# Biochemical events associated with Ficoll washing of ram spermatozoa

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## ABSTRACT

A semen washing technique employing Ficoll density gradient centrifugation was used to separate ram spermatozoa and seminal plasma. While this technique caused a loss of spermatozoan motility, it was shown by $Ca^{2+}$ flux measurements that these immotile ram spermatozoa still had intact plasma membranes. Furthermore, these immotile spermatozoa were shown to be resistant to cold-shock, a treatment which normally disrupts the sperm plasma membrane. Spermatozoan motility was restored by suspending the immotile spermatozoa in diluents containing egg yolk or phospholipids. Ficoll washing appeared to exert its effect on the sperm plasma membrane by an alteration in the membrane lipid components.

## INTRODUCTION

Artificial insemination in the sheep industry has been limited by the lack of effective procedures for either short or long term storage of viable ram spermatozoa. The inherent lability of ram spermatozoa to temperature and/or osmotic trauma is primarily due to a breakdown in the permeability properties of the sperm plasma membrane (Quinn *et al.*, 1969; van Eerten and Forrester, 1980a; Quinn *et al.*, 1980). The susceptibility to cold-shock is demonstrated by a failure to regain full metabolic activity and motility after the thawing of frozen ram spermatozoa.

Harrison (1976) has described an effective method for removing seminal plasma from ejaculated mammalian spermatozoa by centrifugation of semen through a Ficoll density gradient (Ficoll washing). While this method caused only minimal mechanical damage it was noted that several species of spermatozoa, in particular bull spermatozoa, rapidly lost motility after Ficoll washing. Subsequent research by the N.Z. Dairy Board, Newstead, showed that although Ficoll washing rendered bull spermotozoa immotile, the motility could be restored by the addition of an egg yolk diluent (Shannon *et al.*, 1982). Preliminary investigations at Ruakura showed that ram spermatozoa could also be reversibly inactivated using the Ficoll washing method (Tervit *et al.*, 1982).

The objectives of this study were to use a calcium assay procedure to examine the biochemical events induced by Ficoll washing of ejaculated ram spermatozoa, and to identify the egg yolk components which are involved in the restoration of motility to Ficoll inactivated spermatozoa.

## MATERIALS AND METHODS

Semen from fertile Romney rams was collected by means of an artifical vagina and divided into 2 portions. One portion was diluted (1:7) in 14G buffer and washed by centrifugation through a 7% Ficoll solution (Jansen *et al.*, 1981). These Ficoll washed spermatozoa were non-motile and are referred to as inactivated spermatozoa. The other semen portion was used for the preparation of a control sample of spermatozoa. Both inactivated and control spermatozoa were then washed by centrifugation in a sucrose buffer (van Eerten and Forrester, 1980a) and calcium uptake measured using a $Ca^{2+}$ electrode (van Eerten and Forrester, 1980b). Cold-shock treatment (van Eerten and Forrester, 1980a) consisted of running the spermatozoa down the side of an ice-cold test tube. The ability of certain reagents (egg yolk and phospholipids) to reactivate samples of inactivated spermatozoa was studied by suspending Ficoll washed spermatozoa in prewarmed reagent (50:50, v/v). The motility of reactivated samples was assessed both immediately after reagent addition and after 25 min incubation at 37°C. Spermatozoa were scored for forward motility by two independent observers using an Orthoplan phase-contrast microscope. Egg yolk solution was composed of 20% fresh egg yolk in 14G buffer. Phospholipids were added to 14G buffer (10 mg/ml), held at 0°C and sonicated (3 to 15 min) until a clear solution of liposomes was formed. The phospholipid liposomes were centrifuged at 10 000/*g* for 15 min to remove large undispersed aggregates of lipid (Quinn *et al.*, 1980). All solutions used were bubbled with oxygen-free nitrogen for at least 5 min immediately prior to use.

## RESULTS AND DISCUSSION

Sperm plasma membrane integrity can be readily determined by monitoring the changes in $Ca^{2+}$ fluxes on addition of a polyene antibiotic (van Eerten and Forrester, 1980a). In this $Ca^{2+}$ assay, sterol-containing plasma membranes are specifically modified by filipin (Babcock *et al.*, 1975), resulting in the loss of $Ca^{2+}$ -selective impermeability. By comparison, mitochondria membranes remain unaffected by filipin. The mitochondria therefore maintain metabolic integrity, including the ability to respire and actively accumulate $Ca^{2+}$. Experimentally, the large influx of calcium observed after the addition of filipin to intact ram ejaculated spermatozoa is the result of active mitochondrial uptake together with passive binding by cytosolic components. No changes in $Ca^{2+}$ fluxes occur on addition of filipin to ram spermatozoa with disrupted plasma membranes.

