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Localization of the Cytochrome P450 Side-Chain Cleavage Enzyme in the Inactive Testis of the Naked Mole-Rat

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ABSTRACT—Spermatogenesis was histologically examined in non-breeding male of the naked mole rat (*Heterocephalus glaber*) using a light microscopy. Spermatogonia, spermatocytes and spermatids were confirmed in the seminiferous tubules. However, the spermatogenesis was disordered, and many spermatocytes and spermatids were sloughing. Sperms could not be seen in the lumen of the tubules. The characteristic accumulation of interstitial cells was the most noteworthy. In the immunohistochemistry for cytochrome P450 side-chain cleavage enzyme, immunoreactions were not entirely distributed in each interstitial cell, although positive reactions were scattered in the interstitial cell-mass. The findings indicate that few interstitial cells act as a testosterone-synthesizing apparatus in the characteristic structure with accumulated cell-mass. From the immunohistochemical data we suggest the possibility that spermatogonia and Sertoli cells may secrete 17β-estradiol. We also suggest that 17β-estradiol from spermatogonia and Sertoli cells may inhibit the interstitial cells from synthesizing and secreting testosterone and may suppress the later stages of the spermatogenesis to induce apoptosis of germ cells. The *TUNEL* methods demonstrated that cell death occurred in some spermatocytes in non-breeding males.

Key words: naked mole-rat, spermatogenesis, testosterone, *TUNEL*, 17β-estradiol

INTRODUCTION

The naked mole-rat (*Heterocephalus glaber*) maintains a eusocial system with castes in the reproductive colony where only one female "queen" is reproductively active (Jarvis, 1981, 1984; Brett, 1986, 1991; Jarvis and Benett, 1991; Lacey and Sherman, 1991; Sherman *et al.*, 1992; Nowak, 1999). The queen mates with only a few specific breeding males, and all other males and females live as workers or soldiers in a highly-systematized subterranean nest. The males as workers or soldiers do not show reproductive behavior, but these males bring food and nest-materials to the queen and juveniles and make up the burrow system to help the reproduction of the queen within the eusocial castes. The testis of non-breeding males as workers or soldiers has been examined by light and electron microscopy, and the inactive seminiferous tubules and the accumulated interstitial cells have been observed (Fawcett *et al.,* 1973; Onyango *et al.,* 1993). High levels of plasma testosterone were found in non-breeding males (Onyango *et al.,* 1991), and low levels of urinary testosterone were reported (Faulkes *et al.*, 1991, 1994). However, the relationships between the microscopic findings of the inactive testis and the endocrinological and apoptotic control mechanisms

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have not been clarified in this species. In this study, therefore, we apply the immunohistochemical method on the steroidogenesis and the *in situ* analysis on the cell death to the testis of non-breeding male and elucidate the reproductive strategy of this species based on the endocrinological and apoptotic controls in the testis.

MATERIALS AND METHODS

In this study, we used 3 males of the naked mole-rat (*Heterocephalus glaber*). The animals were introduced into the experimental nest system, maintained as a reproductive colony in Chiba University, and were considered as inactive males through observations of their behavior. Each had head and body length of more than about 90 mm, so the animals could be regarded as adults. The pathological changes could not be discerned in the macroscopic observation of the carcasses.

The inactive testes were measured and observed by naked eyes. Testicular tissues were excised and fixed in 10% formalin solution. After 24 hr of fixation, the tissues were dehydrated in ethanol, treated with xylene and embedded in paraffin. The paraffin blocks were sectioned at 4 μm thickness, stained with haematoxylin and eosin, and analyzed by light microscopy.

