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Histological and Ultrastructural Features of the Leydig Cells and their Association with other Testicular Cells of the Vervet Monkey, *Chlorocebus aethiops*

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ABSTRACT

The study was undertaken to investigate the structure of the Leydig cells of the vervet monkey, *Chlorocebus aethiops,* using both light and electron microscopic techniques and to elucidate the association of these cells with other testicular cells. The Leydig cells occur as a group of polymorphic cells in the intertubular region, and are usually associated with large and small blood vessels and abundant loose connective tissue. Leydig cells have large spherical or slightly elongated nuclei, which possess prominent nuclei and frequently deep indentations of nuclear envelope. The nuclei are characterized by patches of euchromatin and heterochromatin regions with the latter usually occupying the periphery of the organelle. The cytoplasm contains numerous mitochondria containing distinct lamellar cristae, small patches of rough endoplasmic reticulum as short cisternae, a large dense network of interconnecting tubular smooth endoplasmic reticulum, and round or oval lysosomes. Abundant lipid inclusions and collagen fibers are also found in the cytoplasm of these cells. These cells are found associated with the spermatogonia type B, pachytene spermatocytes and Sertoli cells. The results from this study show that there are basic similarities in the Leydig cells of the vervet compared to other mammals, humans and nonhuman primates, however, there also seem to be species differences among the mammalian group. The association of these cells with developing cells, and Sertoli cells was established. However, the association of Leydig cells with spermatogenic cells at different stages of spermatogenesis needs further investigation.

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Authors' Contribution
SLL and GH conceived and designed the study. SSL analyzed the data. Both authors wrote the manuscript.

Key words
Testis, Leydig cells, Vervet monkey, Chlorocebus aethiops.

INTRODUCTION

Chlorocebus aethiops, formerly known as *Cercopithecus aethiops*, is commonly referred to as the African green monkey, vervet, tantulus, savannah, or the grivet monkey. Over the past three decades, the vervet monkey, *Chlorocebus aethiops,* has been constantly used in biomedical research as an animal model for human-related problems such as in virology and reproductive studies (Kushner *et al*., 1982). There is considerable information on some aspects of the reproductive biology of this species, for example, on reproductive behaviour (Rowell, 1970), breeding of vervet monkeys in a closed environment (Seier, 1986), and on sperm motility in the epididymis (van der Horst *et al*., 1999). However, data on the anatomy and physiology of the reproductive system of the vervet monkeys remain scanty.

The mammalian testis consists of germinal and non-germinal cells (Sakai *et al*., 1988), which differ both in morphology and physiological function (Hardy *et al*., 1989; Haider and Servos, 1998; Haider, 2004). The germinal cells will undergo a series of divisions and ultimately develop into spermatozoa. The non germinal cells are the Leydig cells and Sertoli cells. Embryologically, it has been speculated that the peritubular fibroblasts contribute to trigger mechanisms that initiate fetal Leydig cell differentiation *via* local growth factors from the endothelial cells (Skinner, 1991). Two sources for the precursors of fetal Leydig cells have been reported in the literature, *viz.*, mesenchymal fibroblasts from the mesonephros and mesenchymal fibroblasts from the gonadal ridge (Byskov, 1986; Buehr and McLaren, 1992; De Kretser and Kerr, 1994; Merchant-Larios *et al*., 1993; Nishino *et al*., 2001).

Adult Leydig cells are classified into the three stages, *i.e.,* progenitor stage, immature stage, and mature stage (Hardy *et al*., 1989). Progenitor stage includes Leydig cells originating from the mesenchymal-like fibroblasts and produce androsterone as the predominant androgen

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end product. Hardy *et al*. (1989) reported that immature stage includes those cells that produce small amounts of testosterone and metabolize most of the testosterone. The predominant androgen end product is the 5α-androstane-3α, 17β-diol. Mature stage will be formed by cells which actively produce testosterone as an androgen end product and are fully functional in the sexually mature animal.

Leydig cells are the major steroid-secreting cells in the intertubular tissue of the mammalian testis. The clusters of Leydig cells are found to be located in the angular spaces among the seminiferous tubules and are frequently associated with blood vessels. These cells possess a spherical or irregularly polyhedral shape and they have a diameter of approximately 20 µm. The nuclei are round and the cell surface is characterized by microvilli (Dym, 1983). The most prominent cytoplasmic organelles in these cells are smooth endoplasmic reticulum (SER), mitochondria with tubular cristae, lipid droplets, and lipofuscin bodies. These features are characteristics of steroid producing cells (Camatini *et al*., 1981; Dym, 1983). In humans, conspicuous cytoplasmic crystals of Reinke are observed and they are regarded as characteristic features of the Leydig cells. These crystals vary widely in size and form, but are often rectilinear and may be 2 µm long and 3 µm in thickness (Dym, 1983).

