Novel organization of the spermatozoon in two species of murid rodents from Southern Asia

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The morphology and structural organization of unusual spermatozoa from two species of bandicoot rats (*Bandicota* spp) from Southern Asia were studied with the light and transmission electron microscope. Unlike those described for nearly all other eutherian species the heads of *Bandicota savilei* and *B. indica* spermatozoa were bulbous or globular in shape and had no narrow uniformly defined apex nor a clearly delineated perforatorium. The nucleus, which had one or more prominent vacuoles, was capped by a huge, bulky, acrosome with no obvious morphologically recognizable equatorial segment. The postacrosomal dense lamina was extremely short and a posterior ring lay anterior to the caudal pole of the nucleus. In addition, the tail of the spermatozoon was only 28–45 μm in length. The organization of the spermatozoon of these two species of *Bandicota* is thus quite unlike that described for any other murid rodent or even mammalian species. Its highly divergent features suggest that the processes of zona penetration and incorporation of the fertilizing spermatozoon into the egg may differ markedly from those of all eutherians so far investigated.

Introduction

The head of the spermatozoon of eutherian mammals displays several novel features not evident in most other vertebrates or even in monotremes and marsupials. These features include disulfide bonding between the chromatin fibres, a specialized stable posterior or equatorial segment of the acrosome, bilateral flattening and, in most, a periacrosomal matrix that projects rostrally as a stabilized perforatorium (Fawcett, 1970, 1975; Bedford, 1982, 1991; Moore and Bedford, 1983; Yanagimachi, 1988; Eddy, 1988; Bedford and Hoskins, 1990). The species of common laboratory murid rodents are unusual in that the otherwise typically eutherian type of spermatozoon head is falciform in shape (Retzius, 1909; Friend, 1936; Clermont *et al.*, 1955; Austin and Bishop, 1958; Piko, 1969; Lalli and Clermont, 1981). The acrosome forms a dome-shaped crescent over the dorsal convex aspect of the nucleus and a stable equatorial segment, which persists after the acrosome reaction has occurred, is present over much of the lateral surface of the head. Caudal to the acrosome, the postacrosomal dense lamina occupies the space between the plasma membrane and the outer nuclear envelope. The connecting piece of the tail attaches on either the lower ventral surface as in laboratory rats and mice, or posteriorly as in hamsters (Retzius, 1909; Friend, 1936; Austin and Bishop, 1958; Piko, 1969; Lalli and Clermont, 1981; and for reviews Fawcett, 1970, 1975; Yanagimachi, 1988; Bedford and Hoskins, 1990).

Although this overall head organization of the spermatozoon is evident in most other murid rodent species albeit with an addition of two extra ventral processes in spermatozoa of most of the Australian hydromyines (Breed, 1983, 1984; Flaherty and

Breed, 1983; Breed and Leigh, 1991), there are a few species whose spermatozoa heads do not have a falciform shape, for example the harvest mouse, *Micromys minutus*, from Eurasia (Friend, 1936), the heath rat, *Pseudomys shortridgei*, and the delicate mouse, *Pseudomys delicatulus*, from Australia (Breed, 1983), the red veld rat, *Aethomys chrysophilus* type B from southern Africa (Breed *et al.*, 1988), three species of *Lophuromys* from East Africa (Breed, 1991), and a few others from southern Asia (Breed and Yong, 1986; Breed and Musser, 1991). However, although there are considerable variations in the shape of the head of these spermatozoa, all those investigated in detail so far have displayed a basic organization that is typical of eutherian mammals. In the light of this, it is of interest to find that two species of a genus of an otherwise normal murid rodent display bizarre features of organization of the head of the spermatozoon that are quite atypical for most eutherian mammals. The morphology of these spermatozoa is described.

Materials and Methods

Testes and caudae epididymides were obtained from three lesser bandicoot rats (*Bandicota savilei*) from Thailand; individuals were collected in central Thailand in 1984, near Sri Thep, Phetchabun on 31 July 1987, and near Phu Namtok on 3 August 1987. In addition, testes and epididymides were also obtained from a great bandicoot rat (*Bandicota indica*) collected near Ban Yaikata, Lopburi, Thailand on 1 August 1987. Each animal was killed by an overdose of Nembutal (pentobarbitone sodium: Boehringer Ingelheim, Artarmon, NSW) and small pieces of testes and caudae epididymides were removed and immersed in either 10% formalin or, in the case of the three animals collected in 1987, in 3% glutaraldehyde–3% formaldehyde made up in

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Fig. 1a,b: Nomarski optics light micrographs of cauda epididymal spermatozoa from *Bandicota savilei* showing variable but generally bulbous or globular shaped sperm heads. Scale bar = 5 μm .