Calcium uptake by motile ram ejaculated spermatozoa initially reached a level of approximately approx. 600 nmol $Ca^{2+}$ bound/$10^9$ spermatozoa (Fig. 1, line A). This value represented $Ca^{2+}$ binding to the exterior of the spermatozoa. When these spermatozoa were exposed to filipin, a further large increase in $Ca^{2+}$ binding (approx. 1200 nmol $Ca^{2+}/10^9$ spermatozoa) occurred as a result of calcium binding to cytoplasmic components and accumulation in the mitochondria. This $Ca^{2+}$ uptake profile was indicative of metabolically active spermatozoa with an intact plasma membrane. Cold shock treatment of these spermatozoa resulted in a rapid maximal $Ca^{2+}$ binding (approx. 1200 nmol/$10^9$ spermatozoa) with no additional binding being induced by filipin (Fig. 1, line B). This sequence demonstrated that the plasma membranes of these spermatozoa were disrupted by cold shock.

After Ficoll washing of ram semen, <1% spermatozoa showed forward motility, however some 50-70% showed very slow flagellar movement. The $Ca^{2+}$ binding profile of inactivated spermatozoa (Fig. 1, line C, squares) was found to be virtually identical to that of control spermatozoa samples (Fig. 1, line A). By comparison, inactivated spermatozoa were quite unusual in their response to cold shock treatment. The $Ca^{2+}$ binding profile of these spermatozoa (Fig. 1, line C, triangles) showed a retention of filipin sensitivity closely resembling that of the control spermatozoa (Fig. 1, line A) and the inactivated spermatozoa (Fig. 1, line C, squares). This result indicated that after Ficoll inactivation, the plasma membranes remained intact for both control and cold shocked samples of spermatozoa. (The lower binding levels reached by the inactivated spermatozoa were probably an indirect result of Ficoll washing on the sperm surface).

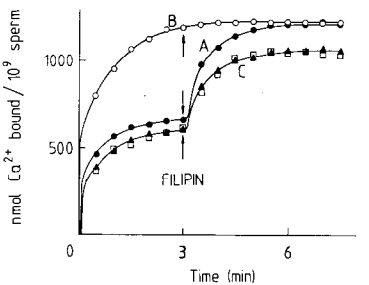


**FIG. 1** Effect of cold shock treatment on $Ca^{2+}$ binding by active and Ficoll inactivated ram spermatozoa. Line A control; Line B cold-shocked control; Line C (□) Ficoll inactivated; Line C (▲) cold-shocked Ficoll inactivated. Filipin (0.2 mM) addition is indicated by the arrows.

These results indicated that neither the mitochondria nor the structural integrity of the plasma membrane of Ficoll washed spermatozoa were perturbed by temperature change. The precise structural modifications of the plasma membrane which accompany Ficoll washing is still uncertain. However, a similar resistance to cold-shock has been observed with ram caudal spermatozoa (Jansen *et al.*, 1981). Also, it is well known that although ram caudal and ram ejaculated spermatozoa are very similar in phospholipid composition, ram ejaculated spermatozoa have a lower cholesterol content (Quinn and White, 1967). Furthermore, susceptibility to cold-shock has been correlated with a high value for both the polyunsaturated:saturated phospholipid ratio (Quinn *et al.*, 1980), and cholesterol:phospholipid ratio (Darin-Bennett and White, 1977), while cold-shock treatment of ram ejaculated spermatozoa causes a release of membrane phospholipids, principally phosphatidyl choline (Darin *et al.*, 1973). All of these reports clearly link cold-shock sensitivity with the lipid component of spermatozoa and strongly suggest that Ficoll washing may alter the plasma membrane lipid composition of ram ejaculated spermatozoa.

In agreement with studies of the N.Z. Dairy Board using bull spermatozoa, we have found the motility of inactiviated ram spermatozoa can be restored by the addition of an egg yolk diluent. In light of the $Ca^{2+}$ binding results described above, we examined the possibility that motility could be restored to Ficoll washed sperm by individual lipids. The results of our preliminary experiments are shown in Table 1. The table shows that motility was restored by several individual phospholipids after 25 min incubation, but that the best result (70% motility) was obtained

with phosphatidyl choline. A complete lipid analysis of the flagellar plasma membrane from both inactivated and control, ram ejaculated spermatozoa is currently being pursued. The effects of Ficoll washing on plasma membranes may not be simply restricted to mammalian spermatozoa since preliminary investigations using erythrocyte membranes indicated that there is a 90% decrease in membrane cholesterol content following Ficoll washing.

**TABLE 1** Reactivation of ram sperm motility by phospholipids

| Reactivation solution | % Forward motility at 0 min | at 25 min |
|---|---|---|
| Egg yolk | 40 | 90 |
| Sphingolipid | 0 | 30 |
| Phosphatidyl ethanolamine | 10 | 20 |
| Phosphatidyl serine | 10 | 30 |
| Phosphatidyl inositol | 10 | 30 |
| Phosphatidyl choline | 10 | 70 |

Our studies have shown that alterations in the structure and function of ram sperm plasma membrane follows Ficoll washing as indicated by the induction of cold-shock resistance and the ability of a single phospholipid to restore motility to the inactivated sperm. The molecular basis of this transition appears to be linked to the lipid component of the plasma membrane, possibly through a change in the phospholipid:cholesterol ratio.

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