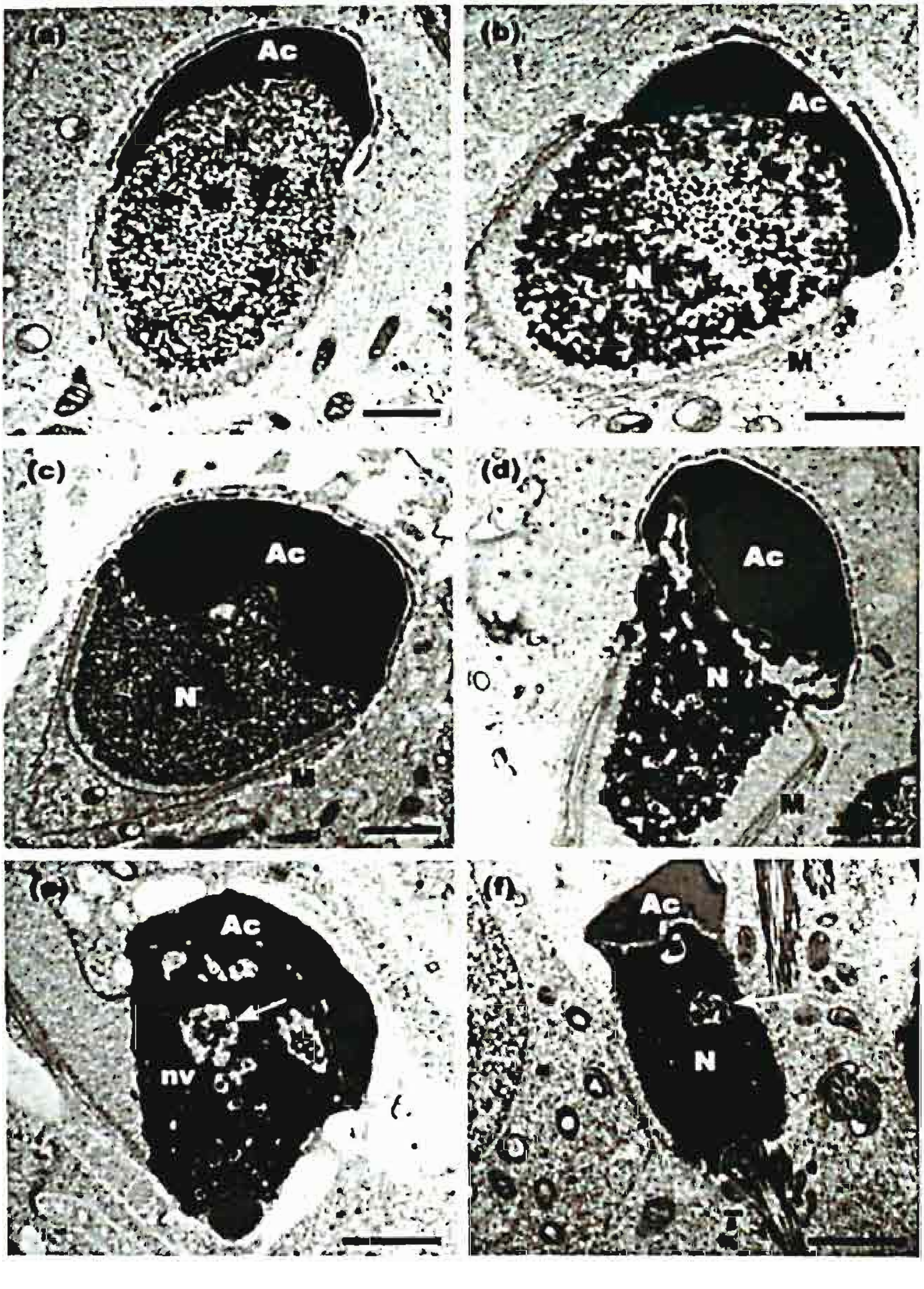
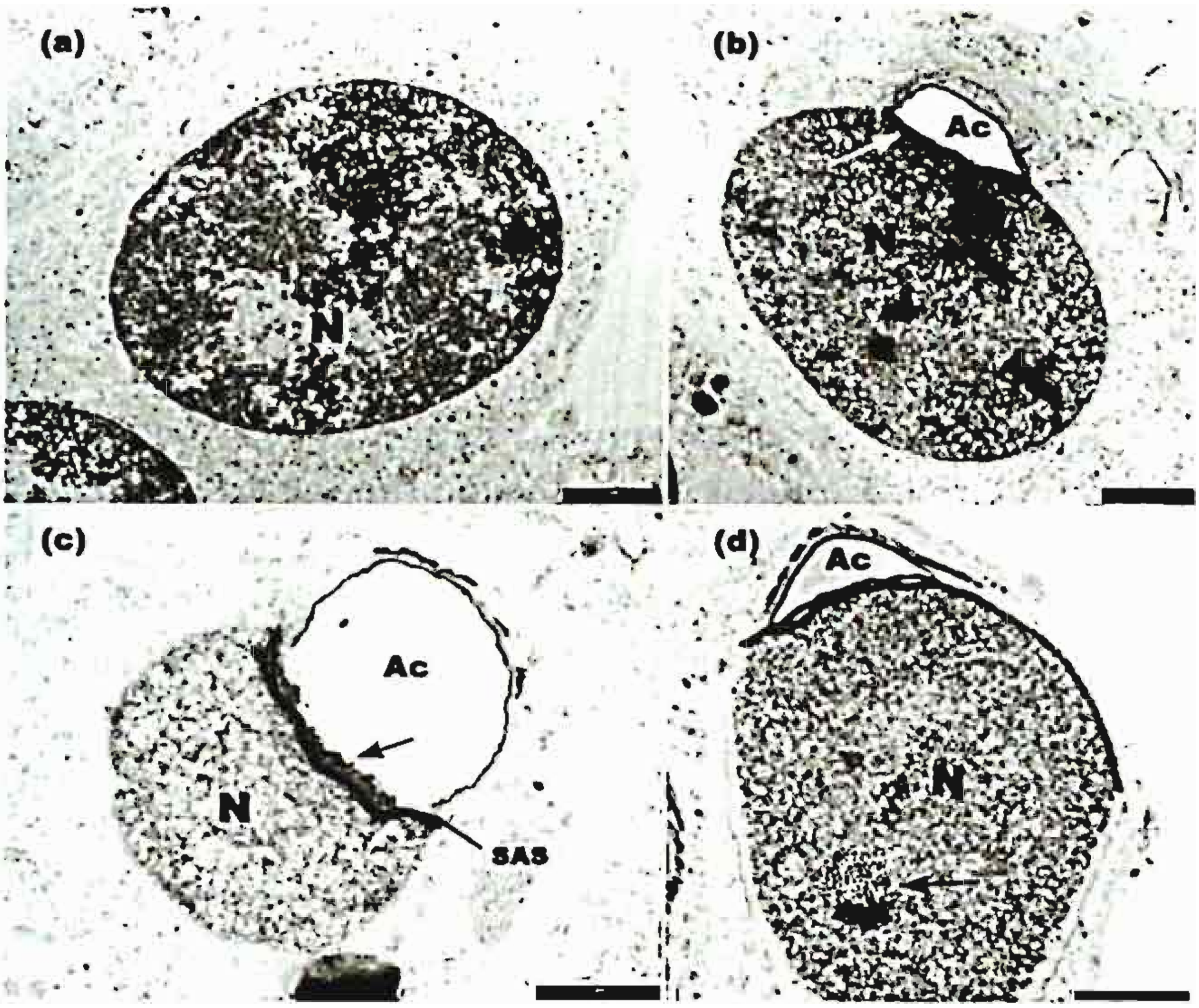
Figs. 7-8. Transmission electron micrographs of four successive phases based upon the principal morphologenetic events which occurred as development proceeds. Fig. 7a-7b = Initial or Golgi phase showed numerous small, electron dense proacrosomal granules (G) appearing between the Golgi apparatus and nucleus (N). Chromatin threads with 15-23 nm in diameter located throughout the nucleus. Fig. 7c-7d = Cap phase a large acrosomal granule attached to the nuclear membrane to form an acrosome (Ac) over the anterior pole of nucleus (N). Thin region of condensed electron dense chromatin just beneath the acrosome (Fig. 7d-arrow) as well as close to other regions of the nuclear envelope (Fig. 7c-arrow). Fig. 7e-7f, and 8a-8c = Acrosome phase the acrosome became oval shape with homogeneous, electron dense material. Shape of nucleus had bi-laterally flattened with gradually condensed chromatin diameter of 39-48 nm foci and chromatin granules ranging from 35-79 nm in diameter located centrally (Fig. 8a-arrow). Fig. 8d-8f = Maturation phase chromatin developing an extremely electron dense nucleus with 46- 117 nm granules in the nucleus and showing the spherical electron dense granules with 73- 148 nm in the nuclear vacuoles (NV) (Fig. 8e-8f-arrow). Abbreviations: PADL = postacrosomal dense lamina, M = manchette. IF = implantation fossa, pr = perinuclear ring, and T = tail. All scale bars = 1 um. and 2 um for Fig. 7f.

Fig.8



Figs. 9-10. Phosphotungstic acid (PTA) stained detecting lysine rich protein in the nuclear chromatin. Early spermatid generally lightly stained chromatin (Fig. 9a), however chromatin lying close to nuclear envelope as well as did some chromatin underlying the acrosome (Ac) stained strongly (Fig. 9b-arrow). Small area of group of chromatin granules had positively stained (Fig. 9c, 9d-arrow). PTA strongly stained material localized within the nucleus (N) and an area underneath the acrosome (Fig. 10a-arrow). With further maturation of spermatid nucleus had poorly stained PTA except for the peripheral nuclear rim (Fig. 10b-arrow), anterior part of nucleus (N) (Fig. 10b-arrowhead), and near the basal plate (Fig. 10c-arrow). Acrosomal material did not stained with PTA except for a small localized area closed to nuclear envelope (Fig. 9c-arrow). Between acrosome and nucleus there was a thin region, subacrosomal space (SAS) stained strongly. Abbreviations: PADL = postacrosomal dense lamina. All scale bars = 1 um, and 0.5 um for Fig. 10a.

Fig.9



Figs. 9-10. Phosphotungstic acid (PTA) stained detecting lysine rich protein in the nuclear chromatin. Early spermatid generally lightly stained chromatin (Fig. 9a), however chromatin lying close to nuclear envelope as well as did some chromatin underlying the acrosome (Ac) stained strongly (Fig. 9b-arrow). Small area of group of chromatin granules had positively stained (Fig. 9c, 9d-arrow). PTA strongly stained material localized within the nucleus (N) and an area underneath the acrosome (Fig. 10a-arrow). With further maturation of spermatid nucleus had poorly stained PTA except for the peripheral nuclear rim (Fig. 10b-arrow), anterior part of nucleus (N) (Fig. 10b-arrowhead), and near the basal plate (Fig. 10c-arrow). Acrosomal material did not stained with PTA except for a small localized area closed to nuclear envelope (Fig. 9c-arrow). Between acrosome and nucleus there was a thin region, subacrosomal space (SAS) stained strongly. Abbreviations: PADL = postacrosomal dense lamina. All scale bars = 1 um. and 0.5 um for Fig. 10a.

Fig.10

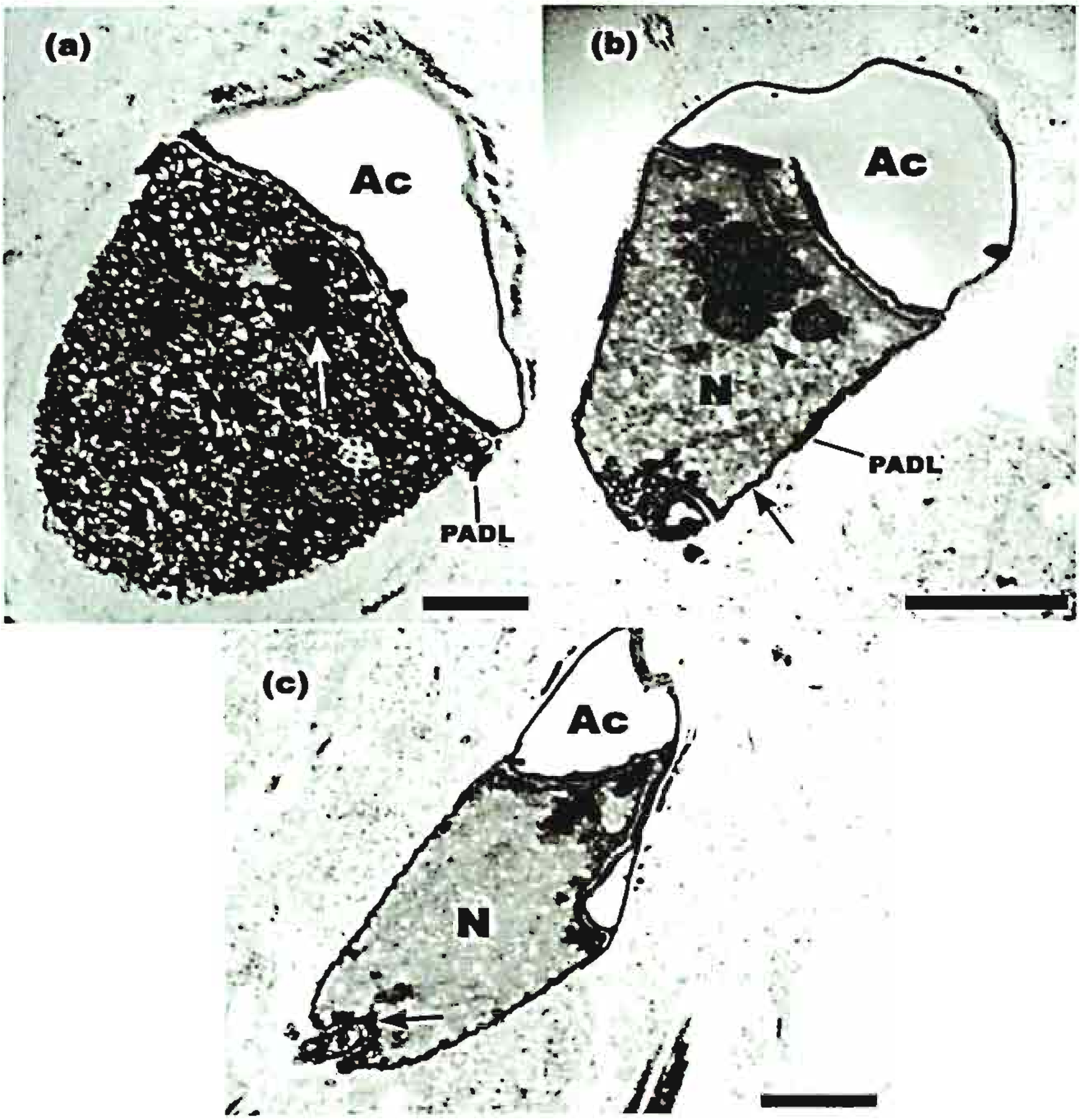


Fig. 11. Light micrographs of paraffin sections of seminiferous tubules immunostained with anti-H1 antibody. A positive staining pattern was visualized at the base of the seminiferous tubule which referred to the spermatogonia. Some of them showed intensely immunoperoxidase reactivity as in Fig. 11A-inserted a, some were not. Pachytene spermatocytes (P) and spermatids are unreactive (Fig. 11A-arrow). A variable staining pattern was depicted among different seminiferous tubule depends on stage of cycle of seminiferous epithelium. Late spermatids showed dense immunoreactivity within the spermatid nuclei as in Fig. 11B-inserted b. Small amounts of spermatogonia cell type are positive staining (Fig. 11B-arrow). No immunoreactivity was found in primary spermatocytes (P). Immunoreactivities of anti-H3 antibody of *B.indica* seminiferous tubules (Fig. 11C, 11D). Pachytene primary spermatocytes showed highly reactivity throughout their nuclei as in Fig. 11C-inserted c2 and also the spermatogonia (Fig. 11C-arrow), whilst round spermatids (inserted c1) showed immunoreactivity at the periphery of nuclei. Intense reactions of anti-H3 antibody presented in late spermatids (Fig. 11D-arrows). Discontinuous thin rims as well as the occasional blebs of reaction product were present at the edge of round spermatid nuclei (Fig. 11D-arrow in inserted d). Spermatogonia and pachytene spermatocytes were unreactive. Light micrographs of seminiferous tubules immunoperoxidase stained with anti-protamine antibody and the control. Fig. 11E was a control section. Fig.11F anti-protamine P2B antibodies reacted only in late spermatids (inserted f1, f2). Others were unreactive. Inserted f2 showed the positively nuclear extension reactivity in late spermatids (Fig. 11F-arrow).