Fig. 2a,b: Fluorescent light micrographs of spermatozoa from *Bandicota savilei* after staining with 4'-6-diamidino-2-phenylindole dihydrochloride showing that the nucleus is rounded basally and rounded or pointed apically. Scale bar = 2.5 μm .

phosphate buffer (0.1 mol l^{-1}). After fixation for 6–24 h, the tissue was placed in $0.1 \text{ mol phosphate buffer l}^{-1}$ and returned to Adelaide by air where it was prepared for transmission electron microscopy and light microscopy. After postfixing with 1% osmium tetroxide for 5–15 min, the tissue was rinsed in buffer, dehydrated, and embedded in epoxy resin. Thick, 0.5–1.0 μm , plastic sections were cut and stained with toluidine blue. When appropriate regions were found, ultra-thin sections were cut and stained with uranyl acetate and lead citrate. They were subsequently observed in a JEOL 100S or 2000FX electron microscope. Sperm smears from the caudae epididymides were also prepared on microscope slides, fixed in the transmission electron microscope fixative, and observed with Nomarski and phase contrast optics. To determine the shape of the nucleus at the lower magnification level of resolution, some smears of spermatozoa were stained with 4'-6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma, St Louis, MO) and observed with an Olympus fluorescence microscope using UGI excitation filter and U dichroic mirror.

Results

The mean weight of the paired testes for the three *B. savilei* was 274 mg (body weight 1263 g) ($=0.46\%$ of body mass), and in

Fig. 3. Transmission electron microscope low power micrograph of lumen of cauda epididymidis from *Bandicota savilei* showing a mixture of variably shaped spermatozoa, a few of which have malformed acrosomes or nuclei or both; there is also a variable amount of cytoplasm with vacuoles, vesicles, and other organelles; note short midpiece (MP) of sperm tail; PP = principal piece. SH: sperm head; V: vacuole. Scale bar = 900 nm.

B. indica the testis weight was 640 mg (body weight 2788 g) ($=0.45\%$ of body mass). Both species thus have low relative testes weights which fall well below the regression line of log testis weight versus body mass for all mammals (Kenagy and Trombulak, 1986).

Cauda epididymal spermatozoa of B. savilei

Nomarski and phase contrast optics demonstrated considerable pleiomorphism in cauda epididymal spermatozoa and, in addition, many abnormal germ cells were evident. In spermatozoa of normal appearance the head was between 4.5 and 5.5 μm in length and breadth was 2.5–3.0 μm . Most sperm heads tapered a little apically (Fig. 1a, b). In the central nuclear region a 1–1.5 μm nuclear vacuole was often seen under the phase contrast microscope. The tail was attached to the mid-basal region of the spermatozoon head and was only about 45 μm in length of which the midpiece made up 10 μm . A cytoplasmic droplet was often present in the region of the connecting piece. After staining with DAPI, the nucleus was also found to be somewhat variable in shape, but was typically fairly broad and bulbous basally, and rounded, or somewhat tapered apically (Fig. 2a, b).

Fig. 4. Cauda epididymal spermatozoa of *Bandicota savilei* showing the presence of a large nuclear vacuole (NV) with some electron-dense material; lateral to the nucleus (N) the acrosome (Ac) is variable in length but similar in width throughout; there is no indication of an equatorial segment; the nuclear envelope (NE) forms a pocket in the region of the connecting piece (CP). Scale bars (a) 140 nm; (b) 120 nm.

Fig. 5. Cauda epididymal spermatozoa of *Bandicota savilei* have a nucleus (N) which is variable in shape anteriorly and is capped by a huge apical acrosomal segment (Ac); the postacrosomal dense lamina (PADL) is very short and a posterior ring situated anteriorly (arrows), caudal to which the plasma membrane is reflected away from the nuclear envelope. Note small electron-dense structure lying close to the condensed chromatin in the caudal region of the sperm head (double arrow). Bar lines: a = 180 nm; b = 200 nm.

