Characteristics of semen collected from the cauda epididymidis of conscious rams

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Summary. A technique which permits collection of the total output of spermatozoa and fluid from the cauda epididymidis of conscious rams is described. The volume of epididymal semen collected varied in an approximately inverse proportion with the frequency of collection, but there was little variation in the concentration of spermatozoa. Chemical analyses showed that cell-free cauda epididymidis fluid contained lactic acid at various concentrations, only traces of glucose but relatively high amounts of phospholipid, pregnenolone, androgen-binding protein and 5 α -dihydrotestosterone (5 α -DHT).

Testicular spermatozoa maintained their metabolic activity and cellular integrity when stored in cauda epididymidis fluid *in vitro* at low temperature. After exposure to cauda epididymidis fluid for 14 days, testicular spermatozoa were predominantly glycolytic in their mode of glucose dissimilation and in this respect they resembled ejaculated spermatozoa.

Introduction

The caudal portion of the epididymis serves as a storage depot where mature spermatozoa are kept for various lengths of time, depending on the frequency of ejaculation. Conditions in the cauda epididymidis are obviously especially conducive to the survival of spermatozoa. In the rabbit, for example, the fertilizing ability of such spermatozoa is unaffected until they are 28 days old (Tesh & Glover, 1969). Understanding of these conditions in the cauda is obviously of paramount importance in the development of methods for the regulation of conception.

White, Larsen & Wales (1959) collected semen from the cauda epididymidis of rams through the fistulated vas deferens, but this approach invariably caused losses of semen and increased the risk of bacterial contamination. An alternative method was subsequently introduced by Amann, Hokanson & Almquist (1963) who obtained epididymal semen from bulls through the catheterized vas deferens. The problems with this preparation have already been described (Sexton, Amann & Flipse, 1971; Voglmayr & Amann, 1973). This technique has now been modified and extended to the ram to permit collections of the total output of spermatozoa and fluid from the cauda epididymidis.

Materials and Methods

Animals

Five entire rams (one Blackface, one Dorset and three Merinos; 3–6 years old) were housed in a barn where they were exposed to ambient temperature (–5°C to 15°C) and 12 h light/day for at least 6 weeks before they were used. The animals were maintained on an unrestricted diet of hay, supple-

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mented with 500 g of a mixture of oats and molasses per day. They were kept unrestrained except for the first 7 days after surgery when they were made to stand in metabolism cages. Shortly before surgery, ejaculated semen was collected twice from each ram by electrical stimulation (Blackshaw, 1954). With the exception of one ram, whose ejaculates contained a high percentage of immotile spermatozoa, the sperm concentrations, initial motility and percentage of abnormal spermatozoa were in the normal range for ram semen (Mattner & Voglmayr, 1962).

Collection and preparation of semen from the cauda epididymidis

The catheter used for the collection of epididymal semen was made of seamless polyethylene tubing (i.d. 0.58 mm, o.d. 0.96 mm, length 16 cm: Clay Adams). Except for a 2.5 cm-portion, the catheter was supported by Silastic tubing (i.d. 0.76 mm, o.d. 1.65 mm: Dow Corning) which was swollen in toluene before use. After the toluene had evaporated, the Silastic tubing formed a tight sleeve around the catheter. A cuff (5 mm diam.) of Silastic adhesive (Silicone type A: Dow Corning) was placed at the proximal end of the sleeve, 2.5 cm from the tip of the catheter. The catheter was then reflected approximately 4 cm from the cuff to form a smooth loop filled with Silastic adhesive (Plate 1).

Under general anaesthesia (Fluothane: I.C.I.) and aseptic precautions the vas deferens was exposed through a 3 cm-long incision near the midline on the anterior aspect of the neck of the scrotum and through the parietal tunica vaginalis immediately beneath the cutaneous incision. A 1.5 cm-long portion of the vas was then carefully dissected out of its sheath without damaging the blood vessels. The sterilized catheter (Tego 103G: Goldschmidt A.G.) was inserted into the proximal end of the transected vas deferens which was secured to the cuff of the catheter by 4–0 silk sutures. After placing the loop of the catheter between the spermatic cord and the internal spermatic fascia, the reflected part of the catheter was exteriorized through the incision on the anterior scrotum. The free end of the catheter was secured in the screw-cap of a 2 ml-capacity receptacle by placing a cuff of Silastic tubing (i.d. 1.02 mm, o.d. 2.16 mm, length 1 cm), swollen in toluene, around the catheter. The screw cap was attached to a hollow elbow of Silastic adhesive on Dacron mesh (4 × 5 cm) which was affixed to the scrotum by heavy Vetafil (Bengen) sutures (Voglmayr, Kavanaugh, Griel & Amann, 1972). The orifice of the catheter was occluded between collections by means of a modified Micro-gator clip (No. 34: Mueller, Cleveland).

