1. ABSTRACT

Experimental cryptorchidism is a common model for examining the expression and function of heat-sensitive spermatogenesis-related genes in testis. Previous studies have shown that germ cells in cryptorchid testis die mainly in an apoptotic way. The molecular mechanism, however, is still unclear. We have established unilateral cryptorchid monkey model (Cynomolgus Macaque) to identify possible molecules involved in the germ cell apoptosis. The degree of germ cell apoptosis, the morphology of the cryptorchid testis, and the changes in the serum concentration of FSH, LH and testosterone after cryptorchid surgery were analyzed. Sertoli cell marker molecule vimentin, the orphan receptor LRH-1, as well as the mitochondria-related protein HSP60 and Bcl-2 were examined. Our results showed that the weight of the cryptorchid testis decreased in a time-dependent manner started from day 7 after the surgery, while the weight of the scrotal testis had no obvious change. HE staining showed that from day 5, some germ cells were detached from the epithelium. A massive degeneration of the seminiferous epithelium characteristic of epithelial structural disorganization and the formation of multinucleated giant cells as well as vacuoles was observed on day 10 and 15. The cryptorchidism induced a marked germ cell apoptosis on day 3 after the operation, reaching a peak level on day 7. The apoptotic germ cells were mainly primary spermatocytes. Radioimmunoassay results showed that serum testosterone level was significantly decreased (p<0.01) in the unilateral cryptorchid monkeys on day 1 and the low level was maintained to the end of the experiment. LH concentration in the serum decreased significantly on day 3 (p<0.05) and subsequently recovered to the normal level. In contrast, no obvious change in the serum FSH concentration was detected. Immunohistochemistry data showed that the pattern of HSP60 expression was mainly perinuclear in the spermatogonia, spermatocytes and Sertoli cells. Weaker staining was also observed in the Leydig cells. In the cryptorchid testis the staining for HSP60 was obviously stronger in these cells. Vimentin staining was observed mainly in cytoplasm of Sertoli cells in the scrotal testis. The expression of vimentin was collapsed and obviously increased in a time-dependent manner in the cryptorchid testis. Western blotting results indicated that HSP60, Bcl-2, and LRH-1 expression increased significantly in the cryptorchid testis as compared to the scrotal testis. RT-PCR data further verified the increase of hsp60 mRNA in the cryptorchid testis. These observations suggest that multiple molecular pathways participate in the germ cell apoptosis induced by cryptorchidism.
2. INTRODUCTION

During the course of spermatogenesis, spermatogonia proliferate and differentiate into mature spermatooza through a complex series of changes (1). It has been reported that clinical cryptorchidism, artificially induced experimental cryptorchidism, local testicular hyperthermia at 43 °C water, as well as varicocele all result in increased death of germ cells (2-6). Cryptorchidism is a common congenital anomaly usually leading to clinical infertility in man due to severe decrease in sperm production (7-8). Previous studies showed that artificially induced cryptorchidism could decrease sperm production by germ cell apoptosis (9-12). The spermatocytes and spermatids are the most sensitive cell types affected by heat stress (13-15). However, the molecular mechanism is still not well characterized.

HSP60 is a member of heat-shock protein (HSP) family. The proteins of this family act as molecular chaperones that assist other proteins in their folding, transportation and assembly. They also protect cells from environmental hazards, such as heat, radiation, and chemicals (16, 17). HSP60 is one of the best-characterized molecular chaperones in both eukaryotic and prokaryotic organisms (18). It is expressed constitutively and is moderately induced in response to environmental insults such as heat (19). In eukaryotes, HSP60 is encoded by nuclear DNA, synthesized within the cytoplasm and quickly imported into mitochondria matrix where it is assembled into a single toroid ring of the seven subunits (20, 21).

HSP60 has been found specifically expressed in gonad (22-24). In the ovary of postnatal rat, HSP60 was detected in the oocytes at all stages of follicular development, while in the pubertal and mature ovary, its expression was high in cytoplasm of theca cells and corpora lutea (22). In rat and human testis, HSP60 was expressed mainly in cytoplasm of spermatogonia, early spermatocytes and Leydig cells during normal spermatogenesis (23, 24). HSP60 protein was also detected in Sertoli cells of primate testis (25). However, the post-meiotic germ cells in rat and Leydig cells in immature primate testis are negative for HSP60 expression (24, 25).