Despite this information, there is a still a need to compare the male reproductive aspects of the vervet monkey with those found in humans and other mammals, and critically evaluate this nonhuman primate for a possible future experimental model in biomedical research. Therefore, the main objective of the study was to investigate the structure of the Leydig cells in the vervet monkey, using both the light and electron microscopic techniques and study the association of the Leydig cells with other testicular cells. In addition, the study will draw a comparison of the structure of the Leydig cells in humans, mammals and other nonhuman primates.

MATERIALS AND METHODS

Study animals

The study was performed using twenty-nine (29) male vervet monkeys which were obtained from the National Research Institute for Nutritional Diseases at the South African Medical Research Council, Tygerberg, Cape Town, South Africa. The animals were housed permanently indoors and fed with a basic diet consisting of precooked maize meal with a protein-vitamin and mineral concentrate, which was mixed to a stiff porridge with water and fed in the mornings. Wholewheat brown bread was provided at noon, and apples and carrots, washed in chlorinated water, were fed in afternoon. Drinking water was supplied *ad libitum via* an automatic device (Seier, 1986). Ethical clearance was obtained from the Medical Research Council's Ethical Committee for this study.

The ages of the animals ranged from 5-11 years and were described as reproductively mature. Animals were weighed to the nearest 100 g and subsequently anaesthetized with intramuscular injection of Ketamine (Ketalar at 10 mg/kg body mass). Immediately after sacrificing, testes were excised. Harvested tissues were processed for both light microscopy and transmission electron microscopy (TEM).

Light microscopy

The samples were fixed in neutral buffered formalin and Bouin's fluid, dehydrated in ethanol at different concentrations and embedded in paraffin wax. The embedded tissues were sectioned using a microtome (American optical, 820 Spencer rotating microtome) and section thickness was set at 4 µm (Reichert-Jung knife). The sections on the slides were stained by haematoxylin and eosin and mounted. Sections were examined by means of a Universal transmitted-light research microscope (Zeiss, Germany) and photographs were taken at various magnifications using an MC 63 automatic photomicrographic camera (for 35mm film) which was mounted on the microscope.

Small pieces of testicular tissue were fixed in 2.5 % Sorenson's phosphate buffered glutaraldehyde. After fixation, the tissues were post fixed in a mixture of 1% osmium tetroxide and 1.5% potassium ferrocyanide, dehydrated, embedded in Epon and sectioned for electron microscopy. Sections were stained with uranyl acetate and lead citrate, and examined.

For high resolution light microscopy, methacrylate resin embedded tissues were sectioned at a thickness of 1µm. Sections were stained with 1% toluidine blue to provide the fine nuclear details of the cells and less superimposition of cell organelles.

Electron microscopy

For TEM study, samples were fixed in 2.5% buffered gluteraldehyde and then cut in cubes of 1 mm^3 before placed in 1% O_sO_4 for contrasting for at least one hour. The tissues were then rinsed in buffer for 15 min. Specimens were dehydrated in increasing concentrations of ethanol. The specimens were then placed in 1:1 mixture of propylene oxide: resin (EMS Sciences, UK), overnight for infiltration. Next morning the specimens were placed in 1:3 mixture of propylene oxide and resin for 2 h. The specimens were then placed in fresh 100% resin under vacuum for 2 h. The procedure was followed by embedding specimens in fresh resin. The specimens were then placed

in an embedding oven at 70˚C for curing. Gold sections (70 nm) made using ultramicrotome (Reichert Jung Ultracut E, Germany), were placed on copper grids and left to dry. Grids were contrasted or stained using heavy metal stains, both lead citrate and uranyl acetate (EMS Sciences, UK). Grids were viewed by means of a Jeol JEM1010 (Jeol Inc., Tokyo) transmission electron microscope (TEM). Selected areas were photographed and micrographs printed for examination.

