Maturation and regulation of the motility of spermatozoa in the epididymis of the tammar wallaby (*Macropus eugenii*)

J. Clulow, R. C. Jones and R. N. Murdoch

Department of Biological Sciences, University of Newcastle, NSW, Australia 2308

Summary. Demembranated spermatozoa from the rete testis developed vigorous flagellation when reactivated with ATP, but showed no forward progression such as that seen in samples from the cauda epididymidis. The proportion of spermatozoa that were reactivated was smaller for samples from the rete testis than from the cauda epididymidis.

Studies *in vitro* of undiluted micropuncture samples from the epididymis indicated that the activity of spermatozoa is suppressed as they develop the capacity for motility. However, as spermatozoa spontaneously became activated during the collection or subsequent incubation of undiluted samples, it was concluded that the suppressive action is labile.

The activity of spermatozoa *in vitro* was examined in diluted samples from the cauda epididymidis. A concentration of 2.5 mmol extracellular calcium/l was better than lower concentrations. Diluents at pH 5.5 completely inhibited sperm motility when they contained 20 mmol lactate/l (but not glutamate) and the effect was reversed by readjusting the diluent to pH 7.4. However, lactate was not considered to suppress sperm motility *in situ*, as the plasma from the cauda epididymidis contained only 2.7 ± 0.5 mmol lactate/l. There was no effect of sodium concentration (1 and 115 mmol/l), pH (5.5 and 7.4) or amiloride (0 and 1 mmol/l) on sperm motility, indicating that motility is not dependent on the concentration of sodium above 1 mmol/l or on a sodium–proton exchange system.

The relative viscosity of plasma from the cauda epididymidis did not affect the motility of spermatozoa.

Keywords: sperm maturation; sperm activation; sperm motility; marsupial spermatozoa; tammar wallaby

Introduction

Mammalian spermatozoa acquire the capacity for motility during epididymal transit. However, studies of reactivated, demembranated spermatozoa from hamsters (Mohri & Yanagamachi, 1980), rats (Yeung, 1984) and bulls (White & Voglmayr, 1986) showed that testicular spermatozoa have the capacity for the same pattern of motility as spermatozoa from the cauda epididymidis, indicating that this aspect of sperm development may involve only changes to the plasma membrane. As the epididymal maturation of marsupial spermatozoa involves more extensive modifications to the tail than that of eutherian spermatozoa (Harding *et al.*, 1975, 1979), we compared the effect of reactivating demembranated spermatozoa from the rete testis and cauda epididymidis of the tammar.

There is considerable variation in reports of the initial activity of spermatozoa collected from the cauda epididymidis of prototherian and eutherian mammals. For example, it has been concluded

that spermatozoa are immotile in samples from rats, hamsters, guinea-pigs and elephants (R. C. Jones, 1973, 1978; Morton *et al.*, 1978; Carr *et al.*, 1985; Turner & Reich, 1985) and highly motile in samples from rabbit, ram, rhesus monkey and echidna (R. Jones, 1978; Morton *et al.*, 1978; Djakiew & Jones, 1983; Usselman & Cone, 1983; Turner & Reich, 1985). Weak motility has been described in samples from dogs (Carr *et al.*, 1985), whilst samples from bulls are reported to have moderate to strong motility (Morton *et al.*, 1978; Pholpramool *et al.*, 1985) or low motility (Cascieri *et al.*, 1976; Carr & Acott, 1984; Carr *et al.*, 1985). This report shows that sperm motility is suppressed during transit through the epididymis of tammar and that the suppression is labile.

As there are no reports on the regulation of motility of marsupial spermatozoa *in situ* (Jones, 1989), the factors that have been implicated in regulating the activity of eutherian spermatozoa were studied in tammar. These factors include extracellular sodium (Lee *et al.*, 1981; Wong *et al.*, 1981; Wong & Lee, 1985), intracellular pH (Babcock *et al.*, 1983; Acott & Carr, 1984; Carr & Acott, 1984, 1989; Carr *et al.*, 1985; Vijayaraghavan *et al.*, 1985; Lindemann & Kanous, 1989), and the viscosity (Usselman & Cone, 1983; Carr *et al.*, 1985), lactate concentration (Acott & Carr, 1984; Carr *et al.*, 1985; Turner & Reich, 1985; Carr & Acott, 1989) and calcium concentration (Morton *et al.*, 1978; Garbers & Kopf, 1980; Lee *et al.*, 1981; Tash & Means, 1983; Lindemann & Kanous, 1989) of the extracellular fluid.