In testicular biopsies from patients with disturbed fertility, the expression of HSP60 decreases with loss of spermatogenic function (24). In infertile men, a decrease in the number of HSP60-expressing spermatogonia paralleled with low spermatogenic efficiency (24). Data obtained from other related studies have demonstrated that HSP60 expression is related to cell death. In cultured epithelium cells HSP60 could result in a marked cell death probably mediated by MAP-2 (26).

Moreover, data obtained from cardiovascular and heart cells showed that extra-mitochondrial HSP60 in plasma membrane could form complexes with both Bax and Bak, but not with Bcl-2 (27, 28). Evidence from cardiac muscle cells indicated that over expression of Hsp60 could increase anti-apoptotic Bcl-xl and Bcl-2 expression, and reduce the protein content of pro-apoptotic Bax, resulting in suppression of cell apoptosis (29). In addition, hyperthermia induced decrease in HSP60 expression, but not in other heat shock proteins (30). These studies strongly suggest that HSP60 may be involved in germ cell apoptosis in testis induced by hyperthermia.

FSH, LH and testosterone play key regulatory roles in spermatogenesis (31-33). Production of male gametes depends on the concerted action of FSH and testosterone (34, 35). Both FSH and testosterone have been found to stimulate all phases of spermatogenesis. In rat, testosterone is an absolute requirement for spermatogenesis to promote adhesion of round spermatids to Sertoli cells. In the absence of testosterone round spermatids are sloughed from the epithelium and spermatid elongation fails. Since male germ cells possess neither FSH nor androgen receptors (35), the action of FSH and testosterone should occur through Sertoli cells. FSH is required for Sertoli cell proliferation and for the maintenance of normal sperm production. The release of mature elongated spermatids from Sertoli cells (spermiation) is also under FSH/testosterone control in rat. Several studies, however, have demonstrated that a high level of testosterone could directly suppress FSH secretion from pituitary (35, 36). Inhibin-beta subunit secreted by Sertoli cells is governed by the stimulatory and inhibitory action of FSH and LH, respectively (35). The data imply that FSH, LH and testosterone play an essential role in spermatogenesis.

Liver receptor homolog-1 (LRH-1; NR5A2) is a member of Ftz-F1 subfamily of nuclear orphan receptors, and is expressed in endoderm of intestine, liver, exocrine pancreas and ovary. LRH-1 has been suggested to play a role in cancer development, reverse cholesterol transport, bile-acid homeostasis and steroidogenesis (37). Studies on breast adipose tissue (38) and mouse ovary (39) have indicated that LRH-1 up-regulates estrogen biosynthesis by enhancing aromatase activity. LRH-1 is expressed mainly in Leydig cells and germ cells, but not in Sertoli cells in rat testis, LRH-1 could increase P450 activity that may play an important role in converting testosterone to estrogen (40).

Vimentin is an important cytoskeleton protein that participates in Sertoli cell shape construction in seminiferous epithelium (41). In Sertoli cell, the 57-kDa vimentin monomers form filaments that surround nuclear expression as a “halo” appearance (42). Vimentin has been reported to play a critical role in surviving of Sertoli cell and germ cells. In cryptorchid testis of immature rat, immunostaining of vimentin revealed loss of intermediate filament extensions, and filaments collapse to a perinuclear localization, which coincided with a massive apoptosis of germ cell (12). In addition, it has been demonstrated that the redistribution of vimentin filaments might depend on hormonal regulation (43). The collapse of the Sertoli vimentin filament cytoskeleton has also been observed after lowering intratesticular testosterone, implying that vimentin expression, to some extent, is related to testosterone changes (44).

The present study was designed to investigate the molecular mechanism of germ cell apoptosis induced by
heat stress. We have chosen 21 adult health monkeys (Cynomolhus Macaque) at the age of 5-8 year old and established an artificial unilateral cryptorchid monkey model. The molecules related to germ cell apoptosis, such as HSP60, LRH-1, Bcl-2, as well as the Sertoli cell marker molecule vimentin were comparatively examined in both the scrotal and the cryptorchid testis in relation to the changes in peripheral serum testosterone, FSH and LH levels.