The immunohistochemical technique was based on our previous reports (Kimura *et al*., 1997; Nishiyama *et al*., 1999). Paraffin was removed by xylene and the sections were treated in a 5% solution of normal goat serum to prevent background staining. The sections were reacted overnight at 4°C to the primary antibody against the rat cytochrome P450 side-chain cleavage enzyme ($P450_{SCC}$) (purchased from Chemicon International Inc. CA, U.S.A.), and diluted (1:200) with BSA/PBS solution to detect steroidogenesis in the testicular cells. Control sections were treated with normal rabbit serum instead of the antibody. Sections were washed with PBS and incubated with biotinylated Goat IgG against rabbit immunoglobulin for 1 hr. We applied the ABC methods to visualize the immunoreactions using 10mg/100ml DAB (3,3' diaminobenzidine) solution including 0.01% H_2O_2.

The *TUNEL* method was also applied by using the Mebstain apoptosis kit (Medical & Biological Laboratories, Nagoya, Japan) to detect DNA fragmentation. After the deparaffinization and the treatment of the protainase K and H_2O_2, the sections were reacted with biotinylated dUTP in the TdT reacting solution, and incubated with streptavidin-peroxidase conjugate. The immunoreactions were also visualized using DAB.

RESULTS

The naked mole-rat possessed a testis bilaterally within the abdomen (Fig. 1), but did not possess a scrotum. The length of the testis in the non-breeding males was 4-5 mm, and the width 3-4 mm. The epididymis was dorsally attached to the testis.

Masses of the abundant interstitial cells were observed, and seminiferous tubules were sparsely distributed among them (Fig. 2). Active spermatogenesis was not observed, and we could not confirm the spermatozoa in the lumen of the seminiferous tubules (Figs. 3–5). Spermatids were observed in two individuals (Figs. 3 and 4), although they were not confirmed in one animal (Fig. 5). In the former, spermatogonia were found in the inner part of the basement membrane. Although the spermatocytes were encountered within the tubules, the arrangement of these cells was disordered. Both leptonema and pachytene stages of the spermatocytes were revealed in the tubules. We also confirmed the well-developed cytoplasmic elongation or network of Sertoli cells, however the spermatocytes and spermatids were largely sloughing. The spermatids with small cytoplasm and nucleus could be observed in the area close to the lumen (Figs. 3–4). The accumulation of the interstitial cells was noteworthy. Enlarged cytoplasm was stained with haematoxylin in the interstitial cell-mass. Each interstitial cell possessed an oval nucleus (Fig. 6).

In the immunohistochemical examination, strong reactions could be detected in the cytoplasm of many sper-

