Changes in protein composition of the luminal fluids along the epididymis of the tammar, *Macropus eugenii*

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Summary. Micropuncture samples of luminal fluid were collected from the rete testis and along the epididymis. Quantitative analyses showed that the ductuli efferentes reabsorb about half the protein leaving the testis. Considerable protein is secreted by the caput epididymidis (initial segment) and there is a net loss of protein from the corpus and cauda epididymidis.

Denatured, polyacrylamide gel electrophoresis showed that there are 5 proteins in rete testis fluid which are not present in blood (M_r of 14 700, 22 800, 24 100, 43 200 and 44 800). One of these proteins (M_r 14 700) is lost from plasma in the ductuli efferentes and 2 (M_r 43 200 and 44 800) are lost in the corpus epididymidis. Twelve proteins appear in the epididymal plasma and are not present in rete testis fluid or blood: 6 appear in the caput epididymidis (M_r 30 000, 31 000, 32 300, 17 400, 18 700 and 21 400), 3 in the corpus epididymidis (M_r 12 800, 39 800 and 90 600) and 3 in the cauda epididymidis (M_r 10 900, 56 300 and 63 000). A protein with the same molecular weight as a blood protein (149 500) accumulates in the corpus and cauda epididymidis.

None of the samples of luminal fluid contained particulate matter other than spermatozoa, indicating that the tammar is a useful animal for micropuncture studies.

Introduction

Orgebin-Crist & Jahad (1979) showed that sperm maturation in the epididymis is dependent on the synthesis and secretion of protein by the epididymal epithelium. Epididymal proteins have been implicated in various aspects of sperm maturation including their association with the sperm plasma membrane (Cameo & Blaquier, 1976; Oliphant & Singhas, 1979; Voglmayr *et al.*, 1982) and an increased ability of spermatozoa to bind to the zona pellucida (Saling, 1982; Orgebin-Crist & Fournier-Delpech, 1982) and to fertilize ova (González Echeverría *et al.*, 1984; Moore & Hartman, 1984).

Proteins are reabsorbed (Koskimies & Kormano, 1975; Djakiew & Jones, 1983) and secreted by the extratesticular ducts of mammals. The occurrence of proteins which are not present in blood or rete testis fluid have been demonstrated in luminal fluids from the epididymis of all mammals which have been studied. Using one-dimensional polyacrylamide gel electrophoresis, about 6 epididymal specific proteins were identified in the rat (Jones *et al.*, 1980; Brooks & Higgins, 1980), 6 in the rabbit (Jones *et al.*, 1981), 7 in the hamster (González Echeverría *et al.*, 1982) and 7 in the ram (Jones *et al.*, 1982). The epididymis of only one non-eutherian mammal, the echidna, has been studied (Djakiew & Jones, 1983) and there are no reports of comparable studies on a marsupial.

This paper examines the role of the epididymis of a marsupial in regulating the protein composition of the milieu in which spermatozoa are matured and stored. Jones *et al.* (1984) described the structural differentiation of the epididymis of the tammar into 3 main segments, an initial, middle and terminal segment, and showed that this segmentation corresponds to the anatomical differentiation into the caput, corpus and cauda epididymidis. Jones *et al.* (1984) concluded that the tammar may be a suitable animal for micropuncture studies of the epididymis since it would be

possible to collect micropuncture samples from the initial segment free of the contamination with stereocilia which is characteristic of samples from the other mammals which have been studied (Levine & Marsh, 1971; Koskimies & Kormano, 1975; Hinton *et al.*, 1980; Jenkins *et al.*, 1980; Djakiew & Jones, 1983; Turner, 1984).

Materials and Methods

Animals. Tammar wallabies were obtained from a colony bred at 'Gungahlin', Canberra, A.C.T., from stock originally collected from Kangaroo Island, South Australia.

Collection of micropuncture samples. The preparation of glass micropipettes and the handling of samples of luminal fluid were described in an earlier report (Djakiew & Jones, 1983). Animals were anaesthetized by an intravenous injection of 5-sec-butyl-5-ethyl-thiobarbituric acid, sodium salt (Inactin: Byk Gulden Pharmaceuticals, Konstanz, West Germany), about 50 mg/kg. They were supported on a metal heater and their body temperature was monitored using a rectal probe and maintained at 36°C. The trachea, a jugular vein and femoral artery were cannulated immediately after anaesthesia. The jugular vein was infused (Model 341A syringe pump; Sage Instruments, Cambridge, MA, U.S.A.) with heparinized saline (9 g NaCl/l, 0.24% w/v heparin) at a rate of 8 ml/h and haematocrits were prepared at about hourly intervals to monitor blood volume. The cannula in the femoral artery was connected to a mercury U-tube manometer to monitor blood pressure. For the first 5 animals which were studied the mean $\pm$ s.e.m. haematocrit and blood pressure were initially $45.0 \pm 2.9\%$ and 123.6 ± 4.4 mmHg respectively, and remained fairly constant during the experimental period. The values were much the same in the remaining animals.