3. MATERIALS AND METHODS

3.1. Materials and reagents

Radioimmunoasssay kits for FSH, LH and testosterone were purchased from the National Hormone and Pituitary Distribution Program, National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases, China. Digoxigenin labeled dUTP kit was purchased from Promega Biotechnology, Inc. Anti-Digoxigenin antibody was from Boehringer Mannheim Biotechnology, Inc. Trizol mRNA extraction kit and Super reverse transcriptional enzyme were purchased from GibcoBRL Biotechnology, Inc. Rabbit anti-goat HSP60 antibody, goat anti-rabbit Bcl-2 antibody, goat anti-rabbit vimentin antibody and goat anti-rabbit actin antibody were purchased from Santa Cruz Biotechnology, Inc. Antibody against LRH-1 was generously provided by Dr. D. M. Stocco.

3.2. Animals and tissue preparations

Adult male animals (Cynomolhus Macaque) at the age 5-8 year old were chosen for this study. The animals used for this study was approved by the Academic Committee of Institute of Zoology, Chinese Academy of Sciences and the Organization of Wild Animal Protection in Guangxi province. All the animals were caged individually in the Experimental Animal Research Center in Guangxi province, China. The animals were fed with fresh monkey food, fruit and water.

The animals were randomly separated into seven groups (n = 3). One group of these animals remained intact as the normal control. To induce unilateral cryptorchidism, the animals were anesthetized, and a midline incision was made in the abdomen. The gubernaculums was cut on the other side was remained intact. Both the abdominal testis and the intact scrotum testis of all the animals were collected on days 1, 3, 5, 7, 10 and 15 after operation. The testes were decapsulated and cut into thin sections (6 µm) for immunohistochemistry. The others were quickly frozen in liquid nitrogen and stored under -70°C for Western blot and RT-PCR analysis.

3.3. Histomorphological analysis

Sections were deparaffinized and fixed in xylene, and rehydrated using a series graded ethanol (100%, 95%, 85% and 70%) and water. Using a 0.1% dilute HCL washed away unwanted color from the sections, then followed by a bluing with 0.1% ammonia. Finally the sections were dyed again using 1% eosin solution, dehydrated and cleared in a series of ethanol and xylene, and mounted.

3.4. Radioimmunoasssay

To evaluate the effect of cryptorchidism on hormonal changes in the circulating blood of the experimental monkeys, the blood samples were drawn from the animals on day 1, 3, 5, 7, 10 and 15 respectively after the cryptorchid operation. The control blood samples were also drawn from three intact animals. All serum samples were obtained by centrifugation (3500g, 10 min) and frozen at -20°C till to perform RIA. The serum FSH, LH and testosterone were assayed by the standard RIA procedures as described previously (45).

3.5. In situ 3' end labeling of testicular DNA fragmentation

Sections were deparaffinized in xylene, and rehydrated by a series graded ethanol (100%, 95%, 85% and 70%), and then incubated in a 37°C water bath with proteinase K (20 microgram/ml) for 30 min. Washed in phosphate-buffer saline (PBS), and incubated in transferase buffer for 10 min. Then DNA 3’-end labeling were performed using a terminal deoxynucleotidyl transferase reaction system for 1 h at 37°C in a wet chamber. After washed in Tris buffer, the sections were incubated in a blocking buffer for 30 min at room temperature. Then the anti-digoxigenin antibody conjugated alkaline phosphatase were added onto the sections and incubated for 1 h at room temperature in a wet chamber, washed again in Tris buffer. Finally the sections were visualized with NBT (Nitro blue tetrazolium) and BCIP (5-bromo-4-choro-3-indolyl phosphate) mixture in Tris buffer.

3.6. Immunohistochemistry

Sections were deparaffinized, immersed in PBS, and pretreated with a 3% H2O2 in distilled water to stop endogenous peroxidase activity. The sections were blocked with 10% normal horse serum (NHS) in PBS, then followed by incubation overnight at 4°C with the polyclonal goat anti-rabbit antibody of HSP60 (Santa Cruz, USA) and polyclonal Rabbit anti-goat antibody of Vimentin (Santa Cruz, USA) respectively, both antibodies were diluted using 10% NHS in PBS (HSP60, 1: 400; Vimentin, 1: 200). Subsequently the sections were washed for three times in PBS and incubated for 30 min at room temperature using a 2% NHS diluted biotinylated immunoglobulin (1:300). Sections were washed three times with PBS again and incubated 30 min at room temperature with PBS diluted peroxidase-conjugated streapavidin (1:200), finally the sections were dyed using a DAB kit, dehydrated and cleared in a series of ethanol and xylene, and mounted.