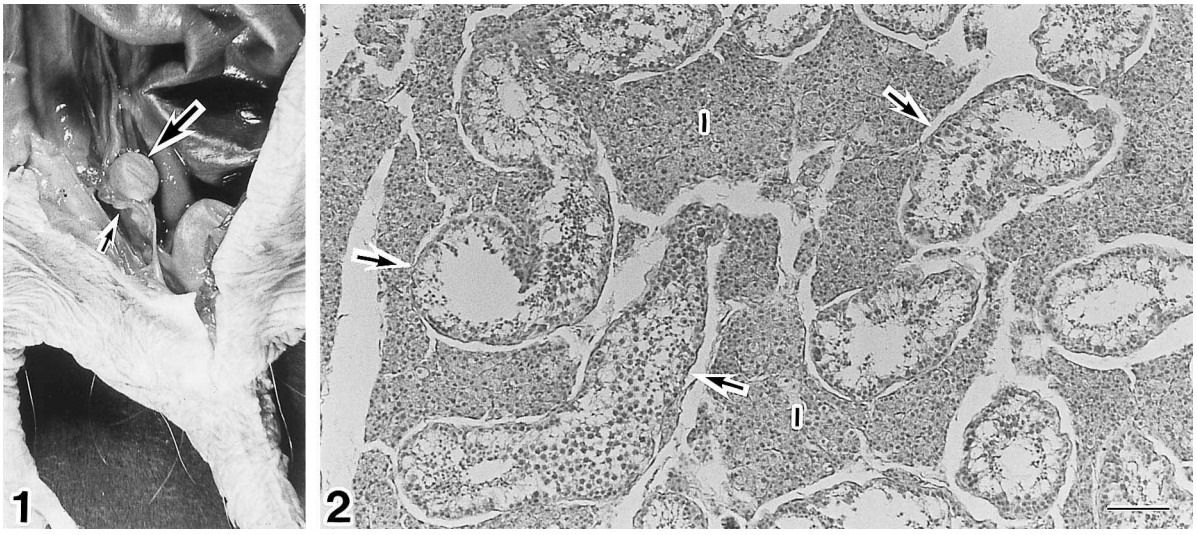

Fig. 1. Ventral aspect of non-breeding male. Cranial direction at the top. The right small testis (large arrow) is observed in intra-abdominal space. The epididymis (small arrow) is dorsally attached to the testis.

Fig. 2. A light micrograph of the testis. The seminiferous tubules (arrows) are observed among masses of the interstitial cell (I). Stained with haematoxylin and eosin. Bar=80 μm.