The left testis and epididymis were freed from the scrotum and prepared for micropuncture by supporting them in a Perspex cup heated to 32°C by water circulating through channels in the base of the cup. The exposed organs were covered with water-saturated paraffin oil and the oil was continuously dripped onto the micropuncture site.

Micropuncture samples were collected from the rete testis, proximal and distal caput, distal corpus and middle and distal cauda epididymidis. The sample sites in the epididymis correspond to regions 1, 8, 18, 20 and 22 in Fig. 1a, b of Jones *et al.* (1984).

The micropuncture and blood samples were centrifuged in constant-bore tubing for 15 min at 12 000 *g* in a micro-haematocrit centrifuge (Hawksley, Lancing, U.K.) to separate cells from plasma. The percentage volumes of sperm and erythrocytes were estimated as the ratio of length of tubing occupied by the cells: total length occupied by the sample. The concentrations of spermatozoa in samples of rete testis fluid and caudal epididymal fluid were also counted with a haemocytometer (6 animals).

The volume of the sample was measured by loading it into a 1 μ l Microcap (Drummond Scientific Co., Broomall, PA, U.S.A.) and measuring its length with a stage micrometer under a dissecting microscope. The fluid was then dispensed into 20 μ l 2.9% (w/v) sodium citrate containing 0.5% (v/v) Triton-X100, mixed with a vortex mixer and loaded into a haemocytometer.

Analysis of proteins. Protein determinations and electrophoreses were carried out as described by Djakiew & Jones (1983). Gels of samples from blood and fluid from the rete testis and cauda epididymidis were loaded with 10 μ g protein per well and stained with Coomassie Brilliant blue R250 (Sigma, St Louis, MO, U.S.A.). Gels of blood and all the micropuncture sites were loaded with 0.5 μ g protein per well and stained with silver reagent (Merrill *et al.*, 1981). Gels stained with Coomassie blue were scanned with a densitometer (R & D Quick Scan densitometer: Helena Laboratories, Beaumont, TX, U.S.A.) and gels stained with silver reagent were photographed. Estimates of apparent molecular weight (M_r) were determined using molecular weight calibration proteins (Pharmacia, Uppsala, Sweden) as standards in graphs of relative mobility of protein versus log of molecular weight.

Analysis of data. Three gels were prepared per sample site per animal for each of the stains. The mean molecular weights for these 3 gels were used to calculate the variance between animals which was used to determine the standard errors shown in the 'Results'.

Results

All of the micropuncture samples were free from contamination with cellular material other than sperm. Mean estimates of spermatozoa and fluid absorption are shown in Table 1. The mean $\pm$ s.e.m. concentration of spermatozoa in rete testis fluid was $59.3 \pm 12.1 \times 10^3$ /ml. Spermatozoa show that the ductuli efferentes reabsorb most of the fluid leaving the testis. The caput epididymidis reabsorbs most of the remaining fluid (10% of the fluid leaving the testis), and the corpus epididymidis reabsorbs about a quarter of the fluid that enters it. At the distal end of the cauda epididymidis the concentration of spermatozoa was $6793 \pm 486 \times 10^3$ /ml.

Quantitative determinations of protein (Table 1) showed that its concentration in rete testis fluid is about 1% of the concentration in blood. In the ductuli efferentes there is a net loss of about

Table 1. Concentration of protein in blood and concentration of spermatozoa and proteins in luminal fluids collected by micropuncture from the rete testis and epididymis

Sample site†	Spermatocrits (%)‡	Absorption of fluid (%)§	Protein conc. (mg/ml)¶
Rete testis	1.5 ± 0.2	—	0.9 ± 0.4
Epididymis			
Proximal caput (1)	11.3 ± 2.5***	87	3.0 ± 0.5L***
Distal caput (8)	50.8 ± 3.4***	78	43.4 ± 7.7G***
Distal corpus (18)	69.9 ± 4.5**	27	32.2 ± 7.0L*
Middle cauda (20)	66.9 ± 1.6	0	18.2 ± 2.3L**
Distal cauda (22)	61.3 ± 2.6	-9	15.6 ± 2.2
Blood	—	—	71.6 ± 3.7

Values are mean ± s.e.m. from 6 animals.

†Numbers in parentheses correspond to regions shown in Fig. 1 of Jones *et al.* (1984).

‡Asterisks indicate that value is statistically different from mean for nearest proximal site: ** $P < 0.01$ and *** $P < 0.001$.

§Expressed as the percentage absorbed between the site under consideration and the site proximal to it.