RESULTS

Light microscopy

Light microscopic studies revealed intertubular area occupied by clusters of polymorphic cells, associated with numerous blood vessels of variable sizes and abundant loose connective tissue (Fig. 1). The seminiferous tubules are connected by interlobular connective tissue and show different developmental stages of spermatogenesis (Fig. 1). The developing germ cells of different stages of spermatogenesis are also observed in the central seminiferous tubule and adjacent tubules (Fig. 1). The lumen of the seminiferous tubules is characterized by different stages of spermatids and mature spermatozoa (Fig. 1A). The dense irregular connective tissue is observed in the interlobular space. The polymorphic cells observed are Leydig cells associated with blood vessels (Fig. 1B). The Leydig cells contain one or more distint

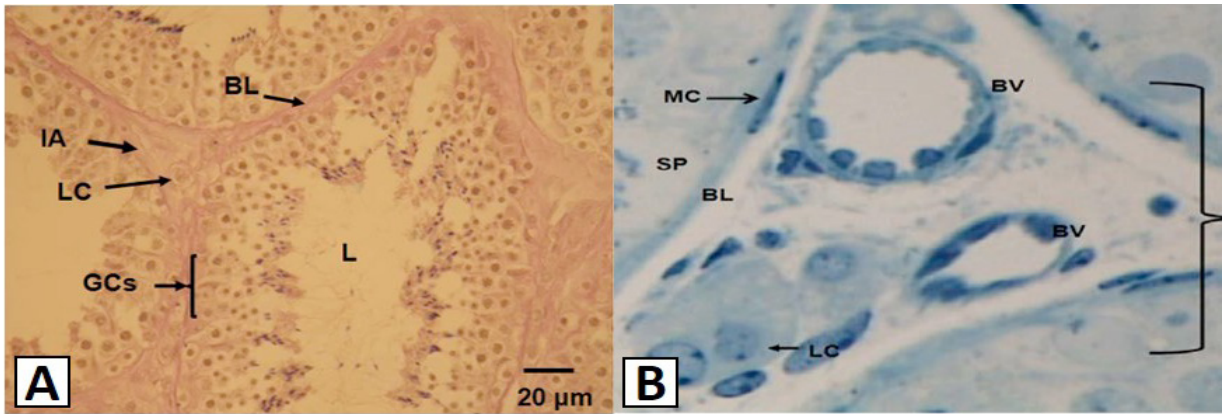

Fig. 1. The light micrograph showing the general layout of the interstitial area (IA) and the seminiferous tubules (A), and Leydig cells (LC) and blood vessels (BV) of the testis (B) of the vervet monkey, *Chlorocebus aethiops*. **A**, Each tubule is surrounded by a basal lamina (BL). The Leydig cells (LC) are found in the interstitial area (IA). Within the seminiferous tubules are developing germ cells (GCs), which will later become the mature spermatozoa. The lumen (L) is occupied by spermatids of different stages; **B**, The myoid cells (MC) are found forming the basement membrane or the basal lamina (BL). On the basement membrane lies the spermatogonium (SP).