Collections were made by palpating the cauda epididymidis for 10 min. In some instances, electrical stimulation was also used (Blackshaw, 1954). The epididymal semen was diluted with 2–3 volumes of 0.9% (w/v) NaCl solution. A small aliquot of the diluted semen was removed to assess the motility of the spermatozoa on a warm microscope stage and to determine the sperm concentration in a haemocytometer (Mattner & Voglmayr, 1962). The spermatozoa were separated from the fluid within 20 min of collection by centrifugation in the cold (2°C) at 2000 g for 20 min and the cell-free fluid was stored at –20°C until used.

Storage and incubation of testicular spermatozoa

After semen had been collected for several weeks, the contralateral rete testis of 3 rams was catheterized to obtain testicular spermatozoa by the procedure described previously (Voglmayr, Scott, Setchell & Waites, 1967). The testicular sperm suspensions were concentrated about 10 times by centrifugation at 200 g for 8 min in a sterile, graduated centrifuge tube of 12 ml capacity. An aliquot of the concentrated sperm suspension was removed to assess the metabolic activity of 'freshly collected' testicular spermatozoa. The remaining sperm suspension (0.5 ml) was mixed with an equal volume of either 0.9% NaCl or cell-free fluid from the cauda epididymidis. The sperm suspensions were cooled over a 2-h period and stored under air at 2–4°C. On Days 4, 8 and 12 of storage, 0.2 ml saline or epididymal fluid was added to the tubes as appropriate. After 14 days storage the sperm samples were equilibrated to room temperature. A small aliquot was used for the preparation of smears stained with nigrosin-eosin at 30°C for 5 min (Hancock, 1952). The percentage of dead and morphologically abnormal spermatozoa was determined from at least 500 counts/slide. The sperm-

Magnified view of the catheter used for collection of semen from the cauda epididymidis of the ram. PC: polyethylene catheter; C: Silastic bulb; S: Silastic sleeve; L: loop filled with Silastic tubing (2.5 mm).

atozoa were washed twice (Voglmayr, Murdoch & White, 1970) and then transferred into 7 ml-capacity Warburg flasks. The flasks contained 0.05 ml 20% (w/v) KOH on filter paper in the centre well and 0.5 μCi [^{14}C]glucose (sp. act. 0.1 $\mu\text{Ci}/\mu\text{mol}$: New England Nuclear) in the side-arm. After equilibration for 10 min the substrate was tipped into the sperm suspension and the reaction mixtures were incubated at 37°C for 2 h in a Gilson differential respirometer adjusted to a shaking rate of 60 cycles/min.

Analytical procedures

Oxygen uptake was measured by standard manometry at 30-min intervals. The glucose utilized and glucose oxidized or converted to lactic acid were calculated from ^{14}C radioactivity in KOH in the centre well or from radioassays and enzymatic estimations of the deproteinized suspending medium before and after incubation (Voglmayr *et al.*, 1970).

Protein was measured with Folin phenol (Lowry, Rosebrough, Farr & Randall, 1951) and glucose by the glucose oxidase method (Glucostat; Worthington Biochemical Company). Lactic acid was determined by the enzymatic method described by Barker & Britton (1957) and free inositol by the biological assay method of Campling & Nixon (1954). The concentrations of testosterone, androstenedione and dihydrotestosterone (5 α -DHT) were determined by radioimmunoassay (Saksena, Lau & Chang, 1976) and pregnenolone by the procedure described by Purvis, Brenner, Landgren, Cekan & Diczfalusy (1975). Androgen-binding protein was measured by the steady polyacrylamide gel electrophoresis technique of Ritzén, French, Weddington, Nayfeh & Hansson (1974). The fluid from the cauda epididymidis was extracted for lipid with 50 volumes of chloroform-methanol (2:1 v/v). The crude lipid extracts were purified as described by Scott, Voglmayr & Setchell (1967) and the phospholipid concentration was calculated from the phospholipid phosphorus determined by the method of Fiske & Subba Row (1925; lipid = $P \times 25$).

Statistical analysis

The results in Table 1 were subjected to analysis of variance. The significance between treatments was assessed by *t* test using the interaction mean square from the analysis of variance to determine the standard error (S.E.M.) and the degree of freedom associated with it.

Results

Composition of semen from the cauda epididymidis

The mean volume of epididymal semen obtained from a single collection was 0.86 ± 0.073 (S.E.M.) ml (range 0.14–2.05) and it varied in an approximately inverse proportion with the frequency of collection (Text-fig. 1). There was little variation in the concentration of spermatozoa ($5.08 \pm 0.31 \times 10^9$ cells/ml) irrespective of the frequency of collection.