Materials and Methods

The source of the animals and the micropuncture procedures were reported previously (Jones, 1987). Spermatozoa were collected from the rete testis, the distal caput epididymidis, the mid-corpus epididymidis and the distal cauda epididymidis corresponding to regions 8, 13 and 22, respectively, described by Jones *et al.* (1984).

Reactivation of demembrated spermatozoa

The method described by White & Voglmayr (1986) was used. It involved washing micropuncture samples twice in calcium-free Krebs–Ringer phosphate, centrifugation for 10 min at 200 *g* and resuspension in extraction buffer (containing 0.1% v/v Triton X-100 and 50 μmol cAMP/l) at a final concentration of 5.0×10^5 spermatozoa/ml for samples from the rete testis, or 1.2×10^6 spermatozoa/ml for samples from the cauda epididymidis. Samples (1 ml) were reactivated in transparent polystyrene dishes (Bunzl, Hendon, USA) by the addition of ATP (to make 1 mmol/l), examined with an inverted microscope (Zeiss, West Germany) at 32°C, and scored according to the rate of progressive motility (scale 0–4) and percentage of motile spermatozoa (Jones *et al.*, 1984).

Incubation of spermatozoa *in vitro*

The whole-droplet technique (Pholpramool *et al.*, 1985) was used to incubate samples at 32°C under water-saturated paraffin oil on a cavitated glass microscope slide (30 nl of undiluted sample was deposited). The overall activity of the samples was scored on a scale of 0–10: the response is referred to as scores of activity to distinguish it from scores of the rate of progressive motility of individual spermatozoa.

In one study, preparations of luminal plasma, separated from spermatozoa by centrifugation at 12 000 *g* for 15 min, were used to dilute micropuncture samples at 1:20. The effect of the concentration of extracellular calcium was examined in another experiment in which the calcium concentration was determined using a published estimate of spermatozoa (Jones, 1987) and concentration of calcium in plasma (Jones & Chulow, 1987). In this study, micropuncture samples were diluted, or not diluted, to obtain estimated calcium concentrations of: 0 (1:20 dilution with Krebs–Ringer phosphate containing 4 mmol EGTA/l), 12 $\mu\text{mol/l}$ (1:20 dilution with Krebs–Ringer phosphate), 0.6 mmol/l (undiluted samples) and 2.5 mmol/l (1:20 dilution in Hepes buffer). In another experiment, the effect of extracellular pH was examined using Mes (pH 5.5) and Hepes (pH 7.4) buffers in diluents containing a permeant (20 mmol lactate/l) and impermeant (20 mmol glutamate/l) acid (Acott & Carr, 1984). For one treatment, micropuncture samples (30 nl) were initially diluted at 1:20 with the Mes buffer, then after 1 h the samples were diluted a second time (1:4) with the Hepes buffer to change the pH of the medium.

Composition of buffers

The Krebs–Ringer phosphate (pH 6.8, 290 mosmol/kg) described by Umbreit *et al.* (1972) was used. The Hepes buffer (295 mosmol/kg) contained 40 mmol Hepes/l, 123 mmol sodium chloride/l, 2.5 mmol calcium chloride/l and

1.2 mmol magnesium sulphate/l and was adjusted to pH 7.4 with potassium hydroxide. The composition of the Mes buffer (290 mosmol/kg) was the same as the Hepes buffer except that it contained 46 mmol Mes and 119 mmol sodium chloride/l and it was adjusted to pH 5.5 with hydrochloric acid. The sodium-free buffers were prepared by replacing sodium chloride with choline chloride. When required by the design of the experiment, amiloride (a gift from Merck, Sharpe and Dohme, Granville, NSW) was added at 1 mmol/l; glutamic and lactic acids were added at 20 mmol/l and EGTA was added to make a final concentration of 4 mmol/l. All diluents contained 10 mmol glucose/l; all the chemicals used were analytical reagent grade.