Fig.11

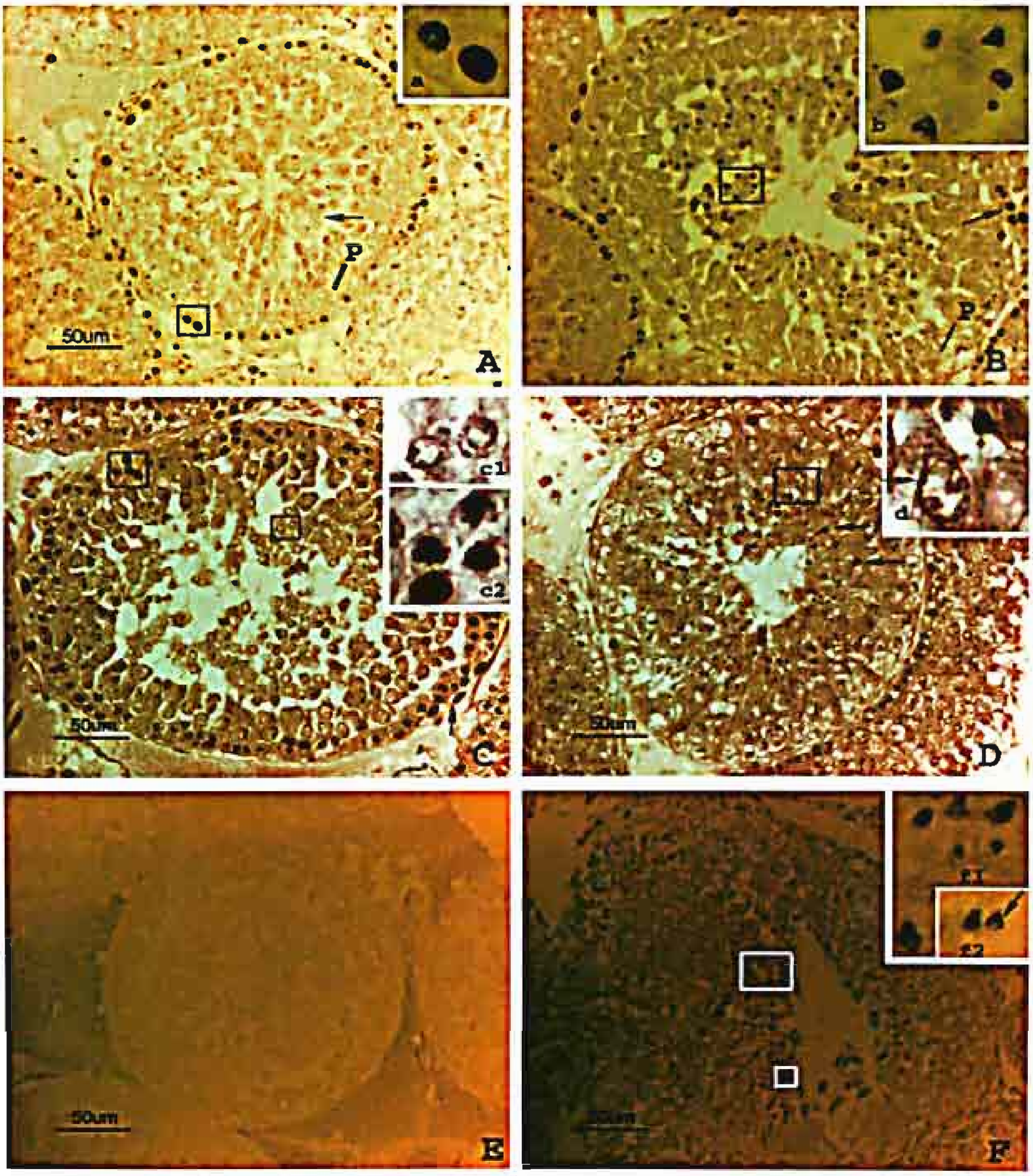


Fig. 12. Transmission electron micrographs (TEM) of gold labeled of spermatid nuclei (a, b, c, and f) and cauda epididymides (d, e) using anti-histone H2A antibody. A number of gold particles were more in nuclei of cauda spermatozoa (d, e) than those in spermatids (a, b, c) but no labeling occurred in nuclear vacuoles (nv) (f). Bar represents 1 um. in fig. (a), 200 nm in fig. (f), and 500 nm in fig. (b-e).

Fig.12

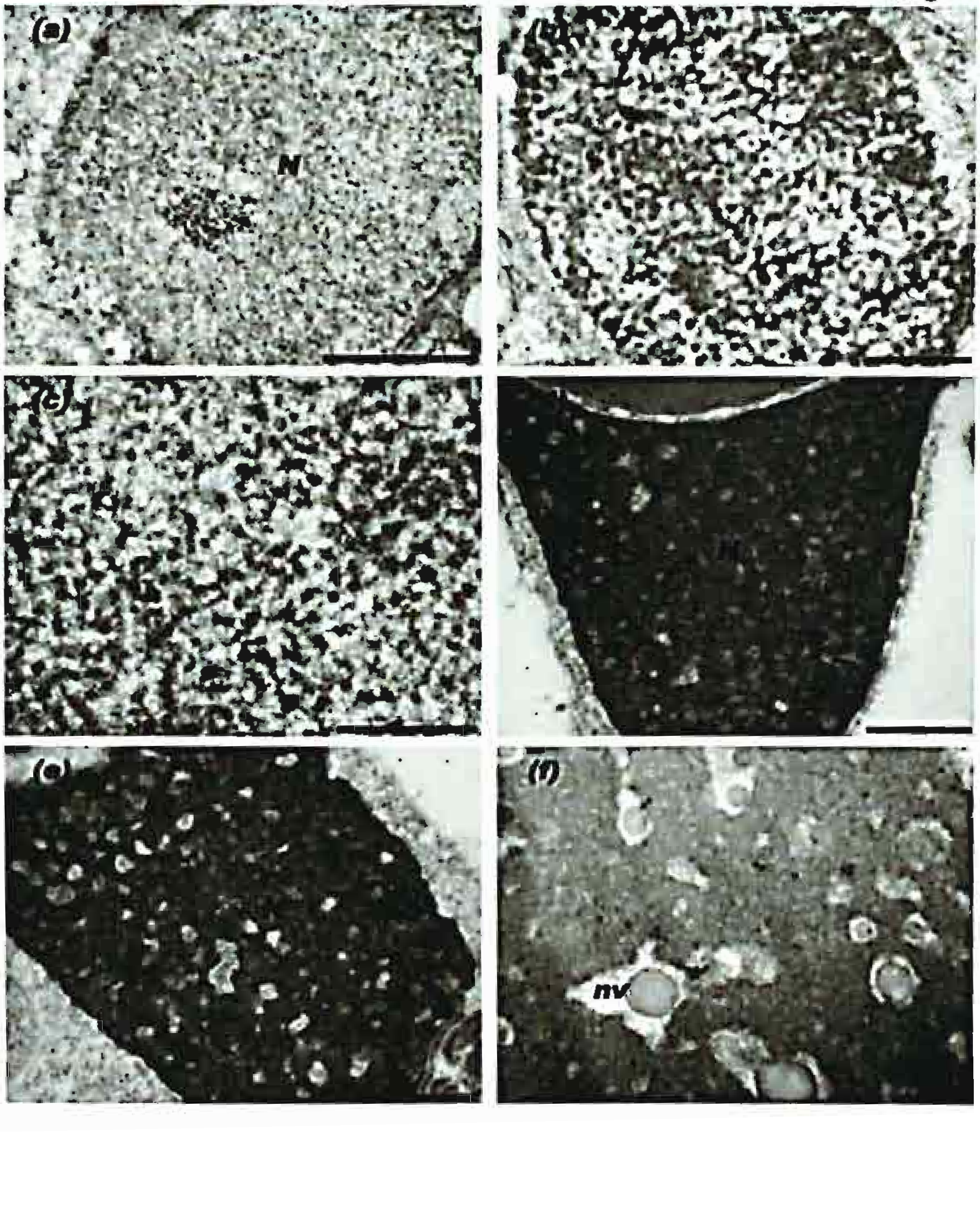


Fig. 13. Immunoelectron micrographs of spermatids and mature sperms stained with anti-histone H2B. A small amount of gold particles were observed in spermatids (a-d), while a slightly increased gold particles in mature sperms (e, f). There is no labeling over the acrosomal region (Ac), nuclear vacuoles (nv), and in part of cytoplasm. Bar represents 200 nm in fig. (e), and 500 nm in fig. (a-d, f).

Fig.13

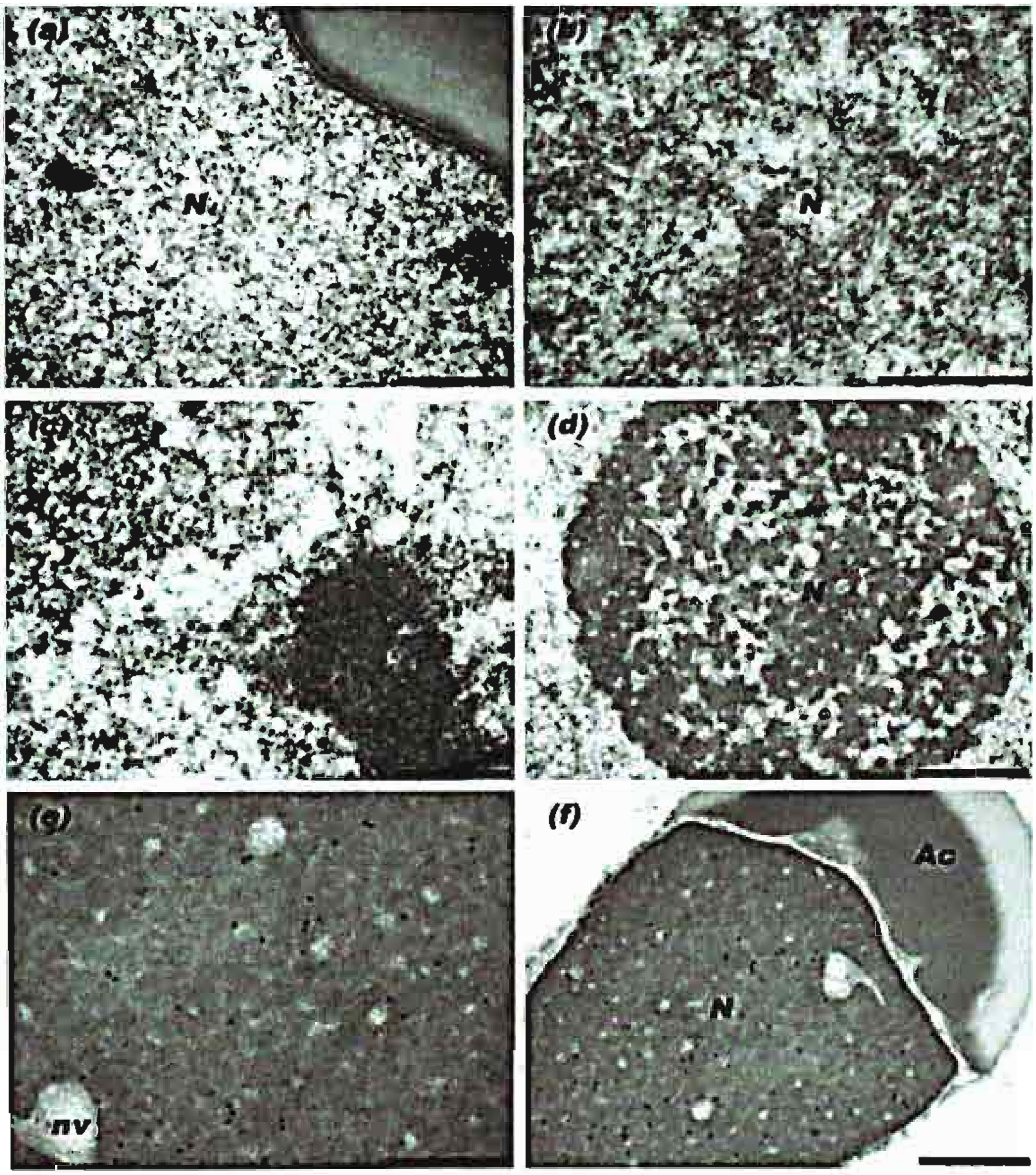


Fig. 14. Immunoelectron micrographs of spermatids and mature sperms stained with anti-histone H3 showing sparse labeling over the dispersed chromatin (a, b), but heavy labeling occurred over condensed chromatin (c, d) in cauda sperm. In the acrosome (Ac) and nuclear vacuoles (nv) showed no labeling (e, f). Bar represents 200 nm in fig. (a, f), and 500 nm in fig. (b-e).

Fig.14

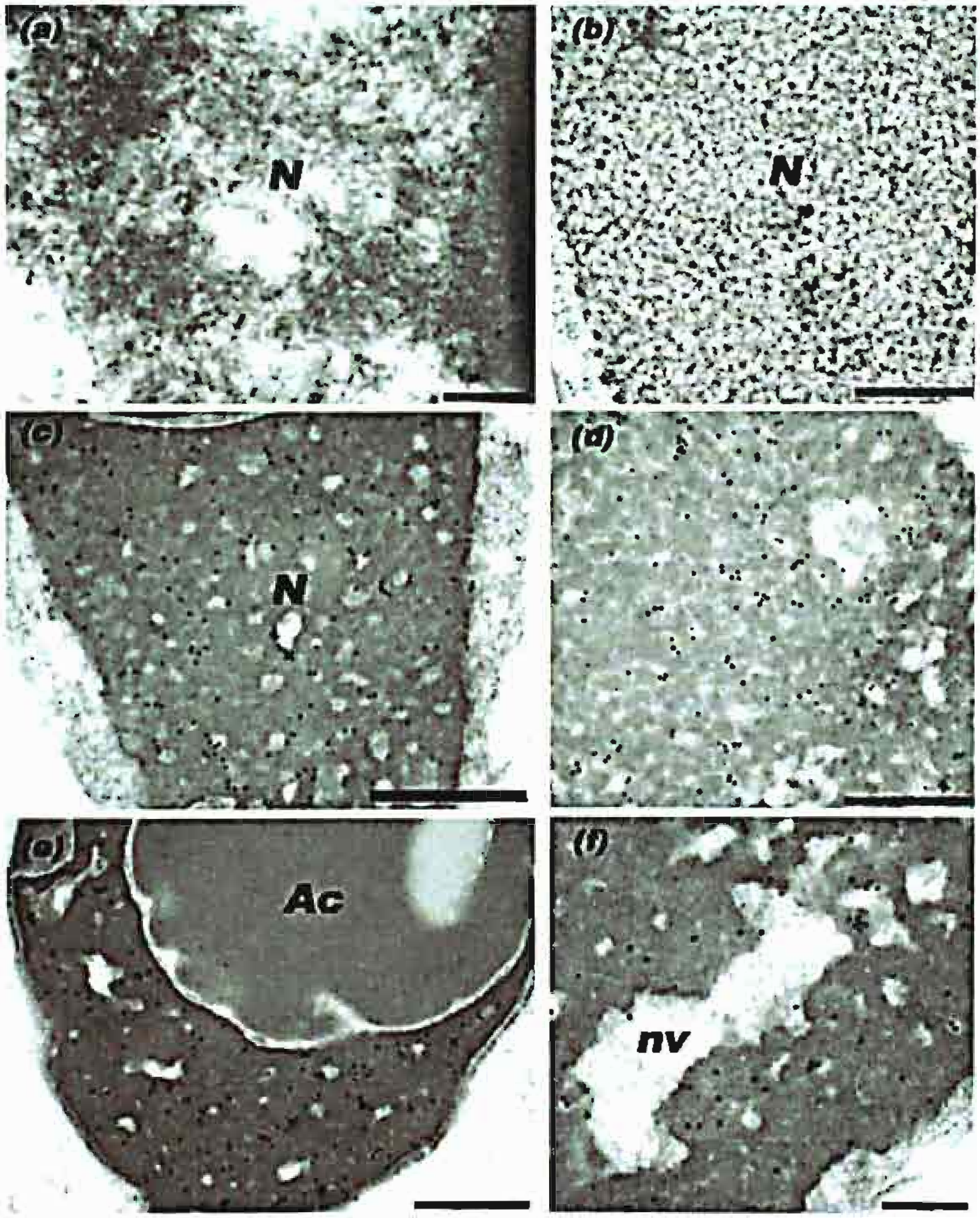


Fig. 15. Immunoelectron micrographs of spermatids and mature sperms stained with anti-histone H4. It showed little labeling over the chromatin in the testicular spermatids (a, b) but more labeling over condensed than uncondensed chromatin (c, d, e), the nuclear vacuoles (nv) showed no labeling (f). Bar represents 1 um. in fig. (b), 200 nm in fig. (e), and 500 nm in fig. (a, c-d, f).