3.7. Western blotting

To evaluate the expression of HSP60, Bcl-2 and LRH-1 proteins during the artificial induced cryptorchidism, the testicular tissues were homogenized in a RIPA buffer using Dounce homogenizer (Fisher
Germ cell apoptosis in cryptorchid testis

Figure 1. Weight Changes in cryptorchid testis during treatment. N, testes before treatment; d1, d3, d5, d7, d10, d15 represent day 1, day 3, day 5, day 7, day 10 and day 15 after the surgery respectively. Each time point represents the average weight from three monkeys.

Figure 2. Histo-morphological analysis of scrotal and cryptorchid testes by HE stain. a: scrotal testis; b-e: cryptorchid testes on day 1, 5, 10 and 15 after surgery respectively. f: a magnification of a (×400). The arrows show the multinucleated giant cell. The seminiferous epithelium of cryptorchid testis was obviously disturbed from day 5 (d) after the surgery, multinucleated giant cells were observed in the cryptorchid testis on day 10 (Figure 2d). Only Sertoli cells and a few spermatogonia were left in the cryptorchid seminiferous epithelium on day 15. Original magnification ×200.

Scientific, Pittsburg, PA, USA), the supernatants were collected by centrifugation and total protein concentration were quantified by spectrophotometer. Samples containing 50μg total protein were mixed with loading buffer (1,4-dithiothreitol 62.5 mmol/L, 5%SDS, and 10% glycerol). After boiled at 95°C for 5 min, the protein were separated by running on a 12% SDS-PAGE gel and transferred onto nitrocellulose membranes. Then the membranes were blocked 1 h using 5% skim milk in phosphorus buffer saline (PH 7.4) at room temperature and sequentially incubated using polyclonal antibodies of HSP60 (Goat anti-rabbit antibody, dilution, 1:1000, Santa Cruz), Bcl-2 (Rabbit anti-goat antibody, dilution, 1:1000, Santa Cruz) and LRH-1 (Goat anti-mouse, dilution, 1:1000) over night at 4°C, respectively. After washing with PBS, the membranes were incubated with the corresponding peroxidase-conjugated second antibodies for 2 h at room temperature and washed again with PBS. The protein bands were visualized with Enhanced Chemiluminescence (ECL) Western-blotting Analysis Kits according to the procedure described by the manufacturer (Pierce Co, Rochford, USA). The final data were acquired by scanning the protein band density.

3.8. RT-PCR

Total RNA were extracted from the tissues of the cryptorchid testis and the scrotal testis that had been frozen in liquid nitrogen using a Trizol RNA extraction kit according the guide manuscript. The cDNA was synthesized from total RNA using Supo-2 reverse transcriptase primed by Oligo (dT). PCR reaction of hsp 60 mRNA was carried out using the following primers: sense, 5′-GCT ACT GTA CTG GCA CGC TCT A-3′; antisense, 5′-TCG CTC AAC AGA ACA TAG GCA-3′, at a procedure of 94°C, 15s denaturing, 56°C, 15s annealing and 72°C, 40s extension, 24 cycles.

3.9. Data analysis

The data from the samples of three individual monkeys were analyzed for each treatment. Experiments were repeated at least three times. One way ANOVO was used for analyzing the data from different groups. Data are presented as means ± S.E.M, probability values less than 0.05 were considered as significant. For immunocytochemistry and in situ hybridization, one representative was shown from at least three similar results.

4. RESULTS

4.1. Changes in testicular weight and histomorphology induced by unilateral cryptorchidism

To examine the effect of abdominal temperature on testicular weight and the tissue morphology, both the cryptorchid and the scrotal testes were collected by surgery at various time points after the unilateral cryptorchid operation. The testes were weighed using a balance. The total average weight of the cryptorchid testis was significantly decreased after day 7 of the operation (Figure 1).