Transmission electron microscopy of thin sections of the tails of the epididymides indicated the presence of a very heterogeneous population of germ cells in the lumen of the duct. Apart from normal spermatozoa there were larger cells with variable amounts of cytoplasm that contained mitochondria, granulo-fibrillar material, one or more large vacuoles, occasional disorganized axonemes, variably condensed nuclei and acrosomal

material of varying shapes and sizes (Fig. 3). Occasionally two sperm heads shared a single tail or one sperm head had two implantation fossae.

The spermatozoa of normal appearance had a nucleus that was round basally. Anteriorly it was variable in shape and there was a very large vacuole present, in some of which electron-dense and flocculant material occurred (Fig. 4a, b). It was capped

Fig. 6. Transverse transmission electron microscope section through midpiece of tail of spermatozoon from *Bandicota savilei* showing crescentic dense fibres with more electron-dense outer cortical regions. M = mitochondria. Scale bar = 100 nm.

Fig. 7a,b: Nomarski optics of cauda epididymal spermatozoa from *Bandicota indica* showing somewhat variable shaped heads. Scale bar = 3 μ m.

by an acrosome that had a massive apical segment of up to 2.5 μ m in length (Figs 3; 5a, b). The principal acrosomal segment was about 0.15 μ m thick and extended for a variable distance lateral to the apical region of the nucleus (Figs 4 and 5), but no equatorial segment was evident. Between the inner acrosomal membrane and the outer nuclear envelope, a thin subacrosomal space was generally present which contained a little flocculant material; there was usually no large accumulation of material anteriorly and thus no clearly defined perforatorium. The postacrosomal dense lamina was only 0.7–1.5 μ m in length and in this region the overlying cell membrane was tightly opposed to the underlying structures (Figs 4a; 5a, b). The postacrosomal dense lamina terminated at the posterior ring which lay 1–2 μ m

anterior to the connecting piece (Figs 4a; 5a, b). Caudal to this the cell membrane was reflected away from the nuclear envelope and between it and the nuclear envelope some cytoplasm with granular material occurred. A small space, with granular material, up to about 200 nm wide, was also often present between the nuclear envelope and the dense chromatin. Occasionally, lying within the nuclear envelope and close to the condensed sperm chromatin, one or two, small dense structures in the posterior region of the head were evident (Fig. 5a). The two membranes of the nuclear envelope were also in less close apposition caudal to the posterior ring. The connecting piece, which contained a typical implantation fossa, basal plate and capitulum, was generally surrounded by cytoplasm in which a group of mitochondria occurred (Fig. 4a). There were about 27 gyres of mitochondria in the mid-piece (Fig. 3) and transverse sections indicated that the outer dense fibres were all of similar size and crescent shape; they had a more electron-dense outer cortical zone (Fig. 6).

Cauda epididymal spermatozoa of B. indica

In *B. indica* the spermatozoa were pleomorphic but generally globular in shape (Fig. 7a, b). The head length varied from 5 to 7 μ m with a maximum width of 3 μ m. The sperm head was rounded anteriorly; posteriorly it tapered to a varying extent. The sperm tail was only 38 μ m in length.

Transmission electron microscope sections of the tails of the epididymides indicated that in the lumen of the duct there were some immature germ cells and spermatozoa with much cytoplasm in which some granular material, vacuoles, cisternae and mitochondria occurred. The subacrosomal space also varied markedly in size between the different spermatozoa.