Assays

Lactate concentrations in blood and epididymal plasma were measured using an enzymatic assay (Bergmeyer, 1974). The viscoelasticity of epididymal plasma was measured as the rate of descent of a steel ball (diameter 765 μ m) through a vertically orientated, glass column (926 μ m internal diameter) filled with the test fluid (Usselman & Cone, 1983).

Statistical analysis

Analyses of variance were carried out using the General Linear Models procedure of the SAS statistics programme (SAS Institute Inc., 1982). The unit of response in the analysis of variance is indicated in the Results where appropriate either as an index computed from the sum of scores made at intervals during a period of incubation (Emmens, 1947), or as the score at a specific time when the activity of samples at one time, or different times, were compared (Jones & Martin, 1965). Error values are standard errors of the mean calculated from the variance between animals, or are indicated in the Results as being calculated from the pooled estimate of error in the analysis of variance.

Results

Reactivation of demembrated spermatozoa

Spermatozoa from the rete testis and cauda epididymidis were reactivated by ATP, but the percentage of motile spermatozoa was lower for samples from the rete testis than from the cauda epididymidis ($P < 0.05$; Fig. 1). The vigour of flagellar beating was correlated positively and closely with the percentage of motile spermatozoa (scores of this response are therefore not shown), and spermatozoa from the rete testis showed no forward progression whilst spermatozoa from the cauda epididymidis did (although the forward progression was hampered by the agglutination of many spermatozoa after the addition of ATP).

Fig. 1. Reactivation of demembrated spermatozoa from the rete testis (■) and cauda epididymidis (□) after 1 mmol ATP/l was added at time 0 to samples of spermatozoa demembrated with 0.1% Triton X-100. Means from five tammaris; error bar indicates the magnitude of the pooled standard error from the analysis of variance.

Activity of undiluted micropuncture samples

Spermatozoa from the rete testis and all regions of the epididymis were immotile as they entered the micropuncture pipette during collection, which used little suction. However, samples became motile within the collection pipette or during subsequent incubation. There was an overall increase in the amount of activity developed by samples taken from successive sites along the genital ducts ($P < 0.001$ for analyses of indices calculated as the sum of individual scores over the 10-h period of incubation), but only weak activity in the proximal samples (Fig. 2). Samples from the mid-corpus were fully active 2 h earlier than samples from the cauda epididymidis, but high activity was sustained longer in the samples from the cauda epididymidis ($P < 0.05$ for mean scores at 6 vs. 1 h).

Fig. 2. Mean scores of activity (1 low, 10 high) of spermatozoa in undiluted micropuncture samples collected from the rete testis (■) and caput (▲), mid-corpus (△) and distal cauda (◻) epididymides and incubated at 32°C for 10 h. Means from four tammaris; error bar indicates the magnitude of the pooled standard error from the analysis of variance.

The suppression of sperm motility was examined further in micropuncture samples from the cauda epididymidis of an additional six tammaris. For the ten animals examined, the mean time taken to achieve maximum activity was 1.3 ± 0.4 h (Fig. 3a), but there was considerable variation between samples. Three patterns of activity were observed in micropuncture samples dispensed under oil (Fig. 3b): (i) initially high activity which continued for 8–10 h (three animals); (ii) initially low activity which declined and then recovered (four animals); and (iii) initially zero activity, then high activity which developed and continued for at least 8 h (three animals).

Role of native luminal fluids

There was no effect of dilution on the survival of spermatozoa for 12 h at 32°C when micropuncture samples from the rete testis, distal caput epididymidis and cauda epididymidis were incubated undiluted or diluted 20-fold in epididymal plasma from the cauda epididymidis. There was no effect of dilution of samples from the cauda epididymidis in luminal plasma from the rete testis.

Effects of extracellular calcium on spermatozoa from the cauda epididymidis

The activity of spermatozoa incubated *in vitro* for 8 h was dependent on the availability of extracellular calcium ($P < 0.001$ for analyses of indices calculated as the sum of individual scores over the 8 h of incubation; Fig. 4). The activity of samples fell below a mean score of 5.0 in 2 h with no calcium, 5 h with 12 µmol Ca/l, 6.5 h with 0.6 mmol Ca/l and > 8 h for 2.5 mmol Ca/l.