Fig.15

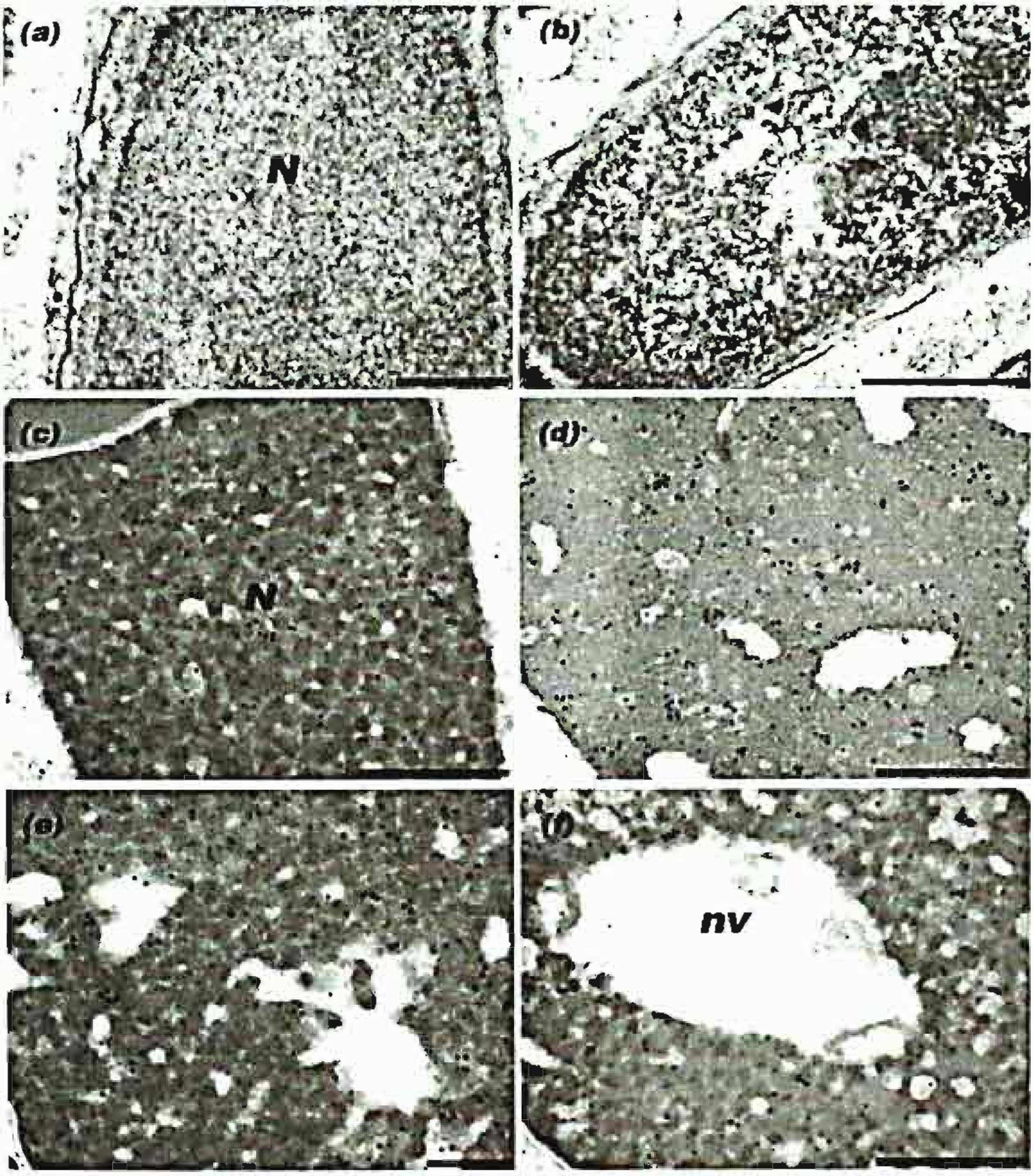


Fig. 16. Gold labeling of transmission electron micrographs using anti-transition proteins TP1 (b-f) and TP2 (a) antibodies. Abundant labeling was clearly evident over the condensing chromatin and also over the condensed chromatin (a-d) but the nuclear vacuoles were conspicuous in their total absent of any labeling (e, f). Bar represents 1 um. in fig. (b, c), 200 nm in fig. (f), and 500 nm in fig. (a, d, and e).

Fig.16

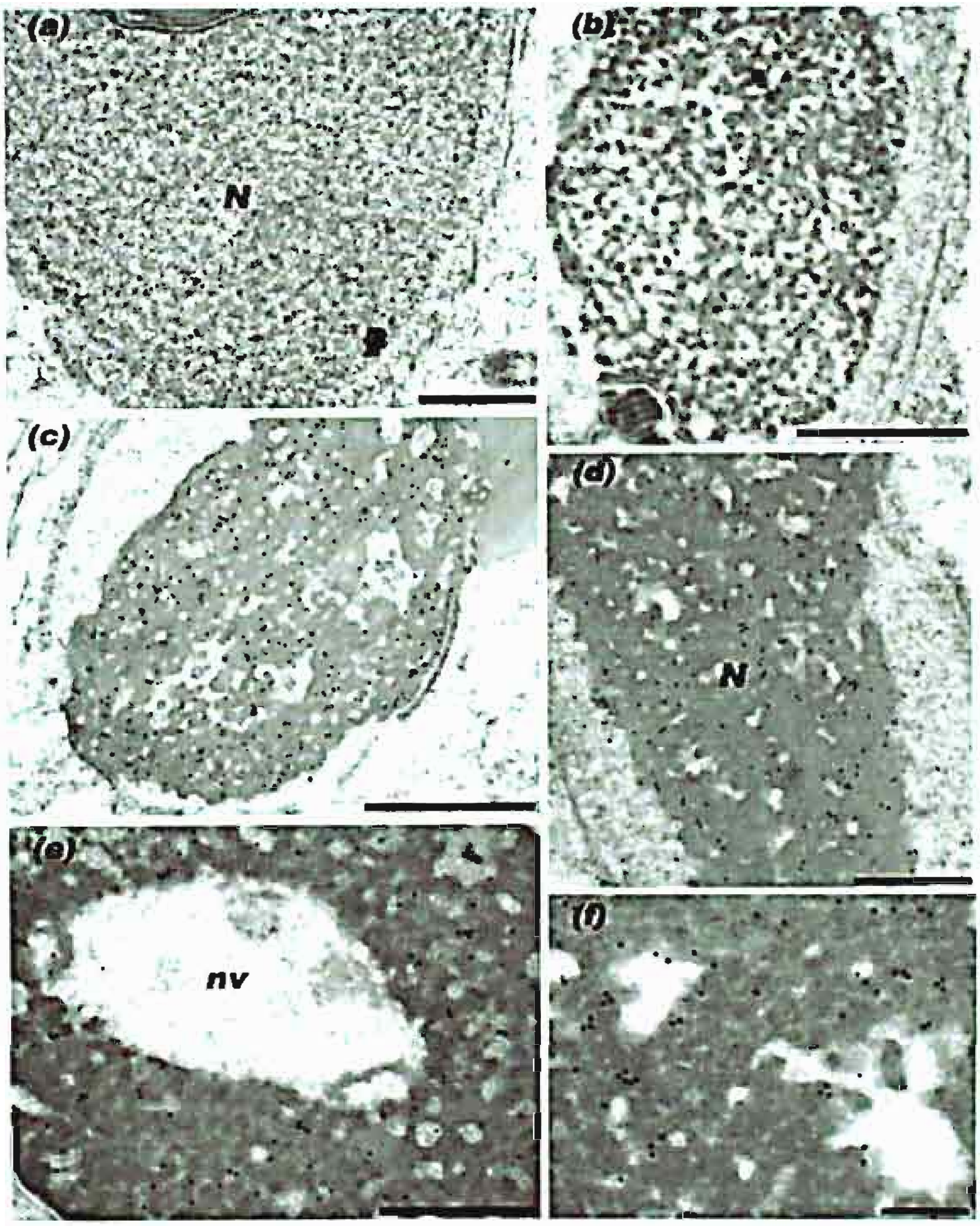
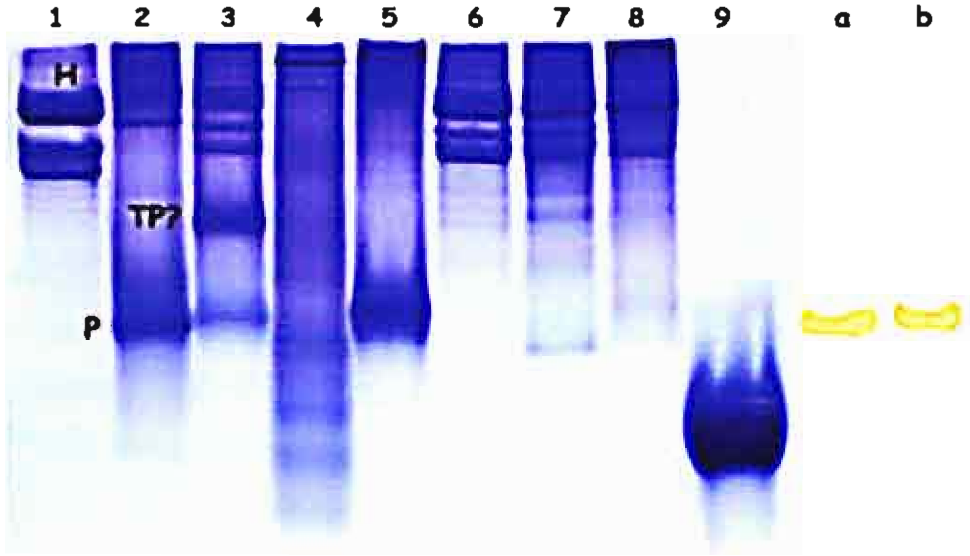


Fig. 17. AUT-PAGE (17%) analysis of the basic nuclear proteins from spermatozoa nuclei of human, *B. indica*, lab rat, and lab mouse (lane 2-5) comparing with the nuclei of testicular cells of *B. indica*, lab rat, lab mouse (lane 6-8), and salmon protamine (lane 9) respectively. Basic nuclear proteins from chick erythrocytes were used as a standard marker (lane 1). Extracted proteins were applied to each well and electrophoresed from top (+) to bottom (-). Each lane showed the Coomassie Blue-stained bands displaying all fractions of histones (H), transition proteins or protamine-like (PL), and protamines (P). Lane a and b = basic nuclear proteins from human and mouse spermatozoa demonstrated the specificity of anti- protamines antibody analyzed by Western blotting.

Fig.17



CONCLUDING REMARKS AND DISCUSSION

Stages of the Seminiferous Epithelium

Characteristics of each cell type were described in detail in this study. The criteria used were cell size and their appearance, the presence of marking organelles such as Golgi body, acrosomal granule, and the condensation of chromatin fibers corresponding with the reduced size of nuclei. As the sperm cell developed and differentiated. Golgi body became visible at stage II. Meanwhile. the acrosomal granule was initially formed in in this stage. This acrosomal granule then contacted with nuclear membrane and it finally became elongated along the nuclear shape. Nuclear chromatin gradually packed tighter and tighter in correlation with the slimmer and small head as the spermatogenic cell became more mature.

Morphological characteristics of spermatogenic cells along with the pattern of cellular association were further used as the criteria to establish seminiferous cycles which were consisted of nine stages. These cellular associations were considerable of heterogenous type and closely similar to that of humans, namely, more than one seminiferous cycle could be found in a cross section of the tubule.

After production in the testis, the sperm of most mammals must be translocated into epididymis to undergo the post-testicular modification in order to gain more fertilizing ability. During this sperm "maturation" process. epididymal sperm are still considerably heterogenous in certain degree depending on species. As assess the sperm head morphology by propidium iodide staining. sperm of *B. indica* revealed a higher degree of heterogeneity among other rodent species. In this context, there were at least seven types of head morphology was notified in bandicoot epididymal sperm.

This study thus showed that this species of bandicoot rat, *B. indica*, has several features of the germ cell organization in the testis that differ from that of the laboratory rat and lesser bandicoot rats. In some regards they showed some similarity to that of humans, apes and New World primates (Chowdhury & Steinberger. 1976; Johnson *et al.*, 1992; Johnson, 1994a; Smithwick *et al.*, 1996; Wistuba *et al.*, 2003). Greater bandicoot rats have previously been found also to have highly derived and pleiomorphic cauda epididymal sperm populations (Breed, 1993, 1998) and, in the present study, the pleiomorphic nature of the sperm nuclei was also confirmed. Furthermore, previous transmission electron microscope studies have shown nuclear vacuoles were sometimes present in late spermatids, testicular spermatozoa, and epididymal spermatozoa and that there was a variable response between the spermatozoa in chromatin dispersion when cauda spermatozoa are incubated in detergents (Breed, 1998). Therefore, the present observations, together with those made previously, indicated that some of the processes of germ cell maturation and the final form of the sperm of the greater bandicoot rat showed some similarity to the situation in humans (Bedford *et al.*, 1973; Fawcett, 1975).

In conclusion, the greater bandicoot rat, that was a common and widespread species throughout much of southeast Asia (Corbett & Hill, 1992; Musser & Carleton, 1993), may turn out to be a useful model for investigations on the processes controlling spermatogenesis and sperm form due to the lack of tight synchrony in both germ cell maturation and final sperm form that occurs. Andrologists in southeast Asia may thus have a very useful model for probing questions on the regulatory processes of male germ cell morphogenesis and maturation both of which are extremely poorly understood phenomena at the present time (Sharp, 1994).

We have published one paper from these data in International Journal of Andrology (see the attached document).

Chromatin Condensation during Spermiogenesis

Although it has been known that the nuclear chromatin undergo high degree condensation during germ cell differentiation, mediating through the replacement of histones with a higher positive charged basic nuclear proteins, termed transition protein and protamine. Sperm of *B. indica* possessed a wide variety of head morphology, therefore, it would be interested to see the pattern of basic nuclear proteins and their localization in developing and differentiated germ cells.

Information of immunohistochemical analysis clearly demonstrated that the remaining of histones in late differentiated spermatids, and protamine was found mainly in late differentiated spermatid. This histone remnant was not coincident with

the presence of many nuclear vacuoles as was addressed by immunoTEM. Question is still remained whether what materials make up of the nuclear vacuoles and why there is still a considerably high level of histone in late differentiated germ cells in *B. indica*? We further confirmed that during germ cell development in the testis, there seem to be a gradual decrease of total histone proteins (observed by the intensity of Coomassie Blue staining when compared to epididymal sperm). Histones were also present in epididymal sperm in considerably high level. It was also apparent that there were transition protein and protamine in epididymal sperm, however, the amount of TP proteins seem to be much higher than protamine. This result was somewhat discrepancy to other mammals studied where protamine was shown to be a predominant basic protein in mature sperm. The onset of transition proteins and protamine was still unclear at the moment and need further investigation.
The experiments will be repeated before any firm conclusion is drawn.

We are preparing in manuscript for publication (about molecular level of chromatin condensation in B. indica) and will be submitted to Biology of Reproduction or Molecular Reproduction and Development.

REFERENCES

Allen, M. J., Bradbury, E. M., & Balhorn, R. (1997). AFM analysis of DNA-protamine complexes bound to mica. Nucleic Acids Research, 25, 2221-2226.

Amann, R. P. (1962). Reproductive capacity of dairy bulls. IV. Spermatogenesis and testicular germ cell degeneration. American Journal of Anatomy, 110, 69-78.

Balhorn, R. (1982). A model for the structure of chromatin in mammalian sperm. Journal of Cell Biology, 93, 298-305.

Balhorn, R., Weston, S., Thomas, C., & Wyrobek, A. J. (1984). DNA packaging in mouse spermatids. Synthesis of protamine variants and four transition proteins. Experimental and Cell Research, 150, 298-308.

Balhorn, R. (1989). Mammalian protamine: Structure and molecular interactions. In K. W. Adolph (Ed.), Molecular biology of chromosome function (pp. 366-395). New York: Springer Verlag.

Balhorn, R., Cosman, M., Thornton, K., Krishnan, V. V., Corzett, M., Bench, G., et al. (1999). Protamine mediated condensation of DNA in mammalian sperm. In C. Gagnon (Ed.), The male gamete: From basic science to clinical applications (pp. 55-70). Vienna: Cache River Press.

Bedford, J.M, Bent, M.J. & Calvin, H. (1973) Variations in the structural characters and stability of the nuclear chromatin in morphologically normal human spermatozoa. Journal of Reproduction and Fertility, 33, 19-29.

Bedford, J. M., & Calvin, H. I. (1974). The occurrence and possible functional significant of -S-S- crosslinks in the sperm, with particular reference to eutherian mammals. Journal of Experimental Zoology, 188, 137-158.

Bedford, J. M. (1982). Fertilization. In C. R. Austin & R. V. Short (Eds.), Reproduction in mammals: Germ cells and Fertilization (2 ed., Vol. 1, pp. 128-163). Cambridge: Cambridge University Press.

Bedford, J. M. (1991). The Coevolution of Mammalian Gametes. In B. S. Dunbar & M. G. O'Rand (Eds.), A Comparative Overview of Mammalian Fertilization (pp. 3-39). New York: Plenum Press.

Bilaspuri, G.S. & Kaur, R. (1994) Spermatogenic cells and stages of the seminiferous epithelial cycle in the Indian Gerbil Field Rat, Tatera indica. Reproduction Fertility and Development, 6, 699-704.

Boonsong, L., & McNeely, J. A. (1977). Mammals of Thailand. In (pp. 397-427). Ladprao, Bangkok: Kurusapha.

Breed, W.G. (1993) Novel organisation of the spermatozoon in two species of murid rodents from Southern Asia. Journal of Reproduction and Fertility, 99, 149-158.

Breed, W.G. (1998) Interspecific variation in structural organisation of the spermatozoon in the Asian Bandicoot rats. Bandicota species (family Muridae). Acta Zoologica, 79, 277-285.

Breed, W. G., & Taylor, J. (2000). Body mass, testis mass, and sperm size in murine rodents. Journal of Mammalogy, 81, 758-768.

Breed, W. G. (2004) The spermatozoon of Eurasian murine rodents: its morphological diversity and evolution. Journal of Morphology, 261, 52-69.

Chowdhury, A.K. & Steinberger, E. (1976) A study of germ cell morphology and duration of spermatogenic cycle in the baboon Papio anubis. Anatomical Record, 185, 155-170.

Clermont, Y. (1963). The cycle of the seminiferous epithelium in man. American Journal of Anatomy, 112, 35-51.

Clermont, Y. & Trott, M. (1969) Duration of the cycle of the seminiferous epithelium in the mouse and hamster determined by means of 3H-thymidine and radioautography. Fertility and Sterility, 20, 805-817.