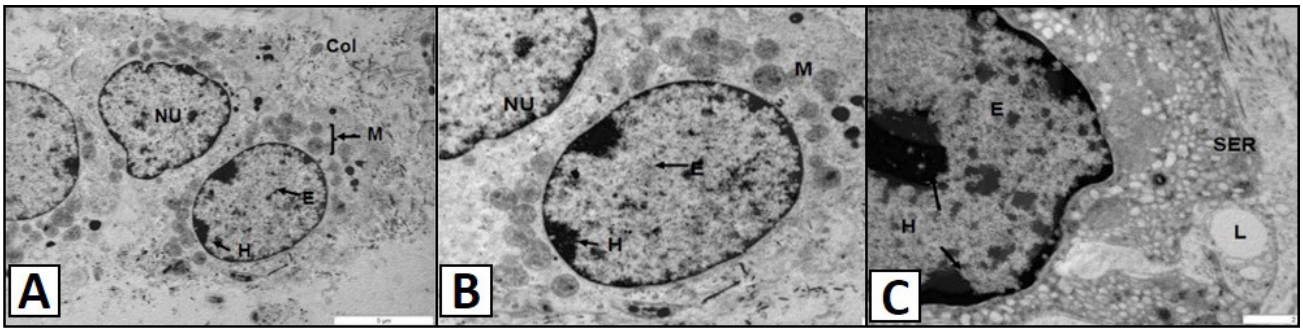

Fig. 2. TEM micrograph showing Leydig cells of the vervet monkey, *Chlorocebus aethiops*. **A,** shows the polygonal nuclei (NU). The nucleus contains patches of euchromatin (E) and heterochromatin (H), with the latter situated at the periphery of the nucleus, close to the nuclear envelope. The cytoplasm contains numerous mitochondria (M) found closer the nucleus. The collagen fibers (COL) are also observed in the apical region of the cell. **B,** shows the round nuclei (NU) of the Leydig cells. The euchromatin (E) and heterochromatin (H) are observed in the nucleus. The cytoplasm contains numerous mitochondria (M) found closer the nucleus. **C,** shows the nucleus and the smooth endoplasmic reticulum (SER) of the Leydig cells. The nucleus contains euchromatin (E) and heterochromatin (H) patches. The lipid droplet (L) is found in association with the SER.

nucleoli (Fig. 1B). Considerable number of myoid cells is observed lining the seminiferous tubules. These myoid cells form one significant layer of the basement membrane. Spermatogonia are observed situated on basement membrane (Fig. 1B).

Electron microscopy

Leydig cells exhibit large spherical or slightly elongated nuclei, which usually possess prominent nucleoli and frequently deep indentations of nuclear envelope (Fig. 2A, B). The nuclei possess fine granulated euchromatin and heterochromatin with the latter adjacent to the nuclear envelope (Fig. 2A, B). Abundant mitochondria containing distinct tubular or lamellar cristae are observed in the cytoplasm (Fig. 2). Mitochondria are found accumulated pericentrally in the cell and in close proximity to the nucleus (Fig. 2A, B). The SER is arranged in concentric lamella around large mitochondria, lysosomes, multivesicular bodies and granular vesicles. Small patches of rough endoplasmic reticulum (RER) are seen as short cisternae, and a large dense network of interconnecting tubular SER is observed in the cytoplasm (Fig. 3A). Rough endoplasmic reticulum is not evenly distributed, only limited and appears as relatively short strand located close to the nucleus and among groups of mitochondria (Fig. 3A). Collagen fibers are observed in the apical and

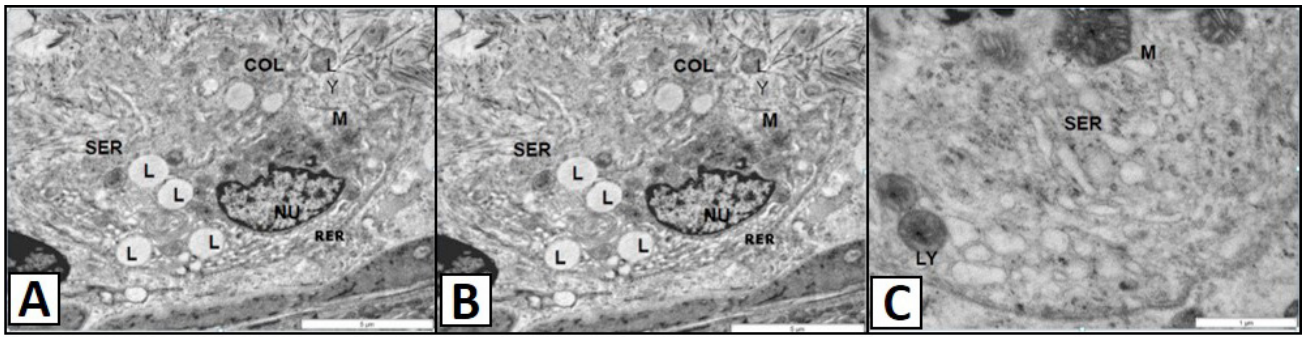

Fig. 3. TEM micrograph showing of the Leydig cells of the vervet monkey, *Chlorocebus aethiops*. **A,** shows the polygonal nucleus (NU) has evenly distributed heterochromatin patches. The rough endoplasmic reticulum (RER) is situated at the basal region of the nucleus. The smooth endoplasmic reticulum (SER) is located at the apical region of the nucleus. The lipid droplet (L) and the lysosomes (LY) are found scattered in the cytoplasm together with collagen fibers (COL). **B,** show the distinct mitochondria of the Leydig cells. B, shows the smooth endoplasmic reticulum (SER) is located at the apical region of the nucleus. The lysosomes (LY) are found scattered in the cytoplasm. **C,** shows the tubular cristae (C) are clearly observed forming the mitochondria. The lipid droplets (L) are found in association with the mitochondria (M).