Fig. 3. Mean scores of activity (1 low, 10 high) of spermatozoa in undiluted micropuncture samples collected from the cauda epididymidis showing the pattern of initiation of motility during incubation *in vitro* at 32°C: (a) means $\pm$ SEM from ten tammaris; (b) patterns of motility initiation for three of the ten tammaris, nos 11 (●), 15 (△) and 16 (○).

Fig. 4. Effects of 0 mmol (●), 12 μ mol (△), 0.6 mmol (▲) or 2.5 mmol (○) extracellular calcium/l on the activity (1 low, 10 high) of spermatozoa from the cauda epididymidis during incubation at 32°C. Means from four tammaris; error bar indicates the magnitude of the pooled standard error from the analysis of variance.

Effects of intracellular and extracellular pH on spermatozoa from the cauda epididymidis

There was no effect of extracellular pH on spermatozoa diluted in acidic (pH 5.5) and/or alkaline (pH 7.4) buffer containing impermeant acid (glutamate; Fig. 5). However, a low pH in the presence of the permeant acid (lactate) caused a decline in activity of spermatozoa ($P < 0.001$) to a

mean score of 0.7 ± 0.2 after 1 h. The response was immediately reversed by adjusting the medium to pH 7.0 by a 1:4 dilution of the diluent with Hepes buffer.

Effects of extracellular sodium on spermatozoa from the cauda epididymidis

The activity of spermatozoa was not dependent on the concentration of extracellular sodium > 1 mmol/l, and a $\text{Na}^+ - \text{H}^+$ exchange system was not involved in the maintenance of motility, because (i) motility in the absence of sodium was unaffected even at low extracellular pH and (ii) amiloride failed to inhibit motility (Table 1).

Lactate and viscosity

Mean lactate concentrations were lower in fluids from the rete testis (1.58 ± 0.37 mmol/l) and cauda epididymidis (2.72 ± 0.53 mmol/l) than in blood plasma (3.93 ± 0.05 mmol/l; $P < 0.01$; means $\pm$ SEM from five tammaras).

It was much easier to draw luminal fluid from the cauda epididymidis of tammaras than from that of rats (R. C. Jones, unpublished data). Measurements of the relative viscosity of plasma from the cauda epididymidis of tammaras were only slightly higher (inverse of rate of fall of steel ball was 1.06 ± 0.26 s/3 cm; mean $\pm$ SEM from four tammaras) than the value obtained for water (< 1 s/3 cm) and much lower than the value for glycerol (130.6 ± 3.2 s/3 cm; mean $\pm$ SEM from six readings).

Discussion

Maturation of spermatozoa in the epididymis

The results obtained in this study using undiluted micropuncture samples (Fig. 2) confirm an earlier finding using diluted samples from minced duct (Jones *et al.*, 1984) that spermatozoa develop their maximum capacity for normal motility in the caput and corpus epididymidis in tammaras. The persistence of strong motility *in vitro* for several hours longer in samples from the cauda than in samples from the mid-corpus epididymidis indicates that a part of the maturation process continues after spermatozoa appear to develop their full capacity for motility.

The reactivation of demembrated spermatozoa from the rete testis by the addition of ATP (Fig. 1) demonstrates that testicular spermatozoa from tammaras are capable of vigorous flagellar beating, so that, as in the eutheria that have been studied (Mohri & Yanagamachi, 1980; Yeung, 1984; White & Voglmayr, 1986), sperm maturation in the epididymis of tammaras must involve changes to the membranes. However, as spermatozoa from the rete testis of tammaras do not develop a pattern of motility involving forward progression as in spermatozoa from the cauda epididymidis, more is clearly involved in epididymal maturation of spermatozoa in this marsupial than in the eutherians that have been studied.

Sperm quiescence and spontaneous activation

This is the first report to document the collection of immotile spermatozoa from the mammalian epididymis which then spontaneously become activated *in vitro*. Accordingly, motility may be suppressed *in situ* in tammaras as spermatozoa develop the capacity for motility. However, the quiescence is highly sensitive to perturbation, and collection by micropuncture was sufficient to initiate motility of some samples even though any contamination with non-luminal fluids would, at the most, be limited to the first 100 nl collected. The suppression of spermatozoa is thought to depend on a physiological mechanism *in situ*, since all the undiluted samples that were studied eventually became highly motile during incubation *in vitro* (Figs 2 and 3), and dilution of the

Fig. 5. Effects of pH on the activity score (1 low, 10 high) of spermatozoa from the cauda epididymidis during incubation at 32°C. Arrow indicates when spermatozoa at pH 5.5 were diluted 1:4 with Hepes buffer to raise the pH of the medium; (Δ) pH 5.5, (■) pH 5.5 and glutamate diluent, (□) pH 5.5 and lactate diluent, (●) pH 7.4. Means from four tammar; error bar indicates the magnitude of the pooled standard error from the analysis of variance.