Clermont, Y., & Antar, M. (1973). Duration of the cyle of the seminiferous epithelium and the spermatogonial renewal in the monkey Macaca arctoides. American Journal of Anatomy, 136, 153-166.

Clermont, Y., Oko, R., & Hermo, L. (1993). Cell biology of mammalian spermiogenesis. In C. Desjardins & L. L. Ewing (Eds.), *Cell and Molecular Biology of the Testis* (pp. 332-376). New York: Oxford University Press.

Corbett, G. B., & Hill, J. E. (1992). The mammals of the Indomalayan Region:A Systematic Review. In Natural History Museum Publications (pp. 488). Oxford: Oxford University Press.

Courtens, J.-L., & Loir, M. (1981). A cytochemical study of nuclear changes in boar, bull, goat, mouse, rat, and stallion spermatids. Journal of Ultrastructure Research, 74, 327-340.

Curtis, G. M. (1918). The morphology of the mammalian seminiferous tubule. American Journal of Anatomy, 24, 339-394.

Dadoune, J. P. (1991). Cytochemical variations in the nucleolus during spermiogenesis in man and monkey. Cell and Tissue Research, 264, 167-173.

Dang, D. C. (1971). Dure'e du cycle de l'e'pithe'lium se'minife're du singe crabier, Macaca fascicularis. Annales de Biologie Animale, Biochimie, Biophysique, 11, 373-377.

de Rooij, D. G., van Alphen, M. M. A., & van de Kant, H. J. G. (1986). Duration of the cycle of the seminiferous epithelium and its stages in the rhesus monkey (Macaca mulatta). Biology of Reproduction, 35, 587-591.

Fawcett, D.W. (1975) The Mammalian spermatozoon. Developmental Biology, 44, 394-436.

Golberg, R. B., Geremia, R., & Bruce, W. R. (1977). Histone synthesis and replacement during spermatogenesis in the mouse. Differentation, 7, 167-180.

Grocock, C.A. & Clarke, J.R. (1975) Spermatogenesis in mature and regressed testes of the vole (Microtus agretis). Journal of Reproduction and Fertility, 43, 461-470.

Grocock, C.A. & Clarke, J.R. (1976) Duration of spermatogenesis in the vole (Microtus agrestis) and bank vole (Clethrionomys glareolus). Journal of Reproduction and Fertility, 47, 133-135.

Hecht, N. B. (1989). Mammalian protamine and their expression. In L. Hnilica, G. Stein & J. Stein (Eds.), Histones and other basic nuclear proteins (pp. 347-373): Boca Raton: CRC Press.

Heller, C. G., & Clermont, Y. (1963). Spermatogenesis in man: an estimate of its duration. Science, 140, 184-186.

Heller, C. G., & Clermont, Y. (1964). Kinetics of germinal epithelium in man. Recent Prog. Horm. Res., 20, 545-575.

Hess, R. A., Schaeffer, D. J., Eroschenko, V. P., & Keen, J. E. (1990). Frequency of the stages in the cycle of the seminiferous epithelium in the rat. Biology of Reproduction, 43, 517-524.

Hilscher, W. (1964). Beitrage zur Orthologie und Pathologie der 'Spermatogoniogenese' der Ratte. Beitrage zur pathologischen Anatomie und allgemeinen Pathologie, 130, 69-132.

Holstein, A. F., & Roosen-Runge, E. C. (1981). *Atlas of human spermatogenesis*. Berlin: Grosse Verlag.

Holt, W. V., & Moore, H. D. M. (1984). Utrastructural aspects of spermatogenesis in the common marmoset (Callithrix jacchus). Journal of Anatomy, 138(1), 175-188.

Johnson, L., Petty, C.S. & Neaves, W.B. (1980) A comparative study of daily sperm production and testicular composition in humans and rats. Biology of Reproduction 22, 1233-1243.

Johnson, L., Chaturvedi, P. K., & Williams, J. D. (1992). Missing generations of spermatocytes and spermatids in seminiferous epithelium contribute to low efficiency of spermatogenesis in humans. Biology of Reproduction, 47, 1091-1098.

Johnson, L. (1994a) A new approach to study the architectural arrangement of spermatogenic stages revealed little evidence of a partial wave along the length of human seminiferous tubules. Journal of Andrology, 15, 435-441.

Johnson, L., Varner, D.D., Roberts, M.E., Smith, T.L., Keillor, G.E. & Scrutchfield, W.L. (2000) Efficiency of spermatogenesis: a comparative approach. Animal Reproduction Science 60-61, 471-480.

Kornberg, R. D., & Lorch, Y. (1999). Twenty-five years of the nucleosome, fundamental particle of the eukaryote chromosome. *Cell, 98*, 285-294.

Kurohmaru, M., Kobayashi, H., Hattori, S., Nishida, T., & Hayashi, Y. (1994). Spermatogenesis and ultrastructure of a peculiar acrosomal formation in the muck shrew, Suncus murinus. Journal of Anatomy, 185, 503-509.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227, 680-685.

Lalli, M., & Clermont, Y. (1981). Structural changes of the head components of the rat spermatid during late spermiogenesis. American Journal of Anatomy, 160, 419-434.

Leblond C.P. & Clermont Y. (1952) Spermiogenesis of rat, mouse, hamster and guinea pig as revealed by the 'periodic acid-fuchsin sulfurous acid' technique.Am.J.Anat. 90, pp.167-216

Lombardi, J. (1998). Gamete and their reproduction. In J. Lombardi (Ed.), *Comparative Vertebrate Reproduction* (pp. 109-130). London: Kluwer Academic Publishers.

Manochantr, S., Sretarugsa, P., Chavadej, C., & Sobhon, P. (2004). Chromatin organization and basic nuclear proteins in the male germ cells of Rana tigerina. Molecular reproduction and development, 9999, 1-14.

Meistrich, M. L., Trostle-Weige, P. K., & Van Beek, M. E. (1994). Separation of specific stages of spermatids from vitamin A-synchronized rat testes for assessment of nucleoprotein changes during spermiogenesis. Biology of Reproduction, 51, 334-344.

Munoz, E.M., Fogal, T., Dominguez, S., Scardapane, L., Guzman, J., Cavicchia, J.C. & Piezzi, R.S. (1998) Stages of the cycle of the seminiferous epithelium of the viscacha (Lagostomus maximus maximus). The Anatomical Record, 252, 8-16.

Musser, G. G., & Carleton, M. D. (1993). Family Muridae. In D. E. Wilson & D. M. Reeder (Eds.), Mammal Species of the World (2 ed., pp. 501-755). Washington D.C.: Smithsonian Institute.

Oakberg, E. F. (1956). Duration of spermatogenesis in the mouse and timing of stages of the cycle of the seminiferous epithelium. American Journal of Anatomy, 99, 507-516.

Oko, R., & Clermont, Y. (1988). Isolation, structure and protein composition of the perforatorium of rat spermatozoa. Biology of Reproduction, 39, 673-687.

Oko, R., Moussakova, L., & Clermont, Y. (1990). Regional differences in composition of the perforatorium and of the outer periacrosomal layer of rat spermatozoa as revealed by immunocytochemistry. American Journal of Anatomy, 188, 64-73.

Ortavant, R. (1954). Contribution a' l'e'tude de las dure'e du processus spermatoge'ne'tique du be'lier a' l'aide de P32. Comptes Rendus Socie'te' de Biologie, 148, 804-806.

Ortavant, R. (1956). Autoradiographie des cellules germinales du testucule de be'lier. Dure'e des phe'nome'nes spermatoge'ne'tiques. Archives d'Anatomie Microscopique et de Morphologie Experimentale, 45, 1-10.

Ortavant, R. (1959). Spermatogenesis and morphology of the spermatozoon. In H. H. Cole & P. T. Cupps (Eds.), Reproduction in Domestic Animals (Vol. 2, pp. 1-50). New York: Academic Press.

Oud, J.J. & de Rooij, D.G. (1977) Spermatogenesis in the Chinese hamster. The Anatomical Record, 187, 113-124.

Paula, T.A.R., Chiarini-Garcia, H. & Franca, L.R. (1999) Seminiferous epithelium cycle and its duration in capybaras (Hydrochoerus hydrochaeris). Tissue & Cell, 31, 327-334.

Peirce, E.J. & Breed, W.G. (1987) Cytological organization of the seminiferous epithelium in the Australian rodents Pseudomys australis and Notomys alexis. Journal of Reproduction and Fertility, 80, 91-103.

Perey, B., Clermont, Y. & Leblond, C.P. (1961) The wave of the seminiferous epithelium in the rat. American Journal of Anatomy, 108, 47-78.

Platz, R. D., Grimes, S. R., Meistrich, M. L., & Hnilica, L. S. (1975). Changes in nuclear proteins of rat testis cells separated by velocity sedimentation. Journal of Biological and Chemistry, 250, 5791-5800.

Ravindranath, N., Dettin, L., & Dym, M. (2003). Mammalian testes: structure and function. In D. Tulsiani (Ed.), *Introduction to mammalian reproduction* (pp. 1-19). Boston: Kluwer Acadamic Publishers.

Redi, C.A., Garagna, S., Heth, G. & Nevo, E. (1986) Descriptive kinetics of spermatogenesis in four chromosomal species of the Spalax ehrenbergi superspecies in Israel. The Journal of Experimental Zoology, 238, 81-88.

Roosen-Runge, E. C., & Giesel, L. O. (1950). Quantitative studies on spermatogenesis in the albino rat. American Journal of Anatomy, 87, 1-30.

Russell, L.D., Ettlin, R.A., Sinha Hikim, A.P. & Clegg, E.D. (1990) Histological and histopathological evaluation of the testis, pp. 286 Vienna, IL, Cache River Press.

Schuler, H.M. & Gier, H.T. (1976) Duration of the cycle of the seminiferous epithelium in the prairie vole (Microtus ochrogaster ochrogaster). Journal of Experimental Zoology 197, 1-11

Schulze, W. & Rehder, U. (1984) Organization and morphogenesis of the human seminiferous epithelium. Cell and Tissue Research, 237, 395-407.

Sharpe, R. M. (1994). Regulation of spermatogenesis. In E. Knobil & J. D. Neill (Eds.), The Physiology of Reproduction (2nd ed., pp. 1363-1434). New York: Raven Press.

Sinha Hikim, A.P., Maiti, B.R. & Ghosh, A. (1985) Spermatogenesis in the bandicoot rat. I. Duration of the cycle of the seminiferous epithelium. Archives of Andrology, 14, 151-154.

Sinha Hikim, A.P., Rajavashisth, T.B., Sinha Hikim, I., Lue, Y., Bonavera, J.J., Leung, A., Wang, C. & Swerdloff, R.S. (1997) Significance of apoptosis in the temporal and stage-specific loss of germ cells in the adult rat after gonadotropin deprivation. Biology of Reproduction, 57, 1193-1201.

Sinha Hikim, A.P., Wang, C., Lue, Y., Johnson, L., Wang, H.-H. & Swerdloff R.S. (1998) Spontaneous germ cell apoptosis in humans: evidence for ethnic differences in the susceptibility of germ cells to programmed cell death. Journal of Clinical Endocrinology and Metabolism, 83, 152-156.

Smithwick, E.B., Young, L.G. & Gould, K.G. (1996) Duration of spermatogenesis and relative frequency of each stage in the seminiferous epithelial cycle of the chimpanzee. Tissue and Cell, 28, 357-366.

Tait, A.J. & Johnson, E. (1982) Spermatogenesis in the grey squirrel (Sciurus carolinensis) and changes during sexual regression. Journal of Reproduction and Fertility, 65, 53-58.

Wanichanon, C., Weerachatyanukul, W., Suphamungmee, W., Meepool, A., Apisawetakan, S., Linthong, V., Sretarugsa, P., Chavadej, J., Sobhon, P. (2001). Chromatin condensation during spermiogenesis in rats. *Science Asia, 27*, 211-220.

Ward, W. S., Partin, A. W., & Coffey, D. S. (1989). DNA loop domains in mammalian spermatozoa. Chromosoma, 98, 153-159.

Ward, W. S., & Coffey, D. S. (1991). DNA packaging and organization in mammalian spermatozoa: Comparison with somatic cells. Biology of Reproduction, 44, 569-574.

Weinbauer, G.F., Aslam, H., Krishnamurthy, H., Brinkworth, M.H., Einspanier, A. & Hodges, J.K. (2001) Quantitative analysis of spermatogenesis and apoptosis in the common marmoset (Callithrix jacchus) reveals high rates of spermatogonial turnover and high spermatogenic efficiency. Biology of Reproduction, 64, 120-126.

Wistuba, J.A, Schrod, A., Grewe, B., Hodges, J.K., Aslam, H., Weinbauer, G.F. & Luitjens, C.M. (2003) Organisation of seminiferous epithelium in primates: relationship to spermatogenic efficiency, phylogeny, and mating system. Biology of Reproduction, 69, 582-591.

Yanagimachi, R. (1994). Mammalian fetilization, chapter 5. In Knobil E. & J. D. Neill (Eds.), The physiology of reproduction, Second edition (pp. 189-317). New York: Raven Press.

Zweidler, A. (1978). Resolution of histones by polyacrylamide gel electrophoresis in the presence of nonionic detergents. *Method Cell Biology, 17*, 223-233.

OUTPUT OF RESEARCH PROJECT

1. List of Publications

1.1 Unusual germ cell organization in the seminiferous epithelium of a murid rodent from southern Asia, the greater bandicoot rat, *Bandicota Indica*. In International Journal of Andrology, 2004.

P. Worawittayawong[1, 2], C.M. Leigh[1], G. Cozens[3], E.J. Peirce[1], B.P. Setchell[1], **P. Sretarugsa**[2], A. Dharmarajan[3], W.G. Breed[1]

[1]Department of Anatomical Sciences, The University of Adelaide, Australia
[2]Department of Anatomy, Mahidol University, Bangkok, Thailand
[3]School of Anatomy and Human Biology, The University of Western Australia, Australia (เอกสารหมายเลข 1)

1.2 Chromatin organization and basic nuclear proteins in the male germ cells of the greater bandicoot rat, *Bandicota indica*.

We are preparing in manuscript for publication and will be submitted to Biology of Reproduction or Molecular Reproduction and Development.

2. Application

This study will provide the better understanding of spermatogenesis in *Bandicoda indica* especially, the process of chromatin condensation during spermiogenesis. Interestingly, the basic nuclear proteins, histones are present in all stages of spermatids and mature sperm as well. Moreover, the transition proteins (TPs) are also found in the epididymal sperm and their amount seem to be much higher than protamines. This is in contrast to the other mammal such as rat, mouse and human which TPs are absent in these species.

Apart from this academic contribution, I do expect that this project will give a chance to the young scientist to gain more experience as well as having a critical thinking in synthesizing and analysing the scientific information.

3. List of presentations

3.1 Oral Presentation:

3.1.1 Is the highly divergent sperm nuclear shape in bandicoot rats due to an unusual process of chromatin condensation?

***P Worawittayawong**[1,2], **P Sretarugsa**[1] , C.M. Leigh[2] , W.G. Breed[2]

[1]Department of Anatomy, Mahidol University, Bangkok, Thailand
[2]Department of Anatomical Sciences, University of Adelaide, Adelaide, Australia
In the 3rd ASEAN Microscopy Conference and 19th Annual Conference of EMST on January 30th – February 1st, 2002 in Chiang Mai, Thailand (เอกสารหมายเลข 2)

3.1.2 Qualitative and Quantitative Production of Spermatozoa in the Asian Bandicoot Rat, *Bandicota indica*.

***P Worawittayawong**[1,2], C Leigh[1], E Peirce[1], **P Sretarugsa**[2], W Breed[1]

[1]Department of Anatomical Sciences, University of Adelaide, Adelaide, Australia
[2]Department of Anatomy, Mahidol University, Bangkok, Thailand
In the 49th AGM The Australian Mammal Society on 7-9 July 2003 at Sydney University, Australia (เอกสารหมายเลข 3)

3.2 Poster Presentation:

3.2.1 Is the highly divergent sperm nuclear shape in bandicoot rats due to an unusual process of chromatin condensation?

***P Worawittayawong**[1,2], **P Sretarugsa**[1] , C.M. Leigh[2] , W.G. Breed[2]

[1]Department of Anatomy, Mahidol University, Bangkok, Thailand
[2]Department of Anatomical Sciences, University of Adelaide, Adelaide, Australia
In RGJ- Ph.D. Congress III on 25-27 April 2002 at Jomtien Palm Beach Resort, Pattaya, Chonburi (เอกสารหมายเลข 4)

3.2.2 A possible cause for the presence of vacuoles in the nucleus of spermatids and spermatozoa of the bandicoot rat (*Bandicota indica*)

***P Worawittayawong**[1,2], C.M. Leigh[1], **P Sretarugsa**[2], W.G. Breed[1]

[1]Department of Anatomical Sciences, University of Adelaide, Adelaide, Australia
[2]Department of Anatomy, Mahidol University, Bangkok, Thailand
In RGJ- Ph.D. Congress V on 23-25 April 2004 at Jomtien Palm Beach Resort, Pattaya, Chonburi (เอกสารหมายเลข 5)

APPENDIX

I J A	5 2 9	B	Dispatch: 9.12.04	Journal: IJA	CE: Vd
Journal Name	Manuscript No.		Author Received:	No. of pages: 10	PE: Valarmathi

เอกสารหมายเลข 1

international journal of andrology, (2004) 10.1111/j.1365-2605.2004.00529.x

Unusual germ cell organization in the seminiferous epithelium of a murid rodent from southern Asia, the greater bandicoot rat, *Bandicota Indica*

P. WORAWITTAYAWONG,*† C. M. LEIGH,* G. COZENS,‡ E. J. PEIRCE,* B. P. SETCHELL,* P. SRETARUGSA,† A. DHARMARAJAN‡ and W. G. BREED*

*Department of Anatomical Sciences, The University of Adelaide, Australia, †Department of Anatomy, Mahidol University, Bangkok, Thailand, and ‡School of Anatomy and Human Biology, The University of Western Australia, Australia

Summary

In the greater bandicoot rat, *Bandicota indica*, of south-east Asia, nine cell associations were documented in the testicular seminiferous epithelium. In about 10% of the tubule cross sections two or more cell associations occurred and, furthermore, some of the generations of germ cells within the cell associations were sometimes either out of phase, or missing, in the tubule cross sections. These features, together with the fact that this species has a highly pleiomorphic sperm head shape, are somewhat reminiscent of those of the seminiferous epithelium in humans and some other primates but not of common laboratory rodents. This species could thus be a good model for investigating irregular patterns of spermatogenesis in naturally occurring wild species of rodent.