We examined tissue histology both in the scrotal and the cryptorchid testes, as shown in Figure 2. HE staining showed that some germ cells were detached from the epithelium of seminiferous tubules starting from day 5 (Figure 2c), a massive degeneration of the seminiferous epithelium characterized by disorganization and multinucleated giant cells as well as vacuoles was observed on day 10 (Figure 2d) in the cryptorchid testis, the seminiferous epithelium became thinner, and the tubular lumen was devoid of spermatids and spermatozoa on day 15 (Figure 2e). In addition, the diameter of the tubules on days 10 and 15 was obviously reduced by the heat stress. Due to loss of the germ cells, tubules only contained Sertoli cells, spermatogonia as well as few spermatocytes. Most of the dead cells were eliminated through phagocytosis by the Sertoli cells (Figure 2e). In contrast, the condensation of the germ cell nuclei and the detachment of the germ cells were negative in the scrotal testes (Figure 2a).
Germ cell apoptosis in cryptorchid testis

4.2. Germ cell apoptosis induced by artificial cryptorchidism

To examine the extent of germ cell apoptosis after artificial-induced cryptorchidism, in situ 3'-end labeling was performed on the paraffin-embedded tissue sections from the scrotal and cryptorchid testis using digoxigenen-dideoxyuridine triphosphate (ddUTP) (Figure 3). The results indicated that cryptorchidism induced a marked germ cell apoptosis on day 3 (Figure 3c) after the operation, and the cell apoptosis reached a peak level on day 7 (Figure 3e). Few ddUTP positive germ cells were observed at sections on day 15 (Figure 3f), probably because of severe loss of germ cells after longer exposure to the abdominal temperature. The apoptotic germ cells were mainly primary spermatocytes.

4.3. Changes in serum level of testosterone, LH and FSH in cryptorchid monkeys

To estimate the possibility that the unilateral cryptorchidism may change the circulating level of testosterone, LH and FSH, we detected the peripheral concentration of these hormones in the cryptorchid monkeys as compared with the intact controls. As shown in Figure 4, the serum testosterone concentration was significantly decreased in the cryptorchid monkeys from day 1 after the operation (p<0.01) and remained low during the treatment as compared to the control (Figure 4A). LH concentration in the serum was only significantly decreased on day 3 after the operation (p<0.05), and was not significantly different from the control thereafter (Figure 4B). In contrast, the serum FSH level (Figure 4C) was not obviously changed by the unilateral cryptorchidism operation.

4.4. Immunohistochemical localization of vimentin in cryptorchid testis

Paraffin sections of the testicular tissues were used for study of immunohistochemical localization of vimentin. As shown in Figure 5, in the scrotal normal testis, vimentin staining was mainly detected in the perinuclear region of the Sertoli cells in the seminiferous tubules. In the cryptorchid testis (Figure 5c-e), a strong vimentin staining was detected in Sertoli cells on day 7 (Figure 5d) and day 15 (Figure 5e) after operation.

4.5. Expression of HSP60 in cryptorchid testis

As shown in Figure 6, immunohistochemical expression of HSP60 was detected in the spermatogonia, some primary spermatocytes and Sertoli cells in the cryptorchid testis. A weak staining was also observed in the Leydig cells, but not in the post-meiotic germ cells (Figure 6b). A higher magnifying section showed that HSP60 had a perinuclear expression pattern in the seminiferous epithelium (Figure 6f). After induced cryptorchidism, the expression of HSP60 was significantly increased in the seminiferous epithelium of cryptorchid testes (Figure 6c-e), particularly in the spermatogonia and Sertoli cells (Figure 6c-d). The strongest staining was observed in the Sertoli cells at the end of the operation (Figure 6e).

Expression of HSP60 protein was also quantitatively examined by western blotting. As shown in Figure 7A, the antibody for HSP60 (a polyclone antibody against goat) recognized a single band at the position of 60kDa, suggesting the antibodies used in this study specifically reacted with HSP60 in the testicular homogenates. The data showed that the abdominal temperature could significantly increase HSP60 expression in the cryptorchid testis in a time-dependent manner (Figure 7B-a), starting from day 1 (p<0.05), reaching the maximum on day 7 after operation, and still remained at a higher level thereafter.

For further analyzing the effect of abdominal temperature on HSP60 expression in the cryptorchid testis, we performed a half quantitative RT-PCR technique to investigate the expression of hsp60 mRNA in the cryptorchid and the scrotal testis (Figure 8). The results showed that increase in temperature could enhance hsp60 mRNA expression in the cryptorchid testis. This result was consistent with the data obtained from the western blotting analysis, further confirming that the abdominal temperature stimulates expression of HSP60.