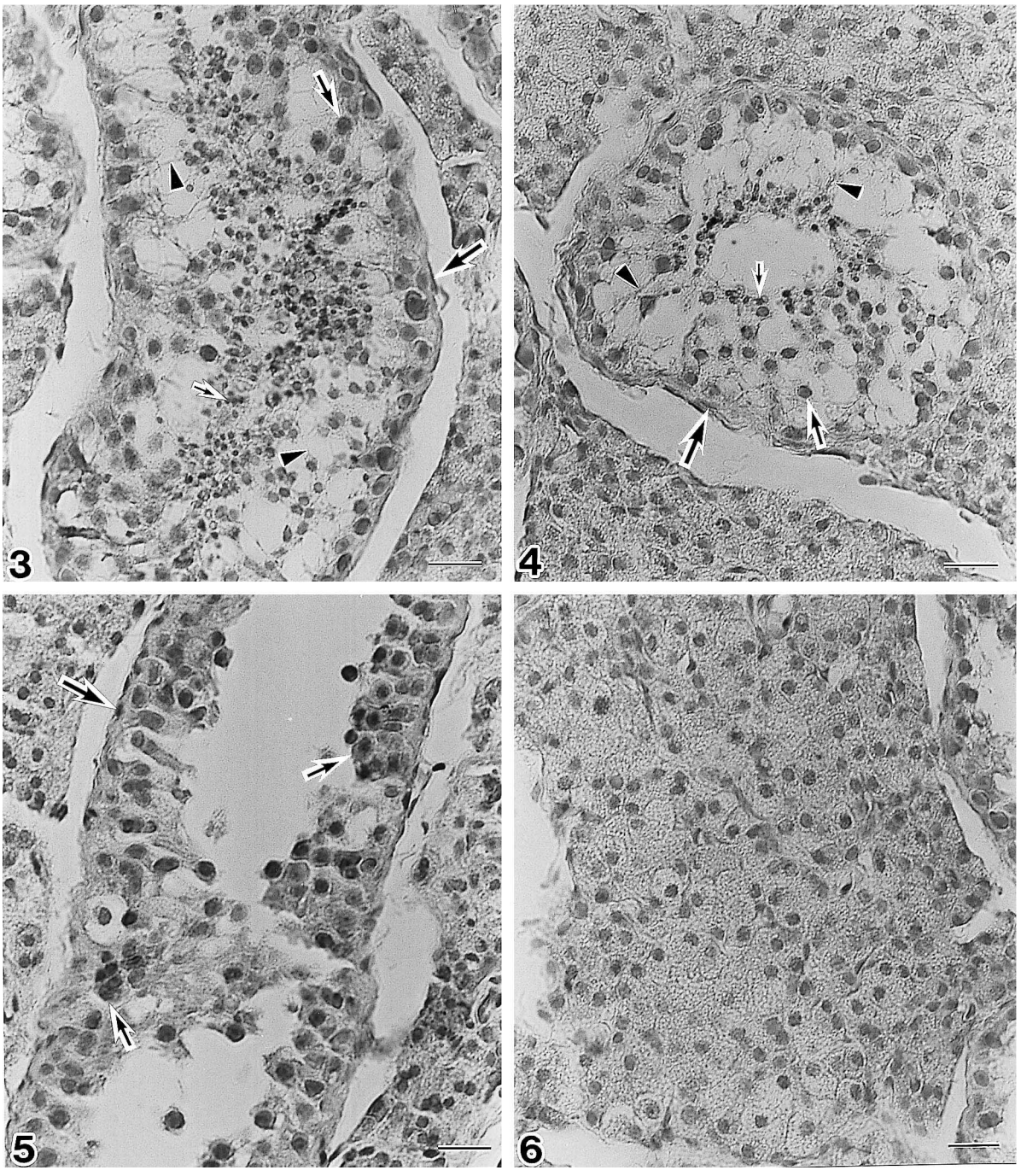

Fig. 3. A light micrograph of the testis. Longitudinal section of the seminiferous tubule. The spermatogonia (large arrow), spermatocytes (intermediate arrows) and spermatids (small arrow) can be observed. The cytoplasm networks of Sertoli cells (arrowheads) are confirmed. The spermatocytes and spermatids are sloughing. Stained with haematoxylin and eosin. Bar=20 μm.

Fig. 4. Transverse section of the seminiferous tubule. The spermatogonia (large arrow), spermatocytes (intermediate arrow) and spermatids (small arrow) can be observed. Arrowheads, the cytoplasm network of Sertoli cells. Stained with haematoxylin and eosin. Bar=20 μm.

Fig. 5. Transverse section of the seminiferous tubule. The spermatogonia (large arrow), spermatocytes (intermediate arrow) are seen. The spermatids cannot be observed in this animal. Stained with haematoxylin and eosin. Bar=20 μm.

Fig. 6. Characteristic masses of the interstitial cells. The cells possess enlarged haematoxylin-stained cytoplasm and an oval distinct nucleus. Stained with haematoxylin and eosin. Bar=20 μm.

matogonia and some Sertoli cells (Fig. 7). The immunoreactions were arranged in the basement membrane of each seminiferous tubule. Only a few obvious DAB reactions were sparsely encountered in the mass of the interstitial cells (Fig. 8). The reactivity could be found within the cytoplasm of each interstitial cell. The reacted cells were restricted and scattered in the cell-mass, and many cells did not show any immunoreaction. The immunoreactions could