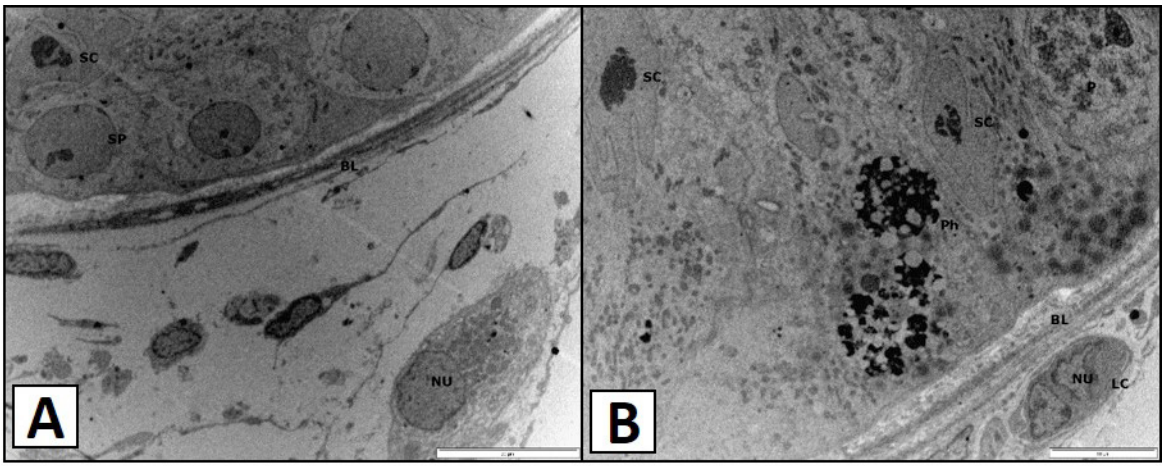

Fig. 4. TEM micrograph showing the distinct nucleus (NU) of the Leydig cells of the vervet monkey, *Chlorocebus aethiops*. **A,** shows the Sertoli cell (SC) and the spermatogonium type B and found lying on the basal lamina (BL). **B,** shows the phagocytic vesicles are observed (Ph) and the spermatocyte at the pachytene (P) is observed. The Sertoli cells (SC) are found lying on the basal lamina (BL).

basal parts of the cytoplasm of the Leydig cells (Fig. 3B). A number of cytoplasmic inclusions are frequently found, including lipid droplets and lysosomes in the Leydig cells (Fig. 3A, B). The distinct tubular cristae which form the mitochondria are observed in the cytoplasm (Fig. 3C). The Leydig cells are associated with the Sertoli cells, which have their bases on the basal lamina. Spermatogium type B is found associated with the Leydig cells (Fig. 4A). The chromosomes in the pachytene stage of the prophase appear in the nucleus of the primary spermatocyte near the Sertoli cells (Fig. 4B). Numerous phagocytic vesicles are found in close proximity to the Sertoli cells (Fig. 4B).

DISCUSSION

Leydig cells are important in the biosynthesis of testosterone from cholesterol. Cholesterol, which is the substrate for all steroid-hormones, is transferred from cellular stores into the outer membrane of mitochondria by protein kinase A (Haider, 2004). Kortner and Arukwe, (2007) reported the roles of steroidogenic acute regulatory protein (StAR) in the movement of cholesterol across the mitochondrial membrane and the final conversion to pregnenolone by cytochrome P450-mediated side-chain cleavage enzyme (P450*scc*), as a strong rate-limiting step in acute steroid production.

Previous research studies reported that the composition of the intertubular tissue observed in the testis of the vervet monkey appears to be similar to that described in humans and other nonhuman primates such as the *Macaca mulatta* (Christensen, 1975; Camatini *et al*., 1981). The histological techniques used in this study revealed that the intertubular tissue of the vervet monkey contains numerous blood vessels of different sizes, Leydig cells, and dense connective tissue. Anbalagan *et al*. (2003), reported distinct centrally placed lymphatic vessels in the testis of the monkey. The ultrastructural characteristics of Leydig cells of *C. aethiops* observed in this study follow the general pattern clearly found in other adult active mammalian Leydig cells, including those found in humans. Smooth endoplasmic reticulum, rough endoplasmic reticulum, and many mitochondria were observed in the cytoplasm of the Leydig cells. The same features were observed by Camatini *et al*. (1981) in their study on the vervet monkey. Camatini *et al*. (1981) further outlined that an extensive and polymorphic aspect of the smooth endoplasmic reticulum could be regarded as a distinguishing characteristic of the Leydig cells in the vervet monkey. Thus, Leydig cells in the testis of the vervet monkey show characteristics of typical steroidogenic cells. Leydig cells in humans possess a tubulovesicular system of the smooth endoplasmic reticulum, and this organelle seems to have a uniform morphology throughout the cell (Camatini *et al*., 1981). However, the SER of the Leydig cells reported in other mammals displayed different forms and shapes (Camatini *et al*., 1981). Numerous complexes of whorl formations with smooth endoplasmic reticulum in lamellar and tubulovesicular arrays have also been described in normal adult mouse Leydig cells (Camatini *et al*., 1981). This finding concurs with results in this study where a well-developed, large dense network of the SER was observed, almost similar to what was described in other mammals.