Keywords: bandicoot rat, seminiferous epithelium, apoptosis

Introduction

In sexually mature, adult, males of most mammalian species germ cell production occurs in a highly regulated and organized way with the resultant spermatozoa having a uniform, and species-specific, shape. In species such as the laboratory rat and mouse, as well as in farm animals, the maturing germ cells within the testicular seminiferous epithelium are organized into a series of characteristic cell associations of various maturational stages that occupy the entire cross-sectional area of a seminiferous tubule. At any one point along a tubule, there is a change in the cell associations over time with the length of time it takes to pass through all of the cell associations resulting in a cycle of the seminiferous epithelium (Leblond & Clermont, 1952; Hess, 1990; Russell *et al.*, 1990). Furthermore, along the length of a tubule at any one time, different cell associations are present with the consequence that a wave of the seminiferous epithelium occurs (Perey *et al.*, 1961). In the human testis however, the germ cell organization is quite different as cross-sections of the seminiferous tubules display multiple cell associations including some that have an atypical composition of germ cell developmental stages (Clermont, 1.2 1964; Heller & Clermont, 1964). Moreover, adjacently located cell associations do not precede or follow each other consecutively in the cycle of the seminiferous epithelium (Heller & Clermont, 1964), although some, but not all, workers believe that a spiral wave is present (Schulze & Rehder, 1984; Johnson, 1994a). Tubule cross-sections with more than one cell association or stage of the cycle of seminiferous epithelium are also common in some other primates including apes and New World monkeys (Smithwick *et al.*, 1996; Weinbauer *et al.*, 2001; Wistuba *et al.*, 2003). Germ cell production in humans is also less efficient than that in laboratory rats (Johnson *et al.*, 1980, 2000) with apoptosis occurring in spermatogonia and

Correspondence: W.G. Breed, Department of Anatomical Sciences, The University of Adelaide SA 5005, Australia. E-mail: bill.breed@adelaide.edu.au

primary spermatocytes as well as in spermatids (Sinha Hikim *et al.*, 1997, 1998). Additionally, in humans, unlike in common laboratory rodents, the final form of the spermatozoon is highly pleiomorphic with some sperm having nuclear vacuoles (Bedford *et al.*, 1973; Fawcett, 1975).

Cell associations within the testes have been described for various rodent species besides common laboratory mice and rats, including hamsters (Clermont & Trott, 1969; Oud & de Rooij, 1977), prairie voles (Schuler & Gier, 1976), field voles (Grocock & Clarke, 1975, 1976), bank voles (Grocock & Clarke, 1976), grey squirrels (Tait & Johnson, 1982), mole rats (Redi *et al.*, 1986), Asian gerbils (Bilaspuri & Kaur, 1994), viscachas (Munoz *et al.*, 1998), capybaras (Paula *et al.*, 1999), lesser bandicoot rats (Sinha Hikim *et al.*, 1985), plains rats (Peirce & Breed, 1987) and hopping mice (Peirce & Breed, 1987). In all of these species, with the exception of the hopping mouse *Notomys alexis* (Peirce & Breed, 1987), a similar germ cell organization to that of the laboratory rat and mouse was found to occur.

A study of the sperm morphology of the three species of Asian bandicoot rats, genus *Bandicota*, has shown that, whereas lesser bandicoot rats have a similar sperm morphology to that of laboratory rats, this is not the case for the greater bandicoot rat, *Bandicota indica* where a very different, and highly variable, sperm head and nuclear morphology is present (Breed, 1993, 1998, 2004). Because of this highly divergent sperm head shape and the fact that these sperm, like those of humans, shows a high degree of pleiomorphism, we, in this study, ask two related questions. First, 'What is the organization of the seminiferous epithelium?' and secondly, 'Do adult males of this species, like humans, show unusually high levels of germ cell apoptosis?'

Materials and methods

Animals

Greater Bandicoot rats, *B. indica*, were collected in Thailand from near Maesod, Tak Province in October 2001 ($n = 22$, 11 females, 11 males) and in Supanburi Province in January 2003 ($n = 21$, 10 females, 11 males). After returning the animals to the laboratory at Mahidol University, Bangkok, they were anaesthetized and their sex, weight and reproductive condition was determined. In the 2001 sample, two of the 11 females were pregnant and two of the 11 males had sperm in the cauda epididymides, whereas in the 2003 sample seven of the 10 females were pregnant, another had recently ovulated, and all 11 males had sperm in the cauda epididymides.

Tissue preparation

From the males with sperm, small pieces of one testis and epididymis were removed and rapidly fixed in 3% glutaraldehyde/3% paraformaldehyde made up in 0.1 M phosphate buffer, pH 7.4, whereas the other testis was fixed whole using the same fixative. The tissue was then returned to The University of Adelaide, South Australia, where the small pieces of testes were post-fixed in 1% osmium, dehydrated and embedded in epoxy resin. Larger pieces of fixed tissue were removed from the whole testis, dehydrated and embedded in paraffin wax. From the epoxy resin-embedded testis samples, 0.5–1 μm thick plastic sections were cut and stained with toluidine blue. From the paraffin-embedded blocks, 6–8 μm thick sections were cut and stained with either H & E or PAS and haematoxylin.

Organization and frequency of stages of the seminiferous epithelium

The testicular seminiferous epithelium in both epoxy-embedded and paraffin sections was analysed for the two animals with sperm collected in 2001 and four of the males collected in 2003. Nine cell associations were characterized, based on the appearance of the younger generation of spermatids within the seminiferous epithelium. The relative frequencies of the different cell associations, and the number of tubular cross-sections displaying more than one cell association, were determined in epoxy sections by scoring at least 130 tubular cross-sections from at least three different locations within the testis for each male.

Germ cell apoptosis

For determining the numbers and distribution of germ cells undergoing apoptosis terminal deoxynucleotide transfer-mediated dUTP nick end-labelling (TUNEL) was carried out on 6 μm thick paraffin sections using an ApopTag Peroxidase in situ apoptosis detection kit (Intergen Co., New York, USA) after 10-min incubation at room temperature for proteinase K digestion (see Lareu *et al.*, 2003).

Sperm morphology

For assessing the pleiomorphism in the sperm head nuclei, cauda sperm from four of the animals collected in January 2003 were placed on glass slides and then stained with dilute 3 propidium iodide. The slides were viewed with an Olympus BH epifluorescent microscope using a 515 nm excitation filter and an IFK 90 nm barrier filter with an absorption wavelength of 535 nm and an emission wavelength of 617 nm. Photographs of the nuclei of cauda epididymal 4 sperm were taken using an Olympus C-4040 zoom digital camera and 196–327 sperm randomly counted from each of these individuals and allocated to the various nuclear morphotypes.

Results

The body weights of the two 2001 individuals studied in detail with sperm were 655 and 561 g respectively and the average paired testes weight 2105 mg, whereas in the 2003 sample mean (±SE) body weight was 489 ± 55 g and paired testes weight 2.075 ± 287 mg ($n = 7$). As these testes

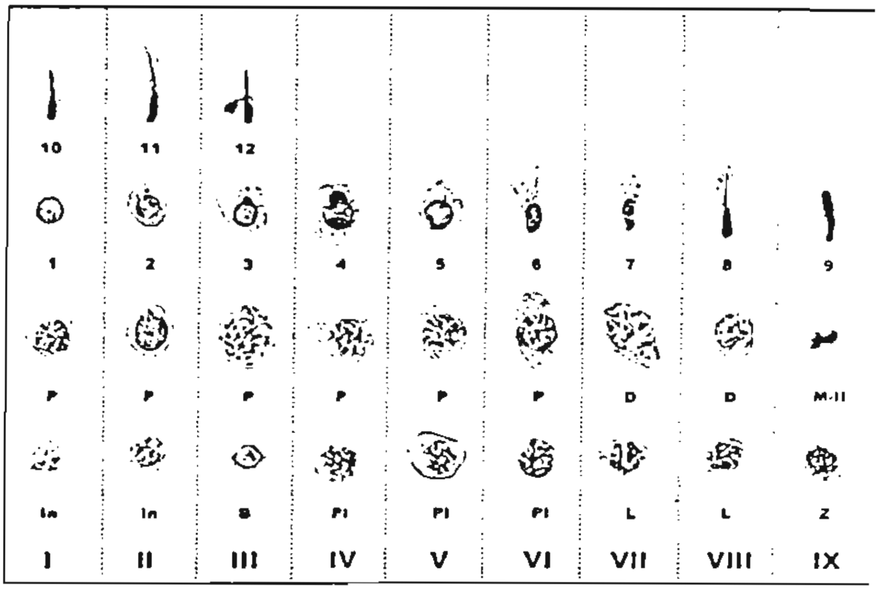


Figure 1. A map of the stages of the cycle of seminiferous epithelium in *Bandicota indica*, showing the phases of germ cell development. Type A spermatogonia have been omitted. In, B = intermediate, and B spermatogonia, Pl = preleptotene; L = leptotene, Z = zygotene; P = pachytene; D = diplotene primary spermatocytes; M-II = meiosis II; 1–12 = steps of spermatid maturation (for more details see text and Figs 2 and 3).

weights are similar to those of mature male greater bandicoot rats caught previously (Breed & Taylor, 2000), and some females in both the 2001 and 2003 samples were pregnant, it is assumed that the males investigated were sexually mature.

Stages of the cycle of the seminiferous epithelium

The classification system used to determine the germ cell associations was that based on the morphological appearance of the younger generation of spermatids in the cell associations with Stage I beginning at the completion of the second meiotic division (see Russell *et al.*, 1990). The cycle of the seminiferous epithelium was divided into 9 cell associations or stages based on spermatid characteristics. In cycle Stages I–III one generation of pachytene primary spermatocytes and two generations of spermatids were present, whereas Stages IV–VIII had two generations of primary spermatocytes but only one generation of spermatids. Stage IX was characterized by the presence of meiotic figures in the older population of spermatocytes or by the presence of secondary spermatocytes.

Morphology and organization of the germ cells of the various cycle stages as seen in the toluidine blue-stained epoxy sections are described below (see Fig. 1 for summary). The cell associations were also identified in PAS and haematoxylin-stained paraffin sections, however cellular preservation and detail were not as good as in the plastic sections and thus the latter were used for describing the cell associations. Type A spermatogonia were present in all cell associations; they were flattened cells displaying extensive cytoplasmic contact with the basement membrane of the seminiferous tubule and had an ovoid nucleus that occupied a major portion of the cell.

Stage I was defined by the presence of small round spermatids without a visible Golgi complex or acrosomal vesicle (Fig. 2a). Type A and occasional intermediate spermatogonia or spermatogonia undergoing mitosis were present at the base of the seminiferous epithelium. The nuclei of intermediate spermatogonia were characterized by patches of heterochromatin around the nuclear envelope (Fig. 2a) whereas those of type A spermatogonia contained little heterochromatin. Pachytene primary spermatocytes had chromatin condensed into thick strands and, above them several layers of early spermatids with round nuclei were present. Situated between, and above, the round spermatids were groups of elongated spermatids in which there were intensely stained nuclei with acrosomes that were generally oriented towards the basement membrane (Fig. 2a).

Stage II was defined by the presence of round spermatids with a Golgi complex adjacent to the nucleus. Acrosomal vesicles or granules were present within the spermatid cytoplasm, but had little contact with the nuclear envelope (arrow – Fig. 2b). Intermediate spermatogonia were present. Round, pachytene primary spermatocytes contained large central, round nuclei with condensed, tightly packed, chromosomes. Early spermatids with central round nuclei were located above the pachytene primary spermatocytes and elongated late spermatids were present, with some of the nuclei having an apical extension (arrowhead – Fig. 2b) or an indented nuclear envelope (asterisk – Fig. 2b). The acrosomes of late spermatids were stained lighter than the nuclei and generally remained oriented towards the basement membrane.

Stage III was characterized by enlargement of the acrosome of the round spermatids. During this stage, the acrosome was round, attached to the anterior pole of the nucleus and spread over about 15° of the nuclear surface (Fig. 2c). Type B spermatogonia were present and characterized by having nuclei with an ovoid to round profile that displayed intensely stained chromatin along the nuclear envelope (arrowhead – Fig. 2c). There were two to three

 Doi: 10.1111/j.1365-2605.2004.00529.x

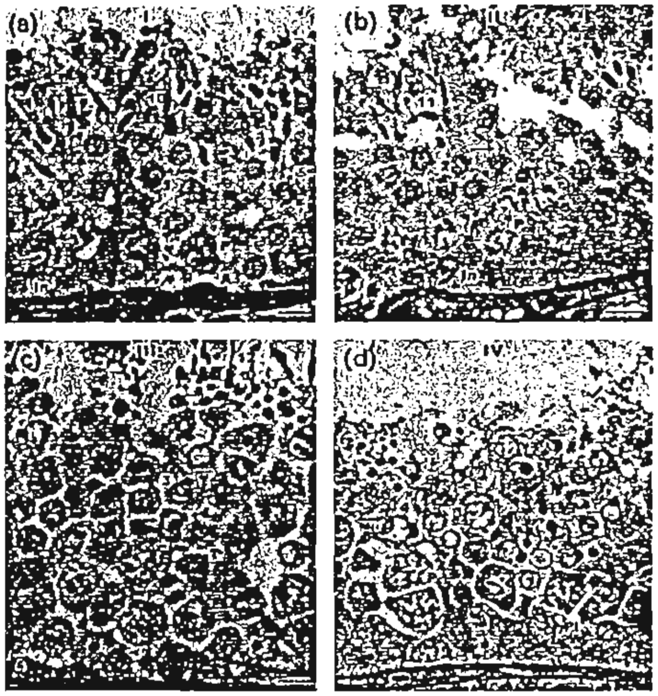


Figure 2. The stages of the cycle of seminiferous epithelium in *Bandicota indica* as shown in plastic sections stained with toluidine blue. (a)–(d) Stages I–IV respectively. A, In, B = A, intermediate, and B spermatogonia; Pl = preleptotene primary spermatocyte; L = leptotene primary spermatocyte; Z = zygotene primary spermatocyte; P = pachytene primary spermatocyte; D = diplotene primary spermatocyte; 1–12 = steps of spermatid maturation. All scale bars = 10 μm.

layers of pachytene primary spermatocytes that had round, heterochromatic, nuclei. Several layers of early round spermatids were located above the pachytene primary spermatocytes and along their nuclear envelopes in some regions there was intensely stained chromatin (Fig. 2c, arrow). The round spermatids were oriented in various directions, in some the acrosome was directed towards the basement membrane whereas in others it faced the lumen (Fig. 2c). In all round spermatids, the acrosome stained strongly with toluidine blue. Late spermatids had their heads located close to the luminal surface of the seminiferous epithelium. In these cells the chromatin was highly condensed and abundant residual bodies were present around the perimeter of the lumen of the seminiferous tubule (Fig. 2c) suggesting the shedding of considerable amounts of germ cell cytoplasm at this time.

In Stage IV the acrosome of the early spermatids was larger than that observed in Stage III and, in some cases, it had become somewhat oval in shape. It extended from 15° to about 45° over the nuclear surface. The spermatid nuclei were mostly round, with intensely stained chromatin along the nuclear envelope (arrow – Fig. 2d) although, in some, the nuclear envelope was flattened beneath the acrosome. These spermatids were located close to the lumen and oriented in various directions. During this stage, mitosis of Type B spermatogonia to form preleptotene primary spermatocytes occurred. The nuclei of the latter cells were generally round in shape, and contained lightly stained fine chromatin threads. Pachytene primary spermatocytes were larger than preleptotene primary spermatocytes and were irregular in shape. Late spermatids had been released into the lumen and were generally not evident although abundant residual bodies could still be seen close to the tubule lumen.

Stage V was characterized by spermatids having an acrosome that was crescent shaped and covered about a quarter of the anterior surface of the nucleus (Fig. 3a). Their nuclei were round or slightly ovoid with lightly stained chromatin. Many residual bodies remained near the luminal surface during this stage. The acrosome, which had reached its maximum size, was very intensely stained and in most cases oriented towards the basement membrane. Preleptotene primary spermatocytes, which were round with central nuclei and lightly stained chromatin, were generally present, whereas the pachytene primary spermatocytes, that were also present, had round nuclei with dense heterochromatin.