The nuclei of the spermatozoa of normal appearance were capped by a huge acrosome (Fig. 8a, b). The nucleus was more or less circular in cross-section for most of its length (Fig. 9a), and there were many small nuclear vacuoles. Apically the nucleus was variable in shape. Sometimes an apical extension occurred (Figs 8a; 9b–h) which was sometimes bilaterally flattened (Fig. 9c) but in many others it was cup-shaped (Fig. 9b), wedge-shaped (Fig. 9d), and even in some two separate nuclear extensions were evident (Fig. 9f). The nucleus was invariably capped by a massive acrosome, the apical segment of which was 2.5–3.0 μ m in length (Figs 8 and 10). The acrosome invariably resulted in the anterior region of the spermatozoon being circular in transverse section, regardless of the nuclear shape (Fig. 9a–i). No equatorial segment of the acrosome could be found, and the subacrosomal space was usually very narrow, although occasional small apical projections with a little flocculant material could be seen (Figs 9h, i; 10a–c). The postacrosomal dense lamina was only 0.5–1.1 μ m in length, and the posterior ring was situated 1.8–2.5 μ m anterior to the connecting piece (Figs 8a, b; 10a–c). Caudal to the posterior ring some granular cytoplasmic material was present between the plasma membrane and nuclear envelope (Figs 8 and 10). In the posterior part of the head of the spermatozoon, one to three electron-dense bodies were sometimes evident close to the condensed chromatin. The condensed nuclear material was sometimes separated from the nuclear envelope by a space of up to 0.3 μ m. The connecting piece was mid-basally attached to the spermatozoon head, and had a typical implantation fossa

Fig. 8a,b: Longitudinal sections through cauda epididymal spermatozoa of *Bandicota indica* showing nucleus (N) of variable shape apically, a huge apical segment of the acrosome (Ac), very short postacrosomal dense lamina (PADL), and posterior ring situated in a remarkably anterior position (arrow). Scale bars: (a) 400 nm; (b) 350 nm.

with basal plate, capitulum, and segmental columns (Fig. 10b). A small pocket of redundant nuclear envelope could also sometimes be seen. Peripheral to the connecting piece some cytoplasm with scattered mitochondria was evident.

Late spermatids

Owing to the highly pleiomorphic nature of the epididymal spermatozoa of the bandicoot rats, and in particular those of *B. savilei*, the morphology of late spermatids was also briefly investigated.

In *B. savilei* spermatids, the acrosomal cap over the anterior pole of the nucleus generally contained homogeneous electron-dense material; localized regions of greater electron density than the rest of the acrosomal matrix and intra-acrosomal vacuoles of various sizes and abundance (Fig. 11a) were sometimes evident. Sometimes vesicles, which had presumably arisen from the Golgi apparatus, had become internalized (Fig. 11a). The acrosomal cap was occasionally shared between two germ cell nuclei or appeared disorganized (Fig. 11b); in some, it also formed a deep invagination of the apical region of the nucleus (Fig. 11c).

As condensation proceeded, prominent chromatin cords or fibres of 50–70 nm diameter became evident and small localized circular regions of thinner fibres of 20–30 nm diameter were also evident (Fig. 11d). In the late spermatids, the chromatin was very electron dense but a nuclear vacuole of variable size was sometimes evident in which there were aggregations of electron-dense, flocculant material (Fig. 12a) or groups of fibres or cords (Fig. 12c). Generally the acrosome became very large (Fig. 12c), but occasionally spermatids were evident that either had a distorted apical segment of the acrosome or appeared to

lack an acrosome altogether, but had what appeared to be an expanded subacrosomal space (Fig. 12b).

Discussion

The study reported here shows that in two species of bandicoot rats from southern Asia the cauda epididymal spermatozoa exhibit a highly unusual form as well as a high degree of pleiomorphism. Although small differences in morphology were evident between the two species, their spermatozoa share a whole suite of divergent features and the late spermatids, as well as the epididymal spermatozoa, often exhibit peculiarities in the acrosome, subacrosomal space and nuclear chromatin organization. These morphological traits presumably first evolved in a recent common ancestor of these two species as a result of a structural, or perhaps regulatory, gene mutation that is expressed during spermiogenesis. Even among the germ cells of normal appearance from the cauda epididymides there is evidence of germ cell immaturity as reflected in the occurrence of nuclear vacuoles, unusual amounts of cytoplasm with various organelles, and the presence in the posterior region of the nucleus of, what appears to be, one or more juxtanuclear bodies. A similar situation has been found in two other species of distantly related murids, the red veld rat of South Africa (Breed *et al.*, 1988) and, to a lesser extent, the heath rat of Australia (Breed, 1983), although the shapes of the sperm heads are, nevertheless, very different both from each other and from those of the bandicoot rats.