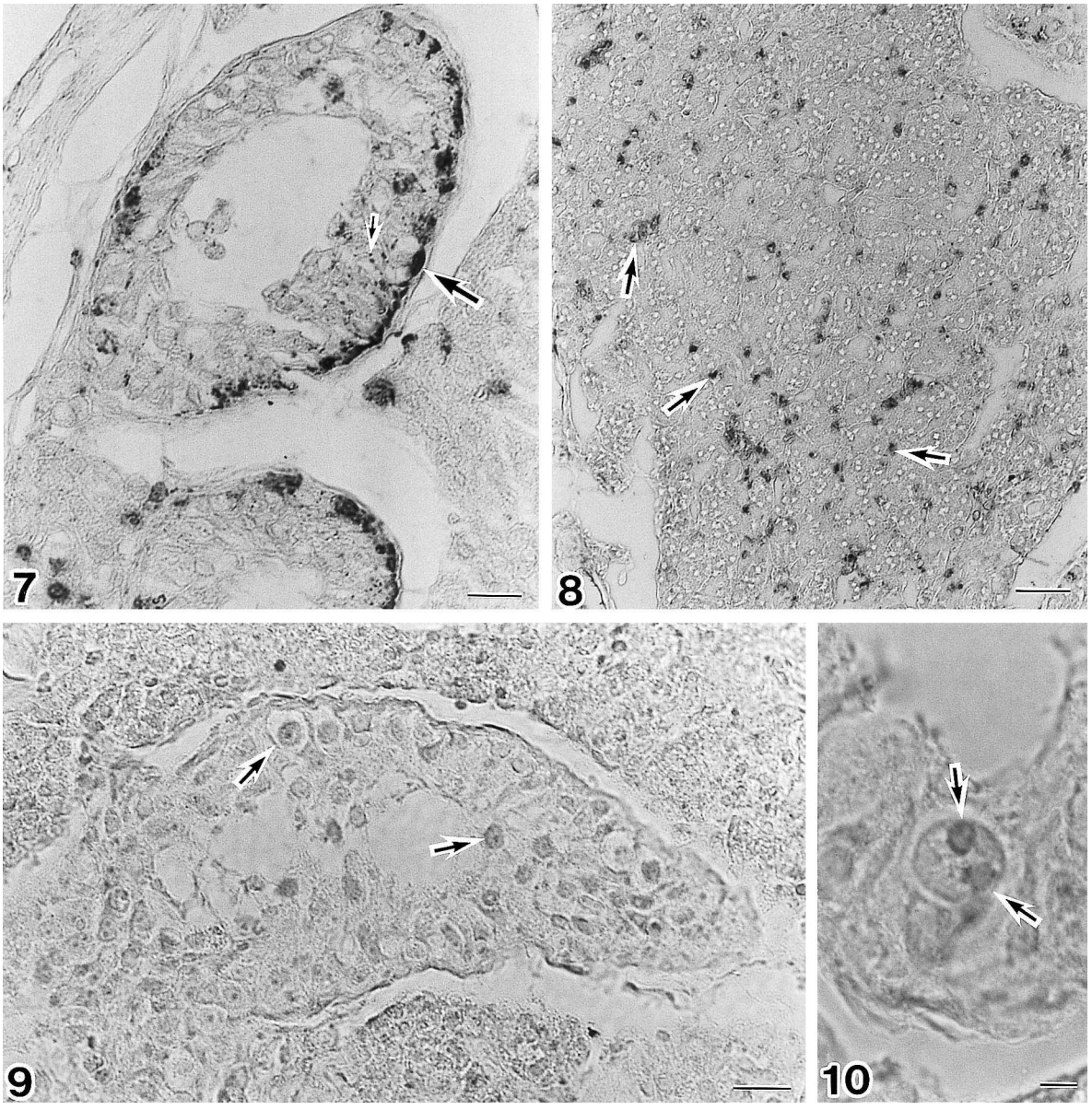

Fig. 7. Immunohistochemical reactions for cytochrome P450 side-chain cleavage enzyme. The strong reactions are detected in the cytoplasm of many spermatogonia (large arrows) and some Sertoli cells (small arrow). Bar=20 μm.
Fig. 8. Immunohistochemical reactions for cytochrome P450 side-chain cleavage enzyme. Only a few obvious DAB reactions (arrows) are sparsely encountered in the mass of the interstitial cells. Bar=20 μm.
Fig. 9. The *TUNEL*-positive reactions (arrows) are confirmed in the nucleus of some spermatocytes. Bar=20 μm.
Fig. 10. The typical apoptotic bodies (arrows) can be detected in the nucleus of some *TUNEL*-positive spermatocytes. Bar=5 μm.

not be seen in the spermatocyte and spermatids in the seminiferous tubules.

The *TUNEL*-positive reactions were confirmed in some spermatocytes (Fig. 9). Other germ cells did not show the *TUNEL* reactions. We observed the reaction in the nucleus, in which typical apoptotic bodies could be detected in some spermatocytes (Fig. 10).

DISCUSSION

We used the words “interstitial cell” in place of Leydig cell, although the cells are obviously equal to the Leydig cells. Their mass appeared so abnormal that we prudently hesitated to name them Leydig.