Inositol was present in cell-free epididymal fluid at a concentration of 179 ± 3.96 $\mu\text{g}/\text{ml}$. The fluid contained only traces of glucose but various amounts of lactic acid. In 4 rams, the mean concentration of lactic acid ranged from 8 to 101 $\mu\text{g}/\text{ml}$ (mean of 5 replicates per ram), whereas in the fifth ram only trace amounts of lactic acid were detectable. In one ram, however, the lactic acid concentration in 2 of the 5 replicate samples exceeded 160 $\mu\text{g}/\text{ml}$. Smears prepared with nigrosin-eosin showed that almost all of the spermatozoa from this animal were stained and tailless.

The mean concentration of total protein in the epididymal fluid was 19.3 ± 1.61 mg/ml. There were 79.9 ± 18.7 pmol androgen-binding protein/ml fluid for 2 of the rams examined. The mean concentration of pregnenolone in epididymal fluid was 9.91 ± 2.043 ng/ml. In contrast, the concentration of pregnenolone in rete testis fluid of 3 rams used in the present study plus 2 other rams was only 0.85 ± 0.144 ng/ml and that of peripheral blood plasma was 0.71 ± 0.123 ng/ml, i.e. <10% the concentration in the cauda epididymidis. The mean levels of testosterone, Δ^4 -androstenedione

Text-fig 1. Volume and sperm concentration of ram semen from the cauda epididymidis obtained through the catheterized vas deferens. Symbols denote single observations from each of 3 rams. Note the relatively large volumes obtained at the first collection after a prolonged interval between collections, see e.g. Ram 2 (○) on Day 11 and Ram 3 (△) on Day 27–32. Epididymal semen from two other rams (not shown) was obtained for 103 and 136 days, respectively.

and 5 α -DHT in the fluid from the cauda epididymidis were 2.58 ± 0.86 ng/ml, 1.30 ± 0.54 ng/ml and 16.93 ± 4.97 ng/ml, respectively. In the cauda epididymidis the concentration of total phospholipid was 1.34 ± 0.127 mg/ml, compared with only 0.042 ± 0.005 mg/ml in the rete testis fluid.

Effect of fluid from the cauda epididymidis on sperm survival

Testicular spermatozoa stored in 0.9% NaCl were markedly different, in morphology and metabolism, from testicular spermatozoa stored in epididymal fluid. As expected, the fluid from the cauda epididymidis had a beneficial effect on the metabolic activity and cellular integrity of the spermatozoa. The nigrosin–eosin mixture stained all of the testicular spermatozoa after storage with saline but only about 10% of the spermatozoa stored in epididymal fluid. The stained spermatozoa invariably displayed a disintegrating cytoplasmic droplet, which apparently lacked a cohesive outer membrane, but there was no change in the position of the cytoplasmic droplet or any significant development of motility in the spermatozoa under either condition of storage.

The oxygen uptake and utilization of glucose by testicular spermatozoa was much greater after storage in epididymal fluid than after storage in saline (Table 1). The glucose uptake of testicular spermatozoa exposed to the epididymal fluid tended to be higher than that of freshly collected testicular spermatozoa, but the relatively high coefficient of variation rendered these differences statistically insignificant. While the % of utilized glucose converted to CO₂ was similar for freshly collected testicular spermatozoa and spermatozoa stored in epididymal fluid, the latter accumulated a much greater proportion of the utilized glucose as extracellular lactic acid than the former. Thus, testicular spermatozoa after exposure to fluid from the cauda epididymidis were more dependent on exogenous glucose than were freshly collected testicular spermatozoa.

Discussion

The procedure which we have described for the collection of semen from the cauda epididymidis of conscious rams has greatly facilitated studies of the function of the epididymis. The catheters

Table 1. Aerobic metabolism of [$U\text{-}^{14}\text{C}$]glucose by washed testicular spermatozoa of the ram after storage in 0.9% NaCl or cauda epididymidis fluid for 14 days

Treatment	Oxygen uptake (μl)	Glucose utilized (μmol)	Glucose oxidized (μmol)	Lactic acid accumulated	
				(μmol)	% of glucose uptake
(A) Freshly collected	30.0	0.35	0.063	0.12	34
(B) After storage in 0.9% NaCl	19.4	0.11	0.009	0.06	54
(C) After storage in epididymal fluid	35.0*	0.50*	0.102*	0.42**	84
Standard error	3.88	0.061	0.0198	0.0101	

Values are / 10^8 spermatozoa and represent the means of 5 replicates.

* Different from B; $P < 0.05$.

** Different from A and B; $P < 0.05$.

remained patent for up to 136 days and abundant quantities of epididymal semen were collected under physiological conditions. The occlusion of the catheter between collections prevented losses of spermatozoa and fluid and eliminated the risks of bacterial contamination inherent in fistulated preparations.