The size of the spermatozoon of the bandicoot rats is also most unusual for a murid rodent. In a previous survey sperm tail lengths for Australian murids were found to be 84–150 μm

Fig. 9a–i: Transverse sections through cauda epididymal spermatozoa of *Bandicota indica*. Basically the nucleus (N) is circular in cross-section (a); apically it is highly variable in shape (b–g) but the cross-section of the sperm head is always circular owing to the presence of the acrosome (Ac); the apical extension of the nucleus may appear cup-like (b), bilaterally flattened (c), convoluted (d, e) or even as two separate extensions (f); the subacrosomal space (SAS) is generally small and contains only a little flocculant material (g, h, i); sometimes invaginations of the outer acrosomal membrane are evident (arrow in g). Scale bars (a) 230 nm; (b) 250 nm; (c) 200 nm; (d) 200 nm; (e) 230 nm; (f) 250 nm; (g) 220 nm; (h) 150 nm; (i) 200 nm.

($n = 26$) (Breed and Sarafis, 1979; Breed, 1980), those from peninsular Malaysia 84–172 μm ($n = 19$) (Breed and Yong, 1986), and from Sulawesi 52–245 μm ($n = 28$) (Breed and Musser, 1991). Thus out of all 73 species of murids so far examined (see Cummins and Woodall, 1985, for review), the tail lengths of 45 μm and 38 μm for these two species of bandicoot rats fall well below those of all other species. In the previous comparative studies of Australian and Asian murids it was found that spermatozoa with the shortest tails invariably had

highly divergent sperm heads which are either spatulate, paddle or pyriform in shape and contained a basally attached connecting piece. The spermatozoa of these two *Bandicota* species add further weight to this association between deviant sperm head shape and short tail length.

In consideration of the evolutionary trends of head shape of murid spermatozoa, Friend (1936) suggested that the 'more simple' nonhooked sperm head was the primitive type. This view was shared by Rolden *et al.* (1992) who argued that murid

Fig. 10. Longitudinal sections through cauda epididymal spermatozoa of *Bandicota indica* showing abundant small vacuoles in nucleus (N), the small subacrosomal space (SAS) that sometimes forms an apical extension (a, c) but contains only a little flocculant material, and very short postacrosomal dense lamina (PADL) with the posterior ring even sometimes being present in the anterior third of the nucleus (arrow); basally the connecting piece (CP) has typical segmental columns and a group of mitochondria. Scale bars: (a) 200 nm; (b) 500 nm; (c) 6 nm.

spermatozoa with round, or oval-shaped heads and short tails were the primitive forms and the 'hooked' type evolved independently on several occasions. The absence of falciform spermatozoa heads outside the cricetid and murid families would suggest that this type of spermatozoon head evolved within the Muroidea (probably independently in the cricetids and murids). Nevertheless, as the apical hook of murid spermatozoa is highly complex in structure with an elaborate and unique organization of both the perforatorium and acrosome (see Piko, 1969; Lalli and Clermont, 1981; Breed, 1983, 1984), and as it is also virtually identical in form in species from Asia, Australia and Africa, it is likely to have evolved in a

common ancestor of these lineages. The species that do not have a spermatozoon with a falciform head shape exhibit a wide range of head shapes varying from pyriform in the Australian delicate mouse, spatulate in the heath rat, wedge-shaped in the South African red veld rat, paddle-shaped in African *Lophuromys* and south-east Asian *Pithecheir*, to now bulbous in the greater and lesser bandicoot rats of southern Asia. Most, perhaps all, of these forms probably evolved, independently, from the falciform sperm head type that is by far the most widespread sperm form within the Muridae.

Eutherian spermatozoa are generally characterized by having a highly resilient or 'keratinoid' nucleus, a bilaterally flattened

Fig. 11. Testicular spermatids of *Bandicota savilei* showing that the acrosomal cap (Ac) may sometimes either contain vacuoles of various sizes in which there may be small vesicles (a), or is disorganized and partly fragmented (b), or it may greatly indent the nuclear envelope (c). During chromatin condensation of the nucleus (N) thick fibres or cords become evident (d) in addition to small circular regions of smaller fibres (arrow). Scale bars (a) 230 nm; (b) 250 nm; (c) 470 nm; (d) 250 nm.