Some of the spermatids of Stage VI had nuclei that were a little bilaterally elongated. They were located near the lumen and had homogeneously stained chromatin. The acrosome

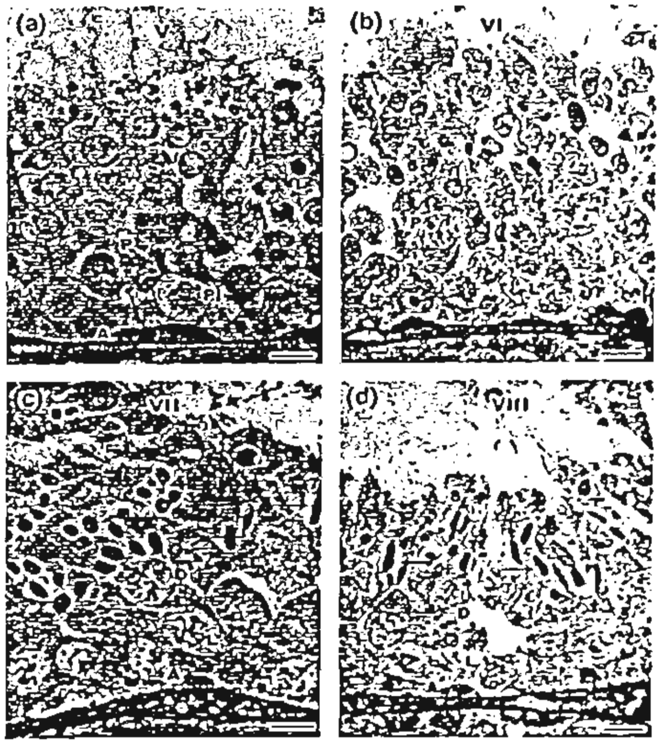


Figure 3. The stages of the cycle of seminiferous epithelium in *Bandicota indica* as shown in plastic sections stained with toluidine blue. (a)–(d) Stages V–VIII respectively. A, In, B = A, intermediate, and B spermatogonia, Pl = preleptotene primary spermatocyte; L = leptotene primary spermatocyte, Z = zygotene primary spermatocyte; P = pachytene primary spermatocyte, D = diplotene primary spermatocyte; 1–12 = steps of spermatid maturation. All scale bars = 10 μm.

was intensely stained and oriented towards the base of the tubule. In these cells the cytoplasm had migrated posteriorly (arrow – Fig. 3b) resulting in the acrosome being generally close to the plasma membrane. Leptotene primary spermatocytes occurred, which were round cells with large round nuclei and tightly packed threads of chromatin. Either pachytene, or occasionally diplotene, primary spermatocytes were present and generally ovoid in shape. Their nuclei were round and contained conspicuous chromatin threads.

In Stage VII, the spermatid nuclei were reduced in size compared with those in Stage VI and they stained more strongly with toluidine blue. They were generally oriented towards the basal lamina and the chromatin was condensed along the nuclear envelope (arrow – Fig. 3c). The acrosome covered the anterior region of nucleus and was deeply stained. Leptotene primary spermatocytes contained round central nuclei with thicker chromatin threads. Diplotene primary spermatocytes were irregular in shape, had spherical nuclei, and were larger in diameter than the pachytene primary spermatocytes at the previous stage. Their chromatin was loosely packed and most of the nucleus was only weakly stained (Fig. 3c).

Stage VIII had spermatids that were elongated and had more strongly stained chromatin than in the previous stage. They were embedded in recesses of the cell membrane of the Sertoli cells and oriented towards the basal lamina. Nuclear vacuoles could be seen (arrowhead – Fig. 3d). Most of the late spermatids had nuclei with apical extension(s) (arrows – Fig. 3d), and acrosomes that were lightly stained. Leptotene and diplotene primary spermatocytes had similar morphologies to those of Stage VII.

Stage IX began with the diplotene spermatocytes undergoing meiosis to form early round spermatids and ended with the completion of the second meiotic division. Zygotene primary spermatocytes were present and round in shape; the strongly stained nuclei showed more tightly packed chromatin than those of leptotene primary spermatocytes. The late spermatids were elongated and located near the lumen, and their chromatin had condensed along the nuclear envelope. The acrosome was strongly stained.

Frequencies (%) of the stages of cycle of the seminiferous epithelium

The frequency of each of the nine cell associations is presented in Table 1. The total number of seminiferous tubule cross sections counted was 1230 from six animals, two of which were collected in 2001 and the other four in 2003. Stage II occurred most often, with an average frequency of 28.5% (range 11.1–41.6%), whereas Stage VIII was the least frequent, and was observed in only three of six animals with a range of 1–3.7%. In three males (Bi 2–003, Bi 5–001, Bi 14–001) Stage II was the most frequent, whereas Stage V was the most common in Bi 7–003, Bi 8–003 and Bi 11–003. Although some variation in the most frequent cell associations was found between animals, in all cases Stages VII, VIII

Table 1. Number and frequency (%) of each germ cell association in seminiferous tubule cross sections of greater bandicoot rats

Animal no.	Number and frequency (%) of tubules with typical cell associations									Total no. with typical cell associations	No. with atypical associations (%)	No. with multi-stage associations (%)
	I	II	III	IV	V	VI	VII	VIII	IX			
Bi 5-001	25 (11.2)	65 (29.0)	36 (16.1)	45 (20.1)	21 (9.4)	8 (3.6)	4 (1.8)	0	20 (8.9)	224	18 (6.5)	34 (12.3)
Bi 14-001	22 (11.9)	77 (41.6)	36 (19.5)	23 (12.4)	19 (10.3)	6 (3.2)	1 (0.5)	0	1 (0.5)	185	62 (22.7)	26 (9.5)
Bi 2-003	26 (11.4)	76 (33.3)	25 (11.0)	47 (20.6)	15 (6.6)	14 (6.1)	4 (1.8)	3 (1.3)	18 (7.9)	228	6 (2.3)	32 (12.0)
Bi 7-003	10 (9.8)	21 (20.6)	14 (13.7)	20 (19.6)	22 (21.6)	11 (10.8)	0	1 (1.0)	3 (2.9)	102	12 (8.5)	27 (19.1)
Bi 8-003	7 (6.5)	12 (11.1)	16 (14.8)	16 (14.8)	26 (24.1)	24 (22.2)	2 (1.9)	4 (3.7)	1 (0.9)	108	22 (15.5)	12 (8.3)
Bi 11-003	11 (10.8)	20 (19.6)	17 (16.7)	12 (11.8)	25 (24.5)	10 (9.8)	6 (5.9)	0	1 (1.0)	102	21 (15.9)	9 (6.8)
Total (mean)	101 (10.6)	271 (28.5)	144 (15.2)	163 (19.1)	128 (13.5)	73 (7.7)	17 (1.8)	8 (0.8)	44 (4.6)	949	141 (11.5)	140 (11.4)

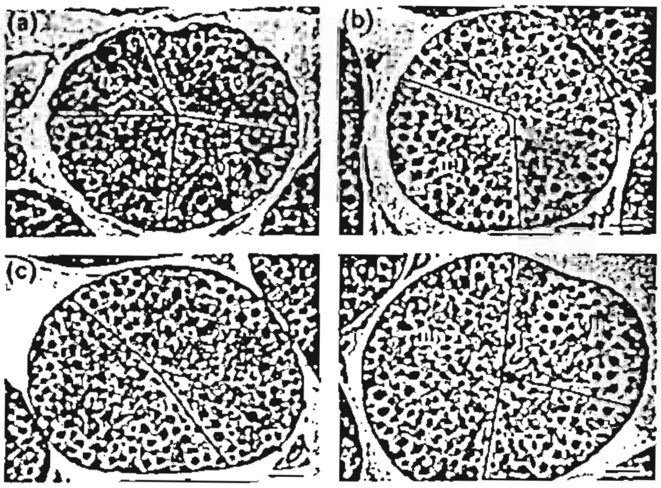


Figure 4. Seminiferous tubules of *Bandicota indica* testis containing more than one cell association in the same cross-section (a) Stages V, VI and IX, (b) Stages II and IV, (c) Stages I and V and (d) Stages III, IV and VII. All scale bars = 25 μm

and IX each comprised <10% of the total number of tubule cross sections and Stage I never exceeded 12% of the total.

In 140 of 1230 tubule cross-sections examined, more than one cell association was present in the tubule cross section. Such tubule cross-sections occurred with an average frequency of 11.4% (range 6.8–19.1% for the different individuals). The most common mixed cell associations in the tubule cross sections were either Stages I and IX, V and VI, or III and IV, but many combinations of cell associations (see Fig. 4a–d) were found to occur occasionally. Three, or even four, stages were present in a single cross section in 14 of the tubule cross-sections examined, e.g. in Bi 2-003 there were Stages V, VI and IX in one tubule cross section (see Fig. 4a).

In 141 of the 1230 tubule cross sections examined there was at least one germ cell population that was different from that normally seen within the cycle stage, with the result that atypical cell associations were present. Such associations occurred in an average of 11.5% (range 2.3–22.7%) of tubules examined for a particular individual. Animals Bi 8-003, Bi 11-003, and Bi 14-001 all had >15% tubule cross-sections with atypical cell associations (Table 1). Most abnormalities were associated with cell association IV. Normally in this stage there is no highly condensed spermatid population close to the lumen as recent release of spermatozoa had generally occurred. However, in some tubule cross sections, late spermatids were still present, and preleptotene primary spermatocytes were absent. In other cases of atypical cell associations a generation of germ cells was absent from the typical cell association (e.g. in Stage III where there were no late spermatids in the cross section of one tubule), or development of a spermatid population appeared to be delayed with respect to the other germ cell types present. For example, Step 9 spermatids generally occurred in Stage IX when the older generation of spermatocytes was undergoing the meiotic divisions but in a few tubule cross-sections more immature spermatids were seen to occur in conjunction with the meiotic figures.

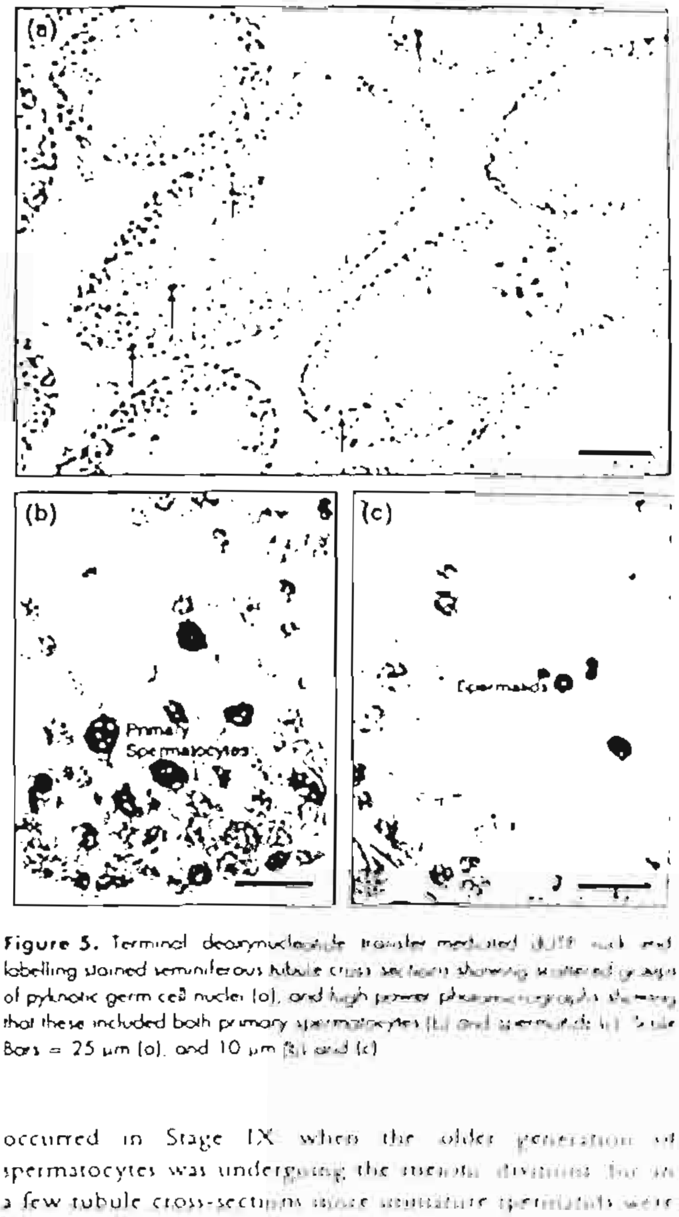


Figure 5. Terminal deoxynucleotide transfer mediated dUTP nick end labelling stained seminiferous tubule cross sections showing scattered groups of pyknotic germ cell nuclei (a), and high power photomicrographs showing that these included both primary spermatocytes (b) and spermatids (c). Scale Bars = 25 μm (a), and 10 μm (b) and (c)

The TUNEL results showed that occasional germ cells were undergoing apoptosis at the time the animals were killed. These cells either occurred singly or as small groups in a particular tubule cross section (Fig. 5a). Although the cells most frequently undergoing apoptosis were leptotene and zygotene primary spermatocytes (Fig. 5b), occasionally apoptotic spermatogonia and spermatids were also seen (Fig. 5c).

Nuclear pleomorphism of mature spermatozoa

Fluorescent microscopy, after staining with the DNA dye propidium iodide, indicated a range of different nuclear shapes occurred, the most frequent of which are shown in Fig. 6. Table 2 shows the frequency of the seven different nuclear morphotypes that were most commonly seen. It is clear that in all individuals a range of different nuclear shapes

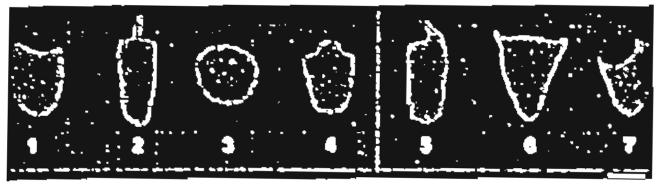


Figure 6. Fluorescence microscopy of nuclei of cauda epididymal sperm from one *Bandicota indica* adult male stained with propidium iodide showing highly variable nuclear shape. Scale bar = 2.8 µm

were evident. In Bi 4-003 and Bi 7-003 morphotype 1 was the most frequent, whereas in Bi 6-003 morphotype 2 predominated, whereas in Bi 11-003 morphotype 3 was by far the most common.

Discussion

Although there is some basic knowledge of the oestrous cycle, pregnancy length and litter size of female greater bandicoot rats (Boonsong, 1984), and the sperm morphology of males (Breed, 1993; 1998), the organization of male germ cells in the testis has not been described for this species. In the present study we have found that the germ cell organization during the cycle of seminiferous epithelium in greater bandicoot rats is more variable that of both laboratory rodents and a closely related species, the lesser bandicoot rat (Sinha Hikim *et al.*, 1985).

In cross sections of seminiferous tubules of the laboratory rodents, as well as of the lesser bandicoot rat, the same cell association generally occupies an entire tubule cross section. In greater bandicoot rats, by contrast, this is sometimes not the case. In all of the individuals of this species that were investigated, around 10% of the tubule cross-sections contained two or more cell associations. In some of these multi-stage tubular cross-sections, the cell associations present were sequential in the cycle of the seminiferous epithelium, and thus represent the spatial transition from one stage to the next. Such an organization is consistent with a segmental arrangement of associations along the length of a tubule as occurs in the laboratory rat (Perey *et al.*, 1961; Russell *et al.*, 1990). In the other multi-stage cross sections however, cell associations adjacent to each other in the epithelium were non-sequential. The organization within these tubular cross sections therefore somewhat resembles that of humans (Clermont, 1963; Schulze & Rehder, 1984; Johnson, 1994b), and some other primate species (Chowdhury & Steinberger, 1976; Smithwick *et al.*, 1996; Wistuba *et al.*, 2003) in which cell associations are confined to smaller regions of the epithelium and may have a spiral wave, in contrast to that of common laboratory rodents or the other species of bandicoot rat, *B. bengalensis*.

The organization of the seminiferous epithelium in the testis of greater bandicoot rats also differs from that of the laboratory rodents and lesser bandicoot rats with respect to the constancy of germ cell composition of particular cell associations. Whereas in the latter species particular maturational stages of germ cells nearly always occur in synchrony with other specific maturational stages (Leblond & Clermont, 1952; Clermont, 1972), in the greater bandicoot rats between 2 and 23% (average 13%) of the tubule cross sections had at least one group of maturing germ cells within a cell association that was out of synchrony with the rest and, occasionally, a particular generation of germ cells appeared to be completely missing. Individual cell associations occupying smaller areas of the epithelium and the mixing of germ cells between associations at their physical boundaries may partially explain an increased incidence of atypical cell associations in this species, however it can not account for missing germ cell generations. Missing germ cell generations could reflect a lack of spermatogonial divisions during a particular cycle, or the death of an entire cohort of cells such that no cells of a particular type are encountered within a section of tissue. Why this might occur more often in this species however, remains to be determined.