The sperm storage sac was observed in non-breeding animals as shown in a previous anatomical study (Faulkes *et al*., 1994). The testicular morphology was reported in non-breeding male (Onyango *et al.,* 1993). The electron micrograph showed many spermatogonia, primary spermatocytes and a few secondary spermatocytes and early stage spermatids. Our findings and the ultrastructural data point out that non-breeding males have a disorder in spermatogene-

sis and that the cell differentiation actually stops at the stages of spermatocytes or early spermatids. Spermatozoa and sperms have been confirmed in previous works (Faulkes and Abott 1997; Clarke and Faulkes 1998; Faulkes *et al*., 1991) unlike our findings. However, the differences are not biologically substantial, since sperms and spermatozoa are considerably few in the non-breeding males in these previous reports.

The important point of this study was to clarify where the steroidogenesis occurs in testicular tissues and to discuss how steroid hormones suppress at least the later stages of spermatogenesis in the inactive testis. Accumulation of the interstitial cells in our study is consistent with previous findings (Fawcett *et al.,* 1973; Onyango *et al*., 1993). The electron micrographs showing smooth endoplasmic reticula and mitochondria have suggested that the interstitial cells may have a function of steroidogenesis in this species (Burgos *et al*., 1970; Christensen, 1975). A large amount of the lipid droplets in cytoplasm indicates that the interstitial cells synthesize and store steroid hormones in inactive males (Onyango *et al*., 1991, 1993).

The interstitial cells with activity of cytochrome $P450_{SCC}$ indicate their ability to synthesize testosterone for stimulation of spermatogenesis to the later stages. In this study, however, immunoreactions to cytochrome $P450_{SCC}$ were not entirely distributed in each interstitial cell, and DAB-positive reactions were scattered in the interstitial cell-mass (Figs. 6 and 8). So, we conclude that few interstitial cells act as a steroid-synthesizing apparatus. We think that testosterone-synthesizing activity may be scarce despite the presence of a large amount of interstitial cells.

The cytochrome P450 aromatase is present in germ and Sertoli cells to synthesize 17β-estradiol from testosterone (Carreau, 2001; Hess *et al*., 2001). 17β-estradiol converted from testosterone suppresses the secretion of gonadotropins to induce the apoptosis of the germ cells (Blanco-Rodriguez and Martinez-Garcia, 1998). Since the occurrences of cytochrome $P450_{SCC}$ were visualized in spermatogonia and Sertoli cells in this study (Fig. 7), we suggest that these cells may synthesize testosterone and 17β-estradiol (Carreau, 2001; Hess *et al*., 2001). It is also suggested that 17β-estradiol from spermatogonia and Sertoli cells may inhibit the interstitial cells from synthesizing and secreting testosterone. We think that the steroidogenic function of the interstitial cell-mass and the occurrence of the later stages of the spermatogenesis may be strongly suppressed by above-mentioned hormonal regulation and that non-breeding males do not have a substantial function to complete the differentiation of germ cells in the seminiferous tubules.

Programmed cell death known as apoptosis is accompanied by condensation of cytoplasm, loss of plasma membrane microvilli, condensation and fragmentation of nuclei, and degradation of chromosomal DNA into oligomers of 180 base pairs. The *TUNEL* method has enabled *in situ* visualization of DNA fragmentation at the single cell level. In spermatogenesis, the apoptosis was encountered in many spermatocytes by the *TUNEL* method in the report on the experimentally-induced cryptorchidism (Itoh *et al.,* 1997). As shown in the cryptorchidism, the *TUNEL* reactions were restricted in the spermatocytes in this study. This suggests that the spermatocytes have biological weaknesses with high frequency of cell death under the endocrinological conditions of non-breeding males.

In the reproduction strategy of the eusocial systems in the naked mole-rat, the males as workers and soldiers must be suppressed by endocrinological controls from the anterior pituitary and the testis. Certainly it has been thought that the pheromonal signals primarily may reach the males to suppress spermatogenesis and to determine animals as workers and soldiers (Jarvis 1994). However the theory of hormonal controls in suppression of spermatogenesis in non-breeding males has not been established. Our results will be effective in elucidating the steroid-hormonal interactions among the characteristic mass of the interstitial cells, germ and Sertoli cells.

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