4.6. Western blot analysis for expression of Bcl-2 and LRH-1 protein in cryptorchid testis

The expression of Bcl-2 and LRH-1 proteins was quantitatively examined by western blotting. As shown in Figure 7A, the anti-body for Bcl-2 (polyclone antibody against rabbit) revealed a single band at the position of 26kDa, suggesting the antibodies specifically reacted with...
Germ cell apoptosis in cryptorchid testis

**Figure 4.** Changes in serum testosterone, LH, and FSH levels in cryptorchid cynomolgus monkeys. (a) Serum testosterone level significantly decreased from day 1 after cryptorchid operation, and remained low thereafter. (b) LH was significantly decreased on day 3, but no significant difference was observed after day five. (c) FSH level in serum was not obviously changed. The RIA data was obtained from the serum samples of three individual monkeys at each point. Data were shown as means ± SD (n=3).

**Bcl-2** in the testicular homogenates. LRH-1 had a main band at 43kDa position (Figure 7A). Our data showed that the abdominal temperature could significantly increase Bcl-2 (Figure 7B-b) expression from day 1 to day 7 in the cryptorchid testis (p<0.05), from day 10 the molecular expression decreased. The expression of LRH-1 (Figure 7B-c) significantly increased on day 3 (p<0.01) and reached a maximum level on day 7, and then decreased gradually.

**5. DISCUSSION**

Germ cell apoptosis during spermatogenesis has been extensively studied either for exploring new method for male contraception or for effective therapy for male fertility defect. The lower scrotal temperature is an important prerequisite for optimal spermatogenesis (46). It has been reported that elevation of testicular temperature by 1 suppressed spermatogenesis by 14% (47). Observations made in infertile men with oligozoospermia or azoospermia suggest that some of the patients may have higher testicular temperature due to an intrinsic defect in scrotal thermoregulation, varicocele, or occupational exposure to high temperatures (6, 48-50). Despite the pathways for germ cell apoptosis have been reported (51, 52), the precise molecular mechanism underlying heat-induced germ cell apoptosis is not well characterized.

Artificially-induced cryptorchidism is a common model for examining changes in heat sensitive related-spermatogenesis genes in testis. It has been well documented that abdominal hyperthermia is the main factor causing germ cell apoptosis in the cryptorchid testis (11, 48, 53). It has been reported that germ cell apoptosis by hyperthermia is mediated mainly by mitochondria-dependent pathway and the pathways elicited by the death receptors (51, 52). We have established various rat and rhesus monkey models to study germ cell apoptosis, and conformably concluded that both heat stress and treatment with higher testosterone could markedly induce germ cell apoptosis (15, 54-57, 65). However, the germ cell apoptosis in the cryptorchid testis occurred much early than that induced by testosterone treatment. The molecular mechanism, however, is not completely understood. In this study, we have chosen the cynomolgus monkey and established a unilateral cryptorchid model to examine the possible mechanism of germ cell apoptosis induced by the heat stress.

It has been reported that HSP60 was expressed in Leydig cells during neonatal and prepubertal period in rat testis (58). In the present study, HSP60 was detected in Sertoli cells, spermatogonia, and a weak staining was also observed in spermatocytes and Leydig cell in the normal monkey testis. However, HSP60 signals were obviously elevated in the cryptorchid testis after the heat shock. The increased HSP60 protein was mainly observed in Sertoli cells and spermatogonia. Despite the increase in expression of HSP60, apoptosis in these cells was rarely detected. Recent studies have suggested an anti-apoptotic role of cytosolic HSP60 in various types of cells under stress conditions (59, 60). Shan et al. (29) provided data to show that HSP60 could modulate post-translation modification of Bcl-2 protein family. Over-expression of HSP60 was associated with enhancing induction of Bcl-xl, and additional suppression of Bax and more profound inhibition of Caspase 3 production. Moreover, HSP60 also down-
Germ cell apoptosis in cryptorchid testis

**Figure 5.** Immunohistochemical staining for vimentin in cryptorchid testis. a, negative control; b, scrotal testis; c-e, cryptorchid testis on day 1, 7, and 15 after surgery; f, a magnification of b; arrows show a perinuclear expression of vimentin in scrotal testis. Vimentin staining in scrotal seminiferous tubules was observed mainly in the perinuclear region of Sertoli cells; Appearance of increased and disorganized vimentin staining in cryptorchid testis on day 7 and 15 was observed. Original magnification ×200.