In the atypical cell associations observed, the germ cell population most commonly out of synchrony with the other cell populations was the most mature spermatid population. The relatively common occurrence of elongated spermatids in stage IV indicates a delay in spermiation, although spermatids at an earlier step in spermiogenesis than that in a typical cell association also occurred. A delay or arrest in spermatid development, resulting in atypical cell associations,

Table 2. Numbers of sperm nuclei in the various different shape categories shown in Fig. 6 for four individual greater bandicoot rats

Animal no.	N^a	Sperm nuclear shapes (see Fig. 6)[b] 1	2	3	4	5	6	7
Bi 4-003	289	92 (32%)	55 (19%)	29 (10%)	14 (5%)	35 (12%)	12 (4%)	52 (18%)
Bi 6-003	186	13 (7%)	73 (39%)	0	56 (30%)	26 (14%)	9 (5%)	9 (5%)
Bi 7-003	327	108 (33%)	0	26 (8%)	56 (17%)	26 (8%)	36 (11%)	75 (23%)
Bi 11-003	276	41 (15%)	14 (5%)	215 (78%)	0	6 (2%)	0	0

[a]Total number of sperm counted for each individual
[b]Numbers refer to head shape shown in Fig. 6

has been documented during the establishment of spermatogenesis in pubertal golden hamsters (Miething, 1998), but disappears as Leydig cell maturation is completed. However, both groups of male bandicoot rats we obtained were collected at the same place and time as pregnant females. Furthermore, since the body and testes weights were typical of sexually mature, adult males of the species (Breed & Taylor, 2000), we thus assume that these males were sexually mature and not just entering the onset of spermatogenesis. Another possible explanation for spermatid variability within an association in this species relates to the pleiomorphism observed in mature spermatozoa. Perhaps differences in the appearance of the elongated spermatid populations in some associations reflect variability in the nuclear form of the spermatozoa.

Several possible explanations have been put forward for the synchronous development of different generations of germ cells within a cell association, including interactions between the germ cells and Sertoli cells that regulate development, factors produced by more mature germ cell populations influencing the development of younger cell generations, and simultaneous activation of different developmental events in the germ cell lineage at a specific locality by periodic stimuli (Russell *et al.*, 1990). Whatever the cause, from its organization, it appears that maturation of germ cells within the seminiferous epithelium of the greater bandicoot rat is not be as tightly synchronized as that of common laboratory rats (Russell *et al.*, 1990) and lesser bandicoot rats (Sinha Hikim *et al.*, 1985). Furthermore, when TUNEL was carried out, it was found that occasional apoptotic cells, including spermatogonia, primary spermatocytes and spermatids, were scattered throughout the germinal epithelium. This is similar to the situation in human testes (Brinkworth *et al.*, 1997; Sinha Hikim *et al.*, 1998), but unlike that in the testes of laboratory rats and mice (Brinkworth *et al.*, 1995; Sinha Hikim *et al.*, 1997; Krishnamurthy *et al.*, 1998).

This study thus shows that this species of bandicoot rat, *B. indica*, has several features of the germ cell organization in the testis that differ from that of the laboratory rat and lesser bandicoot rats. In some regards they show some similarity to that of humans, apes and New World primates (Chowdhury & Steinberger, 1976; Johnson *et al.*, 1992; Johnson, 1994a; Smithwick *et al.*, 1996; Wistuba *et al.*, 2003). Greater bandicoot rats have previously been found also to have highly derived and pleiomorphic cauda epididymal sperm populations (Breed, 1993, 1998) and, in the present study, the pleiomorphic nature of the sperm nuclei was also confirmed. Furthermore, previous transmission electron microscope studies have shown nuclear vacuoles were sometimes present in spermatids and epididymal spermatozoa and that there is a variable response between the spermatozoa in chromatin dispersion when cauda spermatozoa are incubated in detergents (Breed, 1998). Therefore, the present observations, together with those made previously, indicate that some of the processes of germ cell maturation and the final form of the sperm of the greater bandicoot rat show some similarity to the situation in humans (Bedford *et al.*, 1973; Fawcett, 1975).

In conclusion, the greater bandicoot rat, that is a common and widespread species throughout much of south-east Asia (Corbett & Hill, 1992; Musser & Carleton, 1993), may turn out to be a useful model for investigations on the processes controlling spermatogenesis and sperm form because of the lack of tight synchrony in both germ cell maturation and final sperm form that occurs. Andrologists in south-east Asia may thus have a very useful model for probing questions on the regulatory processes of male germ cell morphogenesis and maturation both of which are extremely poorly understood phenomena at the present time (Sharpe, 1994).

Acknowledgements

This work was in part funded by a grant from the Thailand Research Fund through the Royal Golden Jubilee PhD Program (Grant No. PHD/00196/2541) and in part by the Australian Research Council and The University of Adelaide. We thank Leigh Clark for carrying out pilot work on TUNEL for apoptosis and Fiona Khor for initial studies on the germ cell associations of this species.

References

Bedford, J. M., Bent, M. J. & Calvin, H. (1973) Variations in the structural characters and stability of the nuclear chromatin in morphologically normal human spermatozoa. *Journal of Reproduction and Fertility* 33, 19–29.

Bilaspuri, G. S. & Kaur, R. (1994) Spermatogenic cells and stages of the seminiferous epithelial cycle in the Indian Gerbil Field Rat, *Tatera indica*. *Reproduction Fertility and Development* 6, 699–704.

Boonsong, P. (1984) Some biological aspects of the great bandicoot (*Bandicota indica* Bechstein). *Agricultural Research Journal* 2, 135–145.

Breed, W. G. (1993) Novel organisation of the spermatozoon in two species of murid rodents from southern Asia. *Journal of Reproduction and Fertility* 99, 149–158.

Breed, W. G. (1998) Interspecific variation in structural organisation of the spermatozoon in the Asian Bandicoot rats, *Bandicota* species (family Muridae). *Acta Zoologica* 79, 277–285.

Breed, W. G. (2004) The spermatozoon of Eurasian murine rodents: its morphological diversity and evolution. *Journal of Morphology* 261, 52–69.

Breed, W. G. & Taylor, J. (2000) Body mass, testis mass, and sperm size in murine rodents. *Journal of Mammalogy* 81, 758–768.

Brinkworth, M. H., Weinbauer, G. F., Schlatt, S. & Nieschlag, E. (1995) Identification of male germ cells undergoing apoptosis in adult rats. *Journal of Reproduction and Fertility* 105, 25–33.

Brinkworth, M. H., Weinbauer, G. F., Bergmann, M. & Nieschlag, E. (1997) Apoptosis as a mechanism of germ cell loss in elderly men. *International Journal of Andrology* 20, 222–228.

Chowdhury, A. K. & Steinberger, E. (1976) A study of germ cell morphology and duration of spermatogenic cycle in the baboon *Papio anubis*. *Anatomical Record* 185, 155–170.

Clermont, Y. (1963) The cycle of the seminiferous epithelium in man. *American Journal of Anatomy* 112, 35–51.

Clermont, Y. (1972) Kinetics of spermatogenesis in mammals, seminiferous epithelium cycle and spermatogonial renewal. *Physiology Reviews* 52, 198–236.

Clermont, Y. & Trott, M. (1969) Duration of the cycle of the seminiferous epithelium in the mouse and hamster determined by means of 3H-thymidine and radioautography. *Fertility and Sterility* 20, 805–817.

Corbett, G. B. & Hill, J. E. (1992) *The Mammals of the Indomalayan Region: A Systematic Review*, pp. 488. Oxford University Press, Oxford.

Fawcett, D. W. (1975) The Mammalian spermatozoon. *Developmental Biology* 44, 394–436.

Grocock, C. A. & Clarke, J. R. (1975) Spermatogenesis in mature and regressed testes of the vole (*Microtus agrestis*). *Journal of Reproduction and Fertility* 43, 461–470.

Grocock, C. A. & Clarke, J. R. (1976) Duration of spermatogenesis in the vole (*Microtus agrestis*) and bank vole (*Clethrionomys glareolus*). *Journal of Reproduction and Fertility* 47, 133–135.

Hess, R. A. (1990) Quantitative and qualitative characteristics of the stages and transitions in the cycle of the rat seminiferous epithelium: light microscopic observations of perfusion-fixed and plastic-embedded testes. *Biology of Reproduction* 43, 525–542.

Johnson, L. (1994a) A new approach to study the architectural arrangement of spermatogenic stages revealed little evidence of a partial wave along the length of human seminiferous tubules. *Journal of Andrology* 15, 435–441.

Johnson, L. (1994b) Efficiency of spermatogenesis. *Microscopy Research and Technique* 32, 385–422.

Johnson, L., Petty, C. S. & Neaves, W. B. (1980) A comparative study of daily sperm production and testicular composition in humans and rats. *Biology of Reproduction* 22, 1233–1243.

Johnson, L., Chaturvedi, P. K. & Williams, J. D. (1992) Missing generations of spermatocytes and spermatids in seminiferous epithelium contribute to low efficiency of spermatogenesis in humans. *Biology of Reproduction* 47, 1091–1098.

Johnson, L., Varner, D. D., Roberts, M. E., Smith, T. L., Keillor, G. E. & Scrutchfield, W. L. (2000) Efficiency of spermatogenesis: a comparative approach. *Animal Reproduction Science* 60–61, 471–480.

Krishnamurthy, H., Weinbauer, G. F., Aslam, H., Yeung, C.-H & Nieschlag, E. (1998) Quantification of apoptotic testicular germ cells in normal and methoxyacetic acid-treated mice as determined by flow cytometry. *Journal of Andrology* 19, 71–717

Lareu, R. R., Lacher, M. D., Bradley, C. K., Sridaran, R., Friis, R. R. & Dharmarajan, A. M. (2003) Regulated expression of inhibitor of apoptosis protein 3 in the rat corpus luteum. *Biology of Reproduction* 68, 2232–2240.

Leblond, C. P. & Clermont, Y. (1952) Definition of the stages of the cycle of seminiferous epithelium in the rat. *Annals of the New York Academy of Science* 55, 548–573.

Miething, A. (1998) The establishment of spermatogenesis in the seminiferous epithelium of the pubertal golden hamster (*Mesocricetus auratus*). In: *Advances in Anatomy, Embryology and Cell*
Q7 *Biology*, Vol. 40. Springer, Berlin.

Munoz, E. M., Fogal, T., Dominguez, S., Scardapane, L., Guzman, J., Cavicchia, J. C. & Piezzi, R. S. (1998) Stages of the cycle of the seminiferous epithelium of the viscacha (*Lagostomus maximus maximus*). *The Anatomical Record* 252, 8–16.

Musser, G. G. & Brothers, E. M. (1994) Identification of bandicoot rats from Thailand (Bandicota, Muridae, Rodentia) *American*
Q8 *Museum Novitates* 3110, 1–56

Musser, G. G. & Carleton, M. D. (1993) Family Muridae. In *Mammal Species of the World*, 2nd edn (eds D. E. Wilson & D. M. Reeder), pp. 501–755. Smithsonian Institute, Washington, DC, USA.

Oud, J. J. & de Rooij, D. G. (1977) Spermatogenesis in the Chinese hamster. *The Anatomical Record* 187, 113–124.

Paula, T. A. R., Chiarini-Garcia, H. & Franca, L. R. (1999) Seminiferous epithelium cycle and its duration in capybaras (*Hydrochoerus hydrochaeris*). *Tissue & Cell* 31, 327–334.

Peirce, E. J. & Breed, W. G. (1987) Cytological organization of the seminiferous epithelium in the Australian rodents *Pseudomys australis* and *Notomys alexis*. *Journal of Reproduction and Fertility* 80, 91–103.

Perey, B., Clermont, Y. & Leblond, C. P. (1961) The wave of the seminiferous epithelium in the rat. *American Journal of Anatomy* 108, 47–78.

Redi, C. A., Garagna, S., Heth, G. & Nevo, E. (1986) Descriptive kinetics of spermatogenesis in four chromosomal species of the *Spalax ehrenbergi* superspecies in Israel. *The Journal of Experimental Zoology* 238, 81–88.

Russell, L. D., Ettlin, R. A., Sinha Hikim, A. P. & Clegg, E. D. (1990) *Histological and Histopathological Evaluation of the Testis*, pp 286. Cache River Press, Vienna, IL, USA.

Schuler, H. M. & Gier, H. T. (1976) Duration of the cycle of the seminiferous epithelium in the prairie vole (*Microtus ochrogaster ochrogaster*). *Journal of Experimental Zoology* 197, 1–11.

Schulze, W. & Rehder, U. (1984) Organization and morphogenesis of the human seminiferous epithelium. *Cell and Tissue Research* 237, 395–407.

Sharpe, R. M. (1994) Regulation of spermatogenesis. In: *The Physiology of Reproduction*, 2nd edn (eds E. Knobil & J. D. Neill), pp. 1363–1434. Raven Press, New York.

Sinha Hikim, A. P. & Swerdloff, R. S. (1999) Hormonal and genetic control of germ cell apoptosis in the testis. *Reviews of*
Q9 *Reproduction* 4, 38–47.

Sinha Hikim, A. P., Maiti, B. R. & Ghosh, A. (1985) Spermatogenesis in the bandicoot rat. I. Duration of the cycle of the seminiferous epithelium. *Archives of Andrology* 14, 151–154.

Sinha Hikim, A. P., Rajavashisth, T. B., Sinha Hikim, I., Lue, Y., Bonavera, J. J., Leung, A., Wang, C. & Swerdloff, R. S. (1997) Significance of apoptosis in the temporal and stage-specific loss of germ cells in the adult rat after gonadotropin deprivation. *Biology of Reproduction* 57, 1193–1201.

Sinha Hikim, A. P., Wang, C., Lue, Y., Johnson, L., Wang, H.-H. & Swerdloff, R. S. (1998) Spontaneous germ cell apoptosis in humans: evidence for ethnic differences in the susceptibility of germ cells to programmed cell death. *Journal of Clinical Endocrinology and Metabolism* 83, 152–156

Smithwick, E. B., Young, L. G. & Gould, K. G. (1996) Duration of spermatogenesis and relative frequency of each stage in the seminiferous epithelial cycle of the chimpanzee. *Tissue and Cell* 28, 357–366.

Tait, A. J. & Johnson, E. (1982) Spermatogenesis in the grey squirrel (*Sciurus carolinensis*) and changes during sexual regression. *Journal of Reproduction and Fertility* 65, 53–58.

Weinbauer, G. F., Aslam, H., Krishnamurthy, H., Brinkworth, M. H., Einspanier, A. & Hodges, J. K. (2001) Quantitative analysis of spermatogenesis and apoptosis in the common marmoset (*Callithrix jacchus*) reveals high rates of spermatogonial turnover and high spermatogenic efficiency. *Biology of Reproduction* 64, 120–126.
Wistuba, J.A., Schrod, A., Grewe, B., Hodges, J. K., Aslam, H., Weinbauer, G. F. & Luitjens, C. M. (2003) Organisation of seminiferous epithelium in primates: relationship to spermatogenic efficiency, phylogeny, and mating system. *Biology of Reproduction* 69, 582–591.

Received XX xxxxx 200x; revised XX xxxxx 200x; accepted XX 10 *xxxxx 200x*

เอกสารหมายเลข 2

ELECTRON MICROSCOPY SOCIETY OF THAILAND

3rd ASEAN Microscopy Conference
and
19th Annual Conference of EMST

January 30 - Fe[illegible] 20[illegible]

Chiang Mai [illegible]

Oral Presentation

J.E M.S.T Vol.16 No.1, pp 94-95, 2002
Printed in Thailand

P. Worawittayawong[1,2]
P. Sretarugsa[1]
C.M. Leigh[2]
W.G. Breed[2]

Is the Highly Divergent Sperm Nuclear Shape in Bandicoot Rats due to an Unusual Process of Chromatin Condensation?

[1] Department of Anatomy, Faculty of Sciences, Mahidol University, Bangkok, Thailand
[2] Department of Anatomical Sciences, Adelaide University SA 5005 Australia

The sperm head of mammals is largely composed of a nucleus and acrosome and in most murid rodents it is falciform in shape. Two southern Asian murids in the genus *Bandicota*, however, have evolved a unique sperm head that is conical with a small nucleus capped by a massive acrosome. In the present study we investigate the histochemistry of sperm chromatin during late spermiogenesis. For this, male *B indica* were collected in Thailand in September 1996 and January 2001. Testes were fixed in 3% buffered glutaraldehyde and either osmicated and stained with lead citrate and uranyl acetate, or fixed with alcoholic 3% phosphotungstic acid (PTA) overnight. In conventionally stained material chromatin appeared as large cords within which there were nuclear vacuoles with 50-90 nm electron-dense globules. PTA-stained material showed variable staining; some had peripheral electron-dense chromatin (Fig.1-4), suggesting lysine or thiol-rich nucleoproteins in these regions. How the chromatin is eventually packaged remains undetermined, but these observations indicate some unusual features of chromatin organization during late spermiogenesis; this may relate to the bizarre sperm head shape that eventually results.

Acknowledgement

This work was supported by Royal Golden Jubilee Thai Program, Thailand Research Fund.

