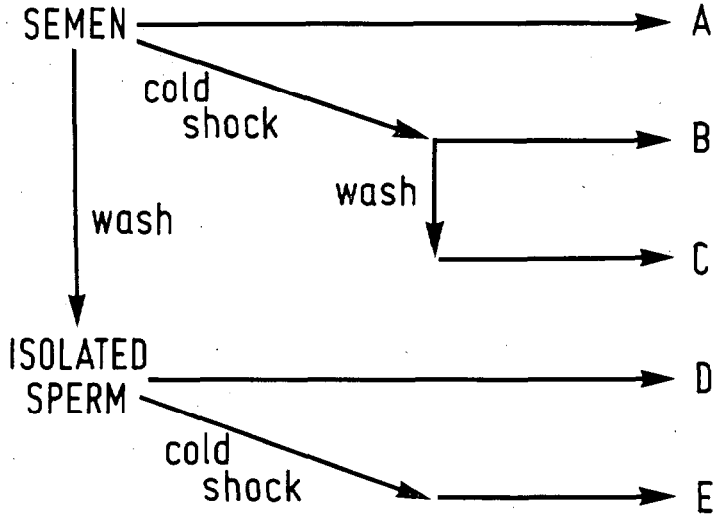

FIG. 2: *The experimental procedure for cold-shock treatment and washing of ram spermatozoa. Samples A to E were analysed for filipin sensitivity and Ca^{2+} accumulation (Table 1).*

rapid accumulation of $^{45}Ca^{2+}$ with no stimulation on the addition of filipin, indicating that the cold shock had disrupted the plasmalemma causing an influx of Ca^{2+} with associated loss of viability. The dramatic loss of motility (from 90% to 2%) supports this observation. Similar changes were also obtained with the isolated washed sperm (fractions D and E). It is interesting that an equivalent situation has been observed during the rapid chilling of bull semen (Karagiannidis, 1976). The results clearly demonstrate that cold-shock treatment of semen or isolated sperm disrupts the plasmalemma and produces an influx of Ca^{2+} which

can only be accumulated if the mitochondria are metabolically intact. Furthermore, the low levels of $^{45}Ca^{2+}$ uptake observed for fraction C (both before and after filipin treatment) suggest that once ram sperm have lost the integrity of the plasmalemma (*i.e.*, by cold shock), subsequent exposure of the cells to centrifugation at 20°C destroys energy-linked mitochondrial activities. However, it should be noted that intact, motile ram sperm can be isolated from the semen using centrifugation techniques, provided physical conditions are carefully controlled (fraction D). The reason for the difference in maximum levels of Ca^{2+} accumulated in semen and washed sperm (fractions A and D) following filipin treatment is uncertain. It may reflect the removal of some inhibitory factor present in the seminal plasma (Babcock *et al.*, 1979); however, when washed sperm were resuspended in seminal plasma there was no observable lowering of Ca^{2+} accumulation before or after filipin treatment.

It is now planned to utilize this Ca^{2+} uptake assay during our investigation of the cryopreservation of ram semen for use in artificial breeding.

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