Table 1. Mean scores of activity of spermatozoa from the cauda epididymidis of the tammar incubated for 1 min or 2 h at 32°C either undiluted or after dilution at 1:20 with Hepes (pH 7.4) or Mes (pH 5.5) buffer containing different concentrations of sodium and 0 or 1 mmol amiloride/l

Composition of diluent	pH	n*	Activity after:	
			1 min	2 h
Samples undiluted	6.3 ^b	8	2.0 ± 1.0	9.0 ± 0.4
115 mmol Na/l	7.4	8	7.1 ± 0.8	8.3 ± 0.8
115 mmol Na/l 1 mmol amiloride/l	7.4	5	9.2 ± 0.4	7.6 ± 1.7
1 mmol Na/l ^a	7.4	5	9.0 ± 0.3	7.6 ± 1.7
1 mmol Na/l ^a	5.5	3	7.3 ± 0.3	9.7 ± 0.3
<i>P</i> (mean 1 vs. mean 2-4)			***	NS

^aDilutents prepared using choline chloride instead of sodium chloride; data from Jones & Clulow (1987) were used to calculate the final concentration of sodium after the 1:20 dilution of semen.

^bFrom Jones & Clulow (1987).

*Number of tammar sampled.

****P* < 0.001.

samples in native epididymal fluid had no suppressive effect. The nature of the suppressive mechanism is not understood.

The demonstration in this study of the tenuous nature of the quiescence of spermatozoa in the epididymis of tammar questions the interpretation of studies on other mammals, which found high motility of undiluted samples from the epididymis (see Introduction).

Regulation of sperm motility *in vitro* and *in situ*

The experiments reported in this study clearly demonstrate that including lactate in a diluent of low pH reversibly suppresses the motility of tammar spermatozoa. In this respect, the tammar closely follows the bovine model (Acott & Carr, 1984; Carr & Acott, 1984, 1989) in which the

reversible loss of motility after the addition of lactate is associated with a reduction in intracellular pH. Alterations to intracellular pH may, therefore, provide a mechanism for the regulation of metabolism and motility in both eutherian and metatherian spermatozoa. However, even though the extracellular pH is acidic (pH 6.3; Jones & Clulow, 1987), the lactate concentrations are low in fluid from the cauda epididymidis of tammar, so that it is unlikely that lactate alone is the regulating factor *in situ*.

That tammar spermatozoa do not require extracellular sodium to maintain motility indicates that they differ from rat spermatozoa, which require > 50 mmol extracellular sodium/l to maintain vigorous motility and can be inactivated by amiloride (Wong *et al.*, 1981). The absence of a combined effect of extracellular protons and sodium indicates that the $\text{Na}^+ - \text{H}^+$ exchange system is probably not important for maintaining intracellular pH in tammar, as it is in the rat. The low viscosity of its epididymal fluid also differentiates tammar from rats; sperm motility *in situ* is inhibited by the high viscosity of epididymal fluid (Usselman & Cone, 1983; Carr *et al.*, 1985; Turner & Reich, 1985). It would be interesting to determine whether bovine spermatozoa, which have a similar pH/lactate response to tammar spermatozoa, are also independent of extracellular sodium for the regulation of intracellular pH.

Extracellular calcium limits the survival of spermatozoa from tammar during incubation *in vitro* and its effect is dependent on concentration (Fig. 4). Studies *in vitro* of the effects of extracellular calcium on eutherian spermatozoa have produced differing results, even for the same species (Tash & Means, 1983; Bradley & Forrester, 1985). Much of this confusion may be due to a failure to take into account the proportion of extracellular calcium that is physiologically available in an ionized form (Tash & Means, 1983; Bradley & Forrester, 1985).

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