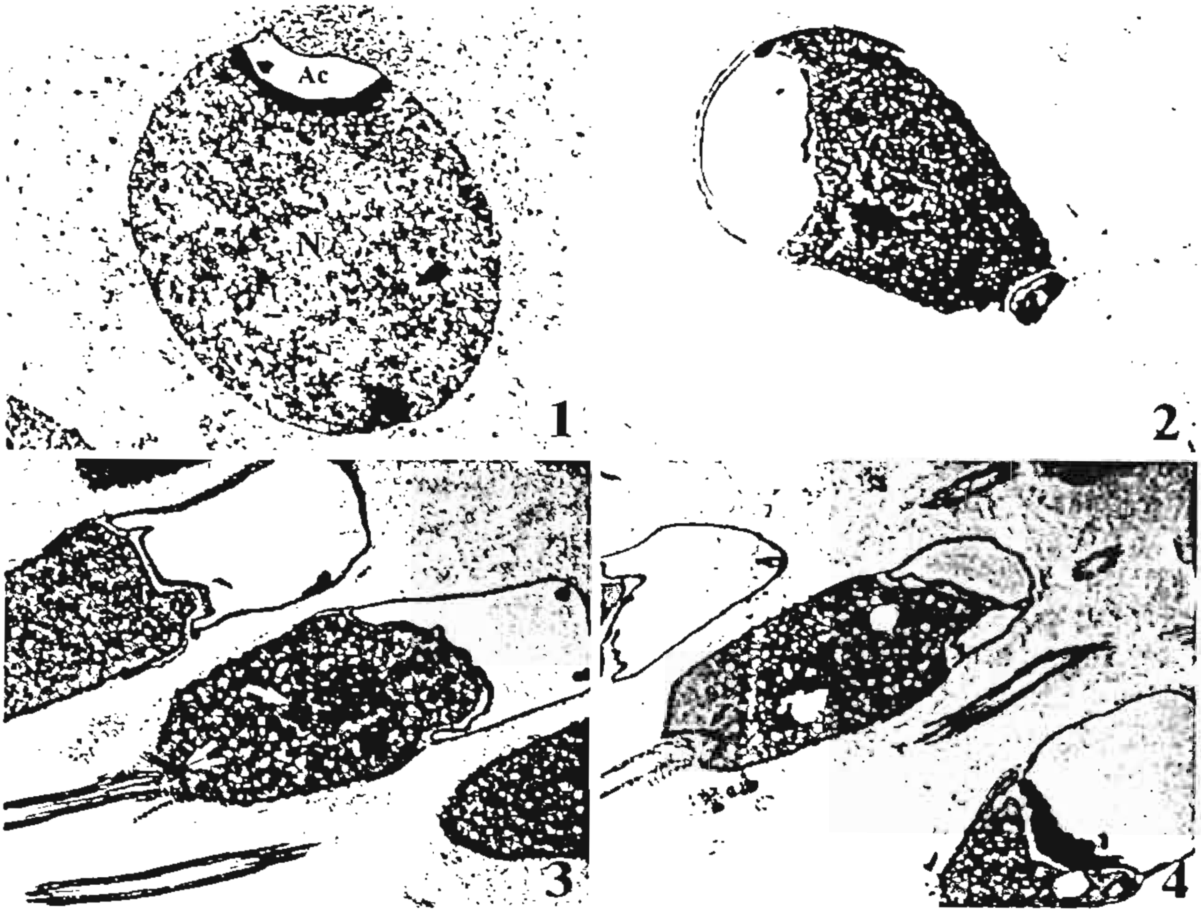


Fig.1 Early spermatid; dispersed chromatin in the nucleus (N) ; with strong PTA stained material under the acrosome (Ac).

Fig.2 Late spermatid; strong PTA stained chromatin in the posterior region of the nucleus; anteriorly a small localized (arrow) region of PTA positive material also occurs.

Fig. 3 Late spermatid or probable spermatozoan; strong PTA stained material in upper middlle part of nucleus (arrow) and near the basal plate (two arrows)

Fig. 4 Very late spermatid or probable spermatozoan; sperm nucleus with no PTA-staining except the region in basal plate (arrow)

เอกสารหมายเลข 3

ABSTRACTS

Forty-ninth AGM

The Australian Mammal Society

7-9 July 2003

Sydney University

Spoken papers pages 1-37
* Asterisks indicate the person delivering the paper

Poster papers pages 39-49

WIGGINS[1,2*], Natasha; McArthur[1,2], Clare; McLean[3], Stuart; and Boyle[3], Rebecca
[1]Cooperative Research Centre for Sustainable Production Forestry
[2]School of Zoology, University of Tasmania, GPO Box 252-05, Hobart, Tas, 7001
[3]School of Pharmacy, University of Tasmania, GPO Box 252-26, Hobart, Tas, 7001

EFFECTS OF TWO PLANT SECONDARY METABOLITES, CINEOLE AND GALLIC ACID, ON NIGHTLY FEEDING PATTERNS OF THE COMMON BRUSHTAIL POSSUM

Plant secondary metabolites (PSMs) can assist in a plant's defence against herbivory. The common brushtail possum (*Trichosurus vulpecula*) is a generalist folivore, adapted to consume a variety of plants. This enables them to encounter a wide range of PSMs, but each at low concentrations. We are interested in the behavioural responses of herbivores to PSMs, and what implications these responses have on their diet. The aim of this investigation was to determine patterns of feeding behaviour in brushtail possums in response to PSMs occurring in foliage they naturally consume.

We introduced two PSMs, cineole and gallic acid, into an artificial diet. Firstly, we tested whether possums altered their feeding behaviour in response to increasing levels of cineole. Diets contained three concentrations of cineole; zero, medium and high. Following this, possums were offered a choice PSM diet (cineole and gallic acid diets simultaneously) or a no-choice PSM diet (containing either cineole or gallic acid). With increasing cineole levels, possums ate less, had smaller feeding bouts, and had a lower rate of intake, but did not extend their total nightly feeding time. Possums offered the choice PSM diet compared with the no-choice diets, ate more, had larger feeding bouts, and tended to increase their rate of intake. These results indicate that PSMs not only constrain overall intake, but that possums alter their feeding behaviour in response to them. Altered feeding patterns may reduce the negative influence of PSMs on intake.

WORAWITTAYAWONG[1,2*], P., Leigh[1], C., Peirce[1], E., Sretarugsa[2], P., and Breed[1], W.
Bolliger Award Candidate
[1]Department of Anatomical Sciences, University of Adelaide
[2]Department of Anatomy, Mahidol University, Bangkok, Thailand

QUALITATIVE AND QUANTITATIVE PRODUCTION OF SPERMATOZOA IN THE ASIAN BANDICOOT RAT, *BANDICOTA INDICA*

The genus of the southern Asian murid rodent *Bandicota* is close to *Rattus*. Nevertheless, previous observations have shown that, in two species, highly divergent spermatozoa from those of *Rattus* occur in which there is generally a globular sperm head and very short tail. The present study on one of these species, *B. indica*, has quantitatively determined in 10 adult males (i) the variation in sperm head shape, and (ii) the organisation of the germ cells during maturation within the seminiferous epithelium. For the former approximately 100 cauda epididymal sperm from each individual were measured and allocated to one of several morphotypes and, for the latter, plastic sections of the testes were cut, stained with toluidine blue, and germ cell maturational stages determined in around 100 tubule cross sections from each individual.

The results showed that, although all sperm had pyriform or globular-shaped heads, there were invariably several different morphotypes present within the mature sperm population. Within the seminiferous tubules, 8 germ cell associations were recognised of varying abundance and sometimes germ cells out of synchrony with the typical cell association were found. These findings highlight that, in this species, sperm form shows marked heterogeneity as well as variable degrees of synchrony between the maturing germ cells within the cell associations. This suggests that the genetic control of sperm form has been relaxed such that synchrony of germ cell maturation does not occur and sperm pleiomorphism is evident in the mature spermatozoon population.

This work was sponsored by the Royal Golden Jubilee Thai Program, Thailand Research Fund.

RGJ - Ph.D. Congress III

เอกสารหมายเลข 4

การประชุมวิชาการ
โครงการปริญญาเอกกาญจนาภิเษก ครั้งที่ 3

25-27 เมษายน 2545
โรงแรมจอมเทียน ปาล์มบีช รีสอร์ท
เมืองพัทยา ชลบุรี

สกว
TRF
สำนักงานกองทุนสนับสนุนการวิจัย
The Thailand Research Fund

ISBN 974-7772-91-4

S3-P1

Is the Highly Divergent Sperm Nuclear Shape in Bandicoot Rats due to an Unusual Process of Chromatin Condensation?

P Worawittayawong[a, b], P Sretarugsa[a], CM Leigh[2] and WG Breed[b]
[a] *Department of Anatomy, Faculty of Sciences, Mahidol University, Bangkok, Thailand.*
[b] *Department of Anatomical Sciences[2], Adelaide University SA 5005 Australia.*

Objective

To investigate the histochemistry of sperm chromatin during spermiogenesis.

Methods

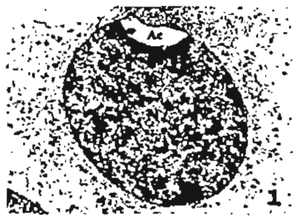
For this, male *B indica* were collected in Thailand in September 1996 and January 2001. Testes were fixed in 3% buffered glutaraldehyde and either osmicated and stained with lead citrate and uranyl acetate, or fixed with alcoholic 3% phosphotungstic acid (PTA) (2) overnight.

Results

The sperm head of mammals is largely composed of a nucleus and acrosome and in most murid rodents it is falciform in shape (1). Two southern Asian murids in the genus *Bandicota*, however, have evolved a unique sperm head that is conical with a small nucleus capped by a massive acrosome. In conventionally stained material chromatin appeared as large cords within which there were nuclear vacuoles with 50-90 nm electron-dense globules. PTA-stained material showed variable staining; some had peripheral electron-dense chromatin (Fig.1-4), suggesting lysine or thiol-rich nucleoproteins in these regions.

Conclusion

How the chromatin is eventually packaged remains undetermined, but these observations indicate some unusual features of chromatin organization during late spermiogenesis; this may relate to the bizarre sperm head shape that eventually results.



Early spermatid ; dispersed chromatin in the nucleus (N) ; with strong PTA stained material under the acrosome (Ac).

Late spermatid ; strong PTA stained chromatin in the posterior region of the nucleus ; anteriorly a small localized (arrow) region of PTA positive material also occurs.

Late spermatid or probable spermatozoon ; strong PTA stained material in upper middle part of nucleus (arrow) and near the basal plate (two arrows).

Very late spermatid or Probable spermatozoon ; sperm nucleus with no PTA-staining except the region in basal plate (arrow).

Keywords: sperm, bandicoot rat, chromatin, condensation.

References

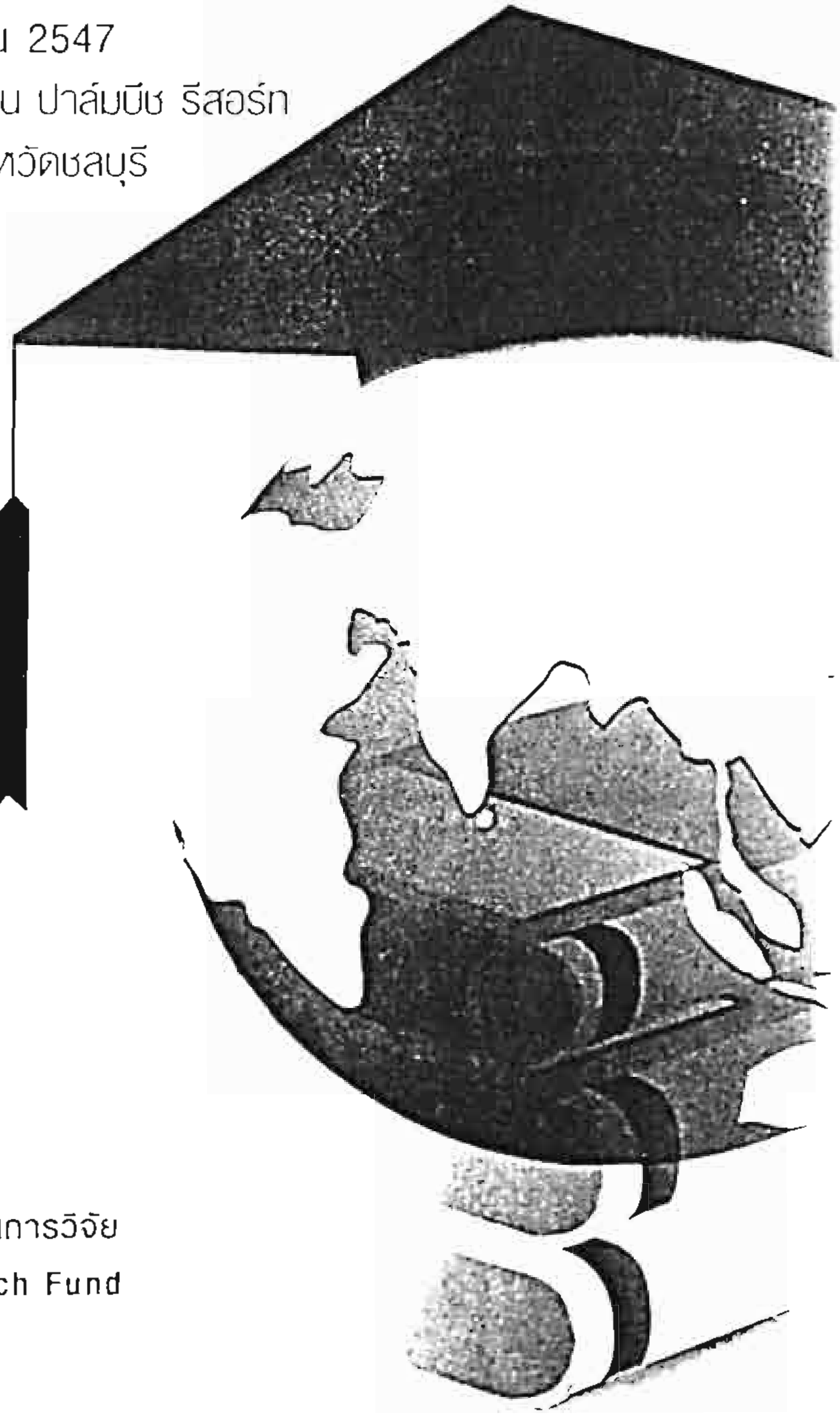
1. WG Breed (1993) Novel organization of the spermatozoon in two species of murid rodents from Southern Asia. *J of Repro&Fer* 99, 149-58.
2. Jean-Luc Courtens and Maurice Loir (1981) A cytochemical study of nuclear changes in boar, bull, goat, mouse, rat, and stallion spermatids. *J of Ultrastructure Research* 74, 327-40.

RGJ - Ph.D. Congress V

เอกสารหมายเลข 5

การประชุมวิชาการ
โครงการปริญญาเอกกาญจนาภิเษก ครั้งที่ 5

23-25 เมษายน 2547
โรงแรมจอมเทียน ปาล์มบีช รีสอร์ท
เมืองพัทยา จังหวัดชลบุรี



สกว
TRF

สำนักงานกองทุนสนับสนุนการวิจัย
The Thailand Research Fund

ISBN 974 9545 80 X

S3-P25

A Possible Cause for the Presence of Vacuoles in the Nucleus of Spermatids and Spermatozoa of the Bandicoot Rat (*Bandicota indica*)

P. Worawittayawong[a,b], C.M. Leigh[a], P. Sretarugsa[b] and W.G. Breed[a]

[a] *Department of Anatomical Sciences, Adelaide University SA 5005*

[b] *Department of Anatomy, Faculty of Sciences, Mahidol University, Bangkok Thailand*

Objective

In this study we ask the following questions: what is the distribution of histones and transition proteins in the spermatid nuclei of the bandicoot rat, and do the nuclear vacuoles exhibit different proteins from the rest of the sperm chromatin?

Method

For routine TEM small pieces of testes were fixed in 2% glutaraldehyde, embedded in araldite, and stained in uranyl and lead citrate. For PTA staining testes were fixed in 4% glutaraldehyde, stained with 3% phosphotungstic acid (PTA) in 100% ethyl alcohol and embedded in Epon (1). For gold labeling with the antibodies to histones and transition proteins, pieces of testes were fixed in 0.5% glutaraldehyde, embedded in Lowicryl K4M, cut, and incubated in either a monoclonal anti-histone (H2A, H2B, H3, or H4) (2) or polyclonal anti-transition protein (TP1 or TP2) (3) antibody raised in rabbits. After washing, incubation in 10 nm gold conjugated goat antirabbit IgG was performed and distribution of gold particles determined.

Results

Routine TEM of spermatids showed that during chromatin condensation fibrillar chromatin with localized electron lucent regions with granules occurred. Further chromatin condensation then took place throughout most of the nucleus but the localized foci of electron lucent regions remained. Alcoholic PTA showed strong positive staining peripherally as well as in a localized area in the anterior region of the nucleus but this region did not appear to coincide with location of nuclear vacuoles. Observations of gold labeling with anti-TP1 showed abundant, widespread, labeling over the condensing chromatin but absence of labeling over the electron lucent nuclear vacuoles. Anti-TP2 and anti-H3 also labeled the condensing chromatin but not material in the nuclear vacuoles.

Conclusions

These results confirm that in the bandicoot rat condensing spermatid nuclei localized foci of electron lucent regions occur. These regions do not stain with alcoholic PTA nor does gold labeling with anti-TP1, anti-TP2, or anti-H3 antibodies occur over these regions unlike the rest of the condensing chromatin. We thus hypothesis that nuclear vacuoles may result from an absence of transition proteins being laid down during spermiogenesis, this may also be the case for vacuoles in human sperm nuclei.

Keywords: sperm, chromatin condensation, histone, Bandicoot rat

Selected References

1. Courtens, J. L. and Loir M (1981). *J. Ultrast. Res* 74. 322-326
2. Biggiogera, M., S. Muller, et al. (1992). *Micr. Res Tech.* 20. 259-267
3. Oko, R. J., V. Jando, et al. (1996) *Biol. Reprod.* 54: 1141-1157.