As expected in many mammals, the close association of the smooth endoplasmic reticulum and mitochondria is involved in androgen synthetic pathways. Mitochondria had extensive cristae as observed by Camatini *et al*. (1981) in vervet monkeys and again in this study. Camatini *et al*. (1981), further reported a very extensive rough endoplasmic reticulum which was found in association with the smooth endoplasmic reticulum network. However, in our study, the RER seemed to be less developed when compared to the SER. The presence of numerous lipid droplets in the cytoplasm of the Leydig cells is a principal feature. This could be attributed to the association of SER and mitochondria with tubular cristae, to which the lipid is intimately related. These droplets could represent a pool of steroid precursors and not storage of newly synthesized steroid as suggested in previous reports (Rothwell, 1973; Rai *et al*., 2004). Svechnikov *et al*. (2010) reported that once cholesterol reaches the inner mitochondrial membrane, it is immediately converted to pregnenolone. Pregnenolone will then leave the mitochondria to the smooth endoplasmic reticulum, where it will be converted to progesterone (Svechnikov *et al*., 2010). Progesterone will then form androstenedione which is a precursor of testosterone.

The results of this study could not show the presence of the crystals of Reinke. These crystals of Reinke are regarded as the distinguishing features of the human Leydig cells. The crystals of Reinke seem to be confined to humans because even in previous studies on the nonhuman primates there seem to be no literature which explained their presence in Leydig cells. However, Prince (1999) reported the presence of inclusions with much similarity to the crystals of Reinke within the Leydig cells of the marmoset monkey. This will suggest that in this respect marmoset monkey could be regarded as been closer to human than the vervet monkey. This was one of the striking differences between the vervet Leydig cells and the human Leydig cells. Since the physiological functions of the crystals of Reinke are not well documented, it could be suggested that these structures could well be one of the few features confined to human Leydig cells. Camatini

et al. (1981) suggested that the existence of some of the morphological features of organelles in the Leydig cells could be attributed to the sexual maturity of the animals under study.

The nuclei of these cells showed euchromatin regions which signified an intense cellular activity. Furthermore, different stages of development of spermatogenesis were also observed which indicated that these cells undergo continuous development in the interstitial region of the testis. This could be due to the testicular activity in the production of the hormone testosterone. The presence of collagen fibers was quite distinct in the cytoplasm of the Leydig cells. Diaz *et al*. (2005) indicated that ultrastructural immunolabeling studies showed that rat Leydig cells exhibit patches of laminin and type IV collagen of their surfaces. It was further reported that collagen type IV could have a modulatory role in Leydig cell steroidogenesis.

There are numerous extensive reports on the description of the Leydig cells and Sertoli cells and their function in different animal groups (Camatini *et al*., 1981; Bakst *et al*., 2007; Chung, 2008; Lebelo and van der Horst, 2010). However, the association and activities of these cells with the developing spermatogenic cells during spermatogenesis remains elusive. This type of association should be investigated to shed some light on the physiological roles of these cells in spermatogenesis. In this study it was clear that there is a strong association between the developing germinal cells, Sertoli cells and Leydig cells. The association of Leydig cells and spermatogenic cells in different stages of spermatogenesis will explain the crucial physiological roles of the cells in male reproduction.

CONCLUSIONS

The ultrastructure of the vervet monkey testis was found to be similar to that reported in other nonhuman primates and showed many similarities with humans. However, this study could not identify the three populations of Leydig cells as reported by other researchers because only the adult, reproductively mature animals were selected for this study. Despite these differences in composition and arrangement of some cellular structures of the Leydig cells of the vervet monkey and humans, the similarities observed in this investigation strongly support the possibility of using a vervet monkey as an experimental model for research on male reproductive physiology and endocrinology.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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