head, a stable inner acrosomal membrane, a perforatorium or apical body at the leading edge, and a posterior, stable, equatorial segment of the acrosome. The first four features have been suggested as being adaptations of the spermatozoon for penetration of the zona pellucida, whereas the cell membrane overlying the equatorial segment of the acrosome initially makes contact with the oolemma at the time of sperm-egg fusion (Bedford, 1982, 1991; Moore and Bedford, 1983; Yanagimachi, 1988; Eddy, 1988; Bedford and Hoskins, 1990). The falciform-shaped sperm heads of murid rodents (Piko, 1969; Lalli and Clermont, 1981; Courtens and Loir, 1981; Breed, 1983, 1984) and even the morphologically divergent spermatozoa of the Australian heath rat and South African red veld rat (Breed, 1983; Breed *et al.*, 1988) are all bilaterally flattened and have a typical equatorial segment of the acrosome. In the two species of bandicoot rats studied here, however, not only does the head of the spermatozoon not have an apical hook, but it is not bilaterally flattened. In these species the spermatozoa have a

nucleus that is rounded in transverse section basally and towards its apical tip is variably shaped. Moreover, the massive acrosome, which is also more or less circular in cross-section, does not appear to have a morphologically distinctive equatorial segment. These structural features are most unusual for spermatozoa of a eutherian mammal. The absence of some of the morphological characteristics considered to be adaptations for penetration of a eutherian zona pellucida based largely on physical factors (Bedford, 1991) suggests a possible different mechanism of zona penetration.

The presence of the very large acrosome in spermatozoa of these bandicoot rats implies that a large quantity of acrosomal enzymes may be available for release at the time of the acrosome reaction. In these animals the lytic activity of acrosomal enzymes may therefore be more important for zona penetration than it is in many other eutherians. The relatively short tail of the spermatozoon and small coarse fibres also suggest modest stiffness of the tail during bending (Fawcett *et al.*, 1971; Fawcett,

Fig. 12. Testicular late spermatids of *Bandicota savilei* showing that the chromatin has condensed but in the anterior region of the nucleus a very large vacuole (NV) with some electron-dense, flocculant material occurs (a). In other spermatids abnormalities may be evident such as a nucleus capped by a huge space with flocculant material and no evident acrosome (b); note also very short postacrosomal dense lamina and three small electron-dense bodies close to the condensed chromatin which may be juxtannuclear bodies; these are sometimes evident in cauda epididymal spermatozoa; generally, an extremely large acrosome becomes evident that overlies the sperm chromatin (c); note nuclear vacuole in which a group of chromatin fibres is present (arrow). Scale bars (a) 220 nm; (b) 220 nm; (c) 440 nm.

1975). Whether the chromatin is cross-linked by disulfide bonds remains to be established, but the nuclear vacuoles suggest a less rigid keratinoid spermatozoon nucleus than those of most eutherians. These features of *Bandicota* spermatozoa collectively suggest less reliance on physical thrust and more on enzymatic digestion for zona penetration. It is already evident that there are differences between species of eutherians in the extent of development of these structures; human spermatozoa are at one end of the spectrum in having no perforatorium and a rather bulbous coronal profile to the anterior part of the nucleus

(Bedford, 1991). It would appear that spermatozoa of bandicoot rats exhibit an even more extreme form of this type.

The spermatozoa of bandicoot rats have one further morphological peculiarity: the apparent absence of a clearly defined equatorial acrosomal segment. This suggests that not only the processes of zona penetration, but also possibly that of sperm-egg fusion, may differ markedly from that of the typical eutherian mammalian pattern. Bedford (1991) proposed that in eutherians the tough nature of the zona has resulted in the evolution of a resilient inner acrosomal membrane. This, he argues,

has brought about a transfer of the fusigenic area of the head of the spermatozoon from the rostral region to the plasma membrane over the equatorial segment. Clearly, these *Bandicota* spermatozoa without the streamlined features of the head, and with the different structure of the acrosome, provide an opportunity to test the relationship between sperm head form, mode of zona penetration, and the site of sperm-egg fusion. A study of these aspects of fertilization in members of this genus of murid rodent from southern Asia could thus prove very instructive in gaining insight into the causation and evolutionary significance of some of the unique features of eutherian spermatozoa.

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