**Figure 6.** Immunohistochemical staining of HSP60 in testes of Cynomolgus macaca. a, negative control; b, scrotal testis; c-e, cryptochid testis on day 1, 7 and 15 respectively after surgery. f, a magnification of b. HSP60 expression could be observed in spermatogonia, spermatocytes and Sertoli cells. A weak expression of this protein could be also observed in Leydig cells. Arrows show a perinuclear expression of HSP60 protein in Sertoli cells. Increased HSP60 expression was observed in cryptorchid testis on day 7 and day 15. Original magnification ×200.
Germ cell apoptosis in cryptorchid testis

Figure 7. Western blot analysis of HSP60, Bcl-2 and LRH-1 expression in cryptorchid testes. (A) Proteins in the testicular extracts were separated by SDS-PAGE and immunoblotted with anti-HSP60, Bcl-2 and LRH-1 polyclonal antibody respectively. (B) Quantitative analysis for HSP60 (B), Bcl-2 (C) and LRH-1 (D). These protein expression was significantly increased in cryptorchid testis. N, scrotal normal testis; d1 to d15: cryptorchid testis on day 1, 3, 5, 7, 10 and 15 respectively after surgery. Experiments from three individual monkeys and repeated three times. Data were shown as means ± SD (n=3). O.D. represents optical density.

regulated Bad expression in the doxorubicin-treated cells (29). These observations suggest that HSP60 has a stronger anti-apoptosis property in cells.

HSP60 has been reported to be specifically expressed in the gonad, and may play an important role in normal cell survival (22-24, 26-29). Evidence has shown that HSP60 could bind to Bax and thus decrease forming complexes with Bcl-2 in the germ cells (27-29). In other words, the increased HSP60 expression in the cryptorchid testis could indirectly increase free Bcl-2 protein and decrease free Bax protein in the germ cells. Bcl-2, an important anti-apoptosis mitochondria protein, was over-expressed in the germ cells of the heat-stressed testis (15, 57, 61). In the present study we did observe that an increase in Bcl-2 expression was correlated with an increase in HSP60 in the cryptorchid testis. Our previous study on Rhesus monkey also demonstrated a decrease in Bax expression in the cryptorchid testis (57). We propose, therefore, that the increased expression of HSP60 in the cryptorchid seminiferous epithelium may release free Bcl-2 by forming complex with Bax which is capable of promoting cell apoptosis via mitochondria pathway (15, 57; 62), whereas Bcl-2 may play a converse role in anti-apoptosis by forming complexes with Bax (63, 64), mainly in Sertoli cells and spermatogonia in the cryptorchid testis.

Recently Zhang et al reported that HSP60 could increase several phosphatase activities in the cultured epithelium cells, such as MAP-2, which is capable of strongly dephosphorylating ERK1/2 activity and leading to cell apoptosis (26). Our unpublished new data demonstrated that ERK1/2 in heat-treated Sertoli cells in vitro or in the cryptorchid testis was significantly phosphorylated. It is, therefore, suggested that increased HSP60 expression in the cryptorchid seminiferous epithelium may play a role in germ cells apoptosis through MAP-2 and ERK1/2 pathway.

In Sertoli cell, vimentin may play a role in forming cellular skeletons, directly and indirectly protect Sertoli cells and germ cells for survive under environmental hazards (12, 41, 42). We observed that the vimentin protein was collapsed and augmented in the Sertoli cells of the cryptorchid testis. This finding is consistent with the previous reports in Rhesus monkey (65) and rats (12). The collapse expression of vimentin in Sertoli cells of the cryptorchid testis might be a defensive response to the “heat-stress” that could severely weaken the contact of the germ cells to the Sertoli cells, leading to germ cell apoptosis in the cryptorchid testis.