¶A net loss (L) or gain (G) of protein from the luminal plasma between the site indicated and the site proximal to it is indicated; * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

half the protein leaving the testis, there is a considerable increase in protein concentration in the caput epididymidis and there is a net loss of protein from the luminal plasma in the corpus and cauda epididymidis.

Figs 1 and 2 show tracings of a Coomassie blue-stained gel and a photograph of a silver-stained gel, respectively. Comparison of these gels shows that the resolution was greatest in the gels stained with silver, but the pale bands corresponding to molecular weights of 30 000, 31 300 and 32 300 in the cauda epididymidis indicate that some of the silver staining may not be quantitative. It was interpreted that rete testis fluid formed 5 bands which were not present in blood (corresponding to M_r values of 14 700, 22 800, 24 100, 43 200 and 44 800). Some of the proteins were removed from the plasma or modified during passage through the sperm ducts: this occurred in the ductuli efferentes for 1 band (M_r 14 700) and the corpus epididymidis for 2 other bands (M_r 43 200 and 44 800).

Twelve bands were present in gels of epididymal fluids which were not present in samples from blood or rete testis fluid. It was interpreted that 6 of these proteins appeared in the caput epididymidis (M_r 30 000, 31 300, 32 300, 17 400, 18 700 and 21 400), 3 in the corpus epididymidis (M_r 12 800, 39 800 and 90 600) and 3 in the cauda epididymidis (M_r 10 900, 56 300 and 63 000). The band corresponding to a protein of M_r 10 900 was present in at least 2 gels per animal in the gels stained with Coomassie blue but was only a weak band (in at least one gel/animal) in the gels stained with silver reagent. One protein which corresponded to a blood protein (M_r 149 500) formed a band in samples from the distal part of the corpus epididymidis and a very dark band in samples from the cauda epididymidis.

Discussion

Technical problems of obtaining uncontaminated samples of luminal fluids from the initial segments of the ductus epididymidis of the rat (Zones 1 and 2; Reid & Cleland, 1957; Fawcett & Hoffer, 1979) have meant that micropuncture samples of luminal fluid have not been collected between the rete testis and regions of the ductus epididymidis distal to the initial segments (see

Fig. 1. Densitometer tracings of denatured linear gradient polyacrylamide gels of blood plasma and micropuncture samples from the rete testis (RTF) and cauda epididymidis (CEF) of tammars. Gels were loaded with 10 g protein and stained with Coomassie blue.

Fig. 2. Photograph of denatured linear-gradient polyacrylamide gel of blood plasma (B) and micropuncture samples of the rete testis (RT) and epididymis of the tammar. pH, proximal caput; dH distal caput; dB, distal corpus; mT, middle cauda; dT, distal cauda. Gels were loaded with 0.5 µg protein. Silver stain. Numerical values are $M_r \times 10^{-3}$.

'Introduction'). Consequently, the functions of the ductuli efferentes and the initial segments have been confounded leading to misinterpretation of data. Therefore, this is the first report on a therian mammal clarifying the reabsorptive role of the ductuli efferentes and distinguishing it from the secretory role of the initial segment. The initial segment of the tammar (caput epididymidis) seems to secrete most of the protein secreted by the epididymis.

Collection and analysis of micropuncture samples of luminal fluids from the epididymis provides information on quantitative changes in protein which are not provided by other methods. However, caution is required in interpreting some of the electrophoretic data. For example, when considering the apparent reabsorption of proteins with M_r values of 24 100 and 22 800 between the proximal and distal ends of the caput epididymidis, it should be remembered that gels were loaded with 0.5 μ g protein from each sample. Since 3 times the concentration of protein was present in samples from the distal compared to the proximal end of the caput epididymidis, the two proteins under consideration would be diluted 3-fold at the distal end even if there was no reabsorption. Also, when bands corresponding to the same molecular weight occur in samples from successive segments of the epididymis (such as those with M_r 30 000, 31 300 and 32 300), microperfusion studies of the second segment are necessary to confirm whether new protein is actually added to the plasma in the second segment.

The proteins accumulated in the epididymis of the tammar are within the same range of molecular weights as the epididymal proteins secreted by other mammals which have been studied (see 'Introduction'). The larger number of proteins identified in the tammar than in the other mammals is not considered to be significant. A reason for this proposal is because gels stained with silver reagent were of higher resolution than gels stained with Coomassie blue so that, for example, bands corresponding to proteins of M_r 30 000 to 32 300 were only identified as 2 bands in gels stained with Coomassie blue. Also, we identified proteins in lightly staining bands such as those with an M_r value of 10 900 and 21 400. Furthermore, these studies have determined only the number of proteins appearing in epididymal plasma which are not in blood or rete testis fluid and so do not distinguish whether they originate from the duct mucosa or from spermatozoa.

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