The amount of epididymal semen (0.86 ml) that can be obtained from a ram in a single day should be compared with the daily volume of up to 50 ml fluid that transports spermatozoa out of the testis (Voglmayr, Waites & Setchell, 1966) and the discrepancy between these volumes is indicative of the resorptive capacity of the epididymis. This selective fluid resorption also explains the marked increase in the concentration of spermatozoa as they are transported through the epididymal duct (Crabo & Gustafsson, 1964). Comparisons with other rams showed that spermatozoa in the cauda epididymidis were about 75 times more concentrated than in rete testis fluid (Voglmayr *et al.*, 1966; Setchell, Voglmayr & Hinks, 1971).

The concentration of inositol in the cauda epididymidis is about 10% of the level normally found in rete testis fluid (Setchell, Dawson & White, 1968; Setchell *et al.*, 1971). Since the output of rete testis fluid per unit time (>120 mg/ram/day) exceeds many times the amount of fluid that emerges from the vas deferens (0.2 mg/ram/day), large quantities of inositol must be either resorbed or utilized by the epididymis. Differences of similar magnitude have already been observed in the bull but, although inositol is known to be utilized by epididymal tissue for synthesis of inositol-containing phospholipids, the significance of inositol uptake in such large quantities is obscure (Voglmayr & Amann, 1973).

The origin of the lactic acid occurring in cauda epididymidis fluid is uncertain, but it may be derived from blood glucose. Allen & Slater (1961) have demonstrated that lactic acid dehydrogenase is located in the apical portion of the epithelial cells lining the epididymal duct, with highest activity prevailing in the corpus and cauda portions. These workers suggested that the functional significance of the specific localization of lactic dehydrogenase lies in the secretion into the lumen of lactic acid. If this is true, lactic acid may serve as an important fuel for mammalian spermatozoa during epididymal transit.

The observed variation in the concentration of lactic acid in the cauda epididymidis might be explained by differences in the metabolic activity of the spermatozoa between samples and it might not be fortuitous that the concentration of lactic acid was highest in epididymal semen containing only few viable spermatozoa. However, it is also possible that manipulation of the cauda epididymidis during semen collection may have produced unusually high amounts of lactic acid in the smooth muscle layers surrounding the epididymal duct, thus contributing to the high lactic acid concentrations found in some samples of epididymal fluid.

The fact that cauda epididymidis fluid contains pregnenolone at relatively high concentration is of particular interest in light of the steroidogenic capacity ascribed to epididymal tissue (Hamilton, 1971). The large pool of pregnenolone would provide a steady supply of precursor for steroid synthesis in the distal regions of the epididymis and could explain why the androgen level in the luminal fluid is much higher than that of peripheral blood plasma.

Androgen-binding protein in the epididymal fluid would also contribute to the high androgen environment, presumably through the concentrating mechanism proposed by Hansson, Ritzén, French & Nayfeh (1975). The androgens that occur in the luminal fluid of the epididymis are unquestionably of importance for maintaining the functional activity of the epithelial lining of the excurrent duct, but they may also exert a direct influence on the spermatozoa (Fährmann & Schuchardt, 1965; Skinner & Rowson, 1968; Voglmayr, 1975). The level of 5 α -DHT in the cauda epididymidis was found to be over 6 times the concentration of testosterone and this high ratio is consistent with previous observations on the rat and the bull showing that 5 α -DHT is the predominant androgen in the distal regions of the epididymis (Ganjam & Amann, 1973; Vreeburg, 1975). Most of the 5 α -DHT in cauda epididymidis fluid is apparently formed from testosterone by the epithelial cells lining the epididymal duct, but not by the spermatozoa since the latter do not possess the 5 α -steroid reductase activity required for the conversion (Vreeburg & Scholte, 1973).

The presence of phospholipid at relatively high concentrations in the fluid from the cauda epididymidis may partly explain the ability of this fluid to prolong the life of the spermatozoa *in vitro*. For instance, certain phospholipids are known to be actively metabolized by ram spermatozoa obtained from any portion of the male reproductive tract (Scott, 1973). The presence of phospholipids in cauda epididymidis fluid would undoubtedly have a beneficial effect on the viability of the spermatozoa *in vitro* by providing substrate for metabolic energy and by protecting the spermatozoa against the damaging effects of low temperature storage (see Scott, 1973, for references). However, phospholipids, together with androgens and other as yet unidentified constituents of epididymal fluid, may also play an active role in promoting the 'maturation' of spermatozoa, and it is of particular interest that testicular spermatozoa, after exposure to cauda epididymidis fluid, utilize more glucose and convert a much greater proportion of the utilized glucose to lactic acid. After storage in the epididymal fluid the testicular spermatozoa do, in fact, become predominantly glycolytic in their mode of glucose dissimulation and in this respect they resemble ejaculated spermatozoa (Voglmayr, 1975).

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