Gonadotropins and testosterone have been reported to play key regulatory roles in spermatogenesis (31-33, 66-68). Production of male gametes depends on the concerted action of FSH and testosterone (34, 35). Both FSH and testosterone have been found to stimulate all phases of spermatogenesis. In our present study, peripheral plasma testosterone level was significantly decreased after the unilateral cryptochid operation in cynomolgus monkeys. A significant decrease in LH concentration on Day 3 after the operation was also noted. In previous studies, low LH level firstly reported in the peripheral plasma of cryptorchid men (69). Several reports also showed that serum levels of testosterone and LH were decreased in the artificial-induced cryptorchid animals (70, 71), while plasma estrogen level in spermatic vein was significantly increased (71-73). Estrogen plays an important role during spermatogenesis, that has been well depicted by Carreau et al (74). Our data in this study showed that the circulation testosterone concentration was significantly down-
Germ cell apoptosis in cryptorchid testis

Figure 8. RT-PCR analysis for changes in HSP60 mRNA in cryptorchid testis. (A) RT-PCR of HSP60: Using total RNA extracted from cryptorchid testis; RT-PCR of actin mRNA as a control; (B) Analysis of HSP60 mRNA by scanning the RT-PCR blot density. The results show that HSP60 mRNA was increased in cryptorchid testis. N, scrotal normal testis; d1 to d15 represent cryptorchid testis on day 1, 3, 5, 7, 10 and day 15 after surgery respectively. Experiments were repeated three times. Data were shown as means ± SD (n=3). O.D., optical density.

Figure 9. A schematic representation of germ cell apoptosis induced by cryptorchidism. Two major, the extrinsic and the intrinsic, pathways for caspase activation in germ cells are presented. The extrinsic pathway can be induced by members of the TNF family of cytokine receptors, such as TNFR and Fas. The intrinsic pathway can also be activated by cryptorchidism, resulting in release of cytochrome C from mitochondria and the subsequent apoptosome formation. Both apoptotic pathways activate the common downstream effector protease, caspase-3, to execute apoptosis. p53 can result in increase of Fas expression and hence induce killing by the Fas/Fas ligand pathway. It can also interfere with Bcl-2 family proteins to regulate the fate of germ cell, including elevations in the levels of pro-apoptotic members such as Bax or down-regulation of anti-apoptotic Bcl-2 expression level. On the other hand, up-regulation of mitochondrial Hsp60 expression by cryptorchidism indirectly increase free Bcl-2 protein and decrease free Bax protein in Sertoli cells and spermatogonia to help the inhibition of activating caspase-3, thus inhibiting germ cell apoptosis. The increased LRH-1 production in the cryptorchid testis might accelerate transformation of testosterone into estrogen leading to the decrease in the serum testosterone concentration, subsequently enhancing germ cell apoptosis by activating testosterone-dependent Fas/FasL death receptor pathway.
Germ cell apoptosis in cryptorchid testis

regulated, while the LRH-1 expression was obviously up-regulated in the cryptorchid testis. LRH-1 has been reported to play an important role in regulating aromatase activity (38), which is a critical enzyme in estrogen synthesis using testosterone as the substrate in testis (40), suggesting that the increased LRH-1 expression in the cryptorchid testis might be responsible for the decrease in the circulating testosterone level. Testosterone may also exert a function for cell survival by suppressing Fas expression (35). Estrogen was reported to inhibit hypothalamus and pituitary function by a negative feedback loop in male animals (75), the increase in estrogen production in the cryptorchid testis might be responsible for the decrease in serum LH level. However, this hypothesis should be further clarified in further work.

In our previous studies, we have demonstrated that administration of exogenous testosterone (TU) to Rhesus monkeys induced germ cell apoptosis by increase in Fas/FasL expression (56). It suggested that the abdominal temperature induced germ cell apoptosis may be also via a testosterone-dependent Fas/FasL death receptor pathway. In addition, lower testosterone may regulate vimentin expression and lead the filaments to collapse (43-44). Therefore, we suggest that the changed expression of vimentin in the cryptorchid testis may be partly resulted from the decreased testosterone.

In summary, the increased expression of HSP60 and Bcl-2 in the cryptorchid testis may suggest a protective mechanism of these molecules against hyperthermia, while vimentin may exert a positive role in forming cellular skeletons, directly and indirectly to protect Sertoli cells and germ cells for survive under environmental hazards during germ cell apoptosis in cryptorchid testis. The increased LRH-1 production in the cryptorchid testis might accelerate transformation of testosterone into estrogen leading to the decrease in the serum testosterone concentration, subsequently enhancing germ cell apoptosis by activating testosteronedependent Fas/FasL death receptor pathway.

Based on the data from the present and our early studies as well as the data available in the literature we proposed a representative diagram to show the possible multiple signal pathways controlling germ cell apoptosis induced by cryptorchidism (Figure 9)

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Germ cell apoptosis in cryptorchid testis


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Germ cell apoptosis in cryptorchid testis


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