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Expression of *Hsp70-2* in Unilateral Cryptorchid Testis of Rhesus Monkey During Germ Cell Apoptosis

Xin-Chang Zhou,[1] Xiao-Bin Han,[1] Zhao-Yuan Hu,[1] Ru-Jin Zhou,[2] and Yi-Xun Liu[1]

[1]State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing, China; and [2]Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, China

We investigated the possible role of *Hsp70-2* in germ cell apoptosis induced by heat stress in monkey unilateral cryptorchid testis. The study focused on *in situ* analysis of the testicular cell DNA fragmentation and on the possible relationship between *Hsp70-2* expression and germ cell apoptosis. The TUNEL result showed that most of the germ cells were labeled in the cryptorchid testis on d 5 after induction of cryptorchidism; that with most of the apoptotic germ cells depleted, only a few germ cells were labeled on d 10; and that almost no apoptotic signal was observed in the cryptorchid testis on d 15 and thereafter. This indicates that the increasing germ cell degeneration in cryptorchid testis may take the form of apoptosis. Using *in situ* hybridization, immunohistochemistry, and Northern blot, we examined the changes of *Hsp70-2* expression in the monkey cryptorchid testis. The level of *Hsp70-2* mRNA decreased slightly, while the expression of HSP70-2 protein was almost unchanged at the early stage of germ cell apoptosis in the cryptorchid testis on d 5 and dropped dramatically along with the loss of apoptotic germ cells in the cryptorchid testis on d 10 after operation. It is therefore suggested that *Hsp70-2* might not take part in inhibiting the apoptosis of germ cells at the early stage during operation-induced cryptorchid testis, and that *Hsp70-2* gene does not belong to the immediate early related gene responsible for germ cell apoptosis induced by heat stress.

Key Words: Apoptosis; cryptorchid testis; heat stress; *Hsp70-2*.

Introduction

HSP70-2 protein is a member of the mouse 70-kDa heat-shock protein (HSP70) family functioning as molecular chaperones *(1)*. *Hsp70-2* is a single-copy gene and was first isolated from mouse genomic library *(2)*. The expression of *Hsp70-2* begins in meiosis prophase in letotene spermatocytes and is developmentally regulated *(3–5)*. Targeted disruption of the gene showed that the male mice homozygous for the mutation were infertile, with the late pachytene spermatocytes undergoing apoptosis *(6)*. In addition, *Hsp70-2* is associated with the synaptonemal complex of the homologous chromosomes in spermatocytes. Although synaptonemal complexes form in spermatocytes of *Hsp70-2*$^{-/-}$mice, the paired chromosomes fail to desynapse in diplotene spermatocytes at the end of the meiotic prophase *(7,8)*.

Spermatogenesis is sensitive to modest elevation of temperature. In most mammals, the testes are maintained 5–7ºC below body temperature by their location outside the body cavity in the scrotum and by a countercurrent heat-exchange process that cools the blood entering the testes *(9)*. The cooler temperature may provide the optional environment for testicular development and function. Spermatogenesis has been reported to be disrupted readily by a modest increase in temperature *(10)*, such as in cryptorchidism, with germ cells undergoing apoptosis *(11)*. In monkey cryptorchid testis, however, whether the degenerated germ cells undergo apoptosis is uncertain. Moreover, the molecular mechanism related to heat-affected spermatogenesis and germ cell apoptosis is not well understood.

The expression of testicular orphan receptor 2 (TR2) *(12,13)* and its role in germ cell apoptosis in cryptorchid testis *(14)* have been investigated in our previous studies. To study further the possible relationship between *Hsp70-2* and germ cell apoptosis induced by heat stress in monkeys, we have established a monkey cryptorchidism model and examined the changes of *Hsp70-2* expression and the testicular apoptotic signal in cryptorchid testis as compared with those in the normal testis by using TdT-mediated dUTP nick end labeling (TUNEL), *in situ* hybridization (ISH), immunohistochemistry, and Northern blot techniques.

Results

In Situ *Analysis of Apoptotic DNA Fragmentation in Germ Cells of Cryptorchid Testis*

To study the specific cells with apoptotic DNA fragmentation of unilateral cryptorchid monkeys, *in situ* 3'-end labeling with digoxigenenin-dideoxyuridine triphosphate (ddUTP) was performed on paraffin-embedded sections of the testes

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Author to whom all correspondence and reprint requests should be addressed: Prof. Yi-Xun Liu, State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100080. E-mail: liuyx@panda.ioz.ac.cn

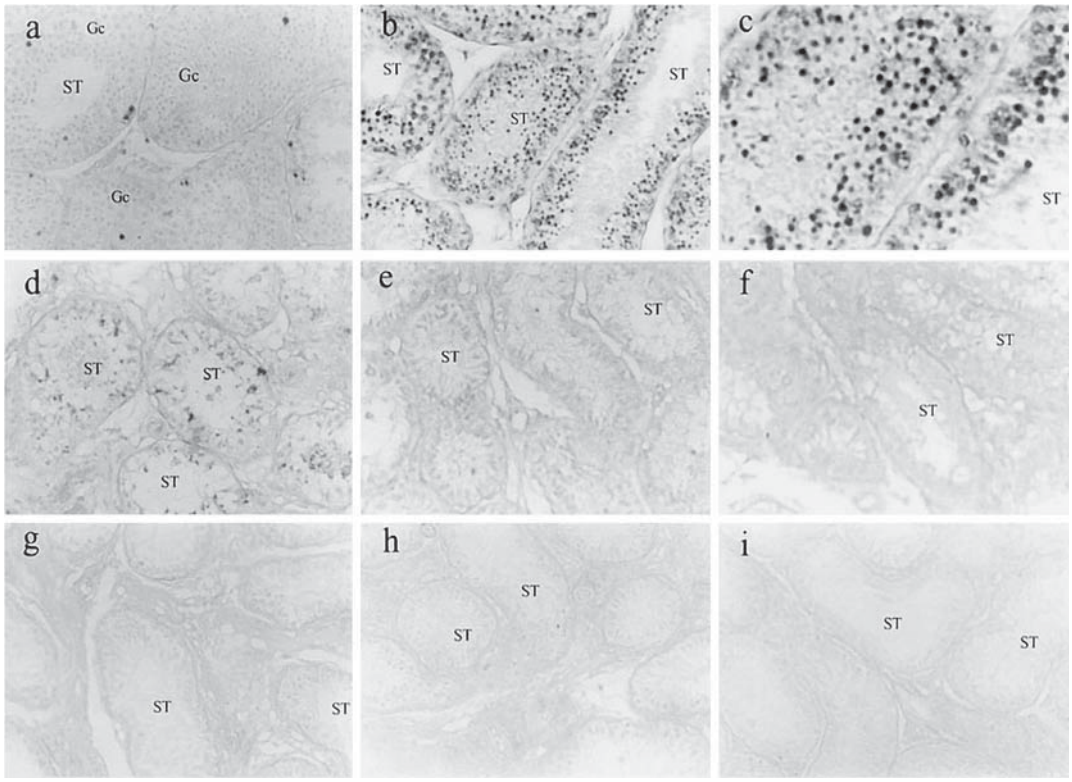

Fig. 1. *In situ* 3'-end labeling of DNA fragmentation on testicular sections from unilateral cryptorchid monkeys. (**A**) Normal testis showed only a few germ cells undergoing apoptosis. Most of the germ cells were labeled on d 5 after induction of cryptorchidism. With a few germ cells labeled, however, most of the apoptotic germ cells were depleted on d 10. Almost no apoptotic signal was observed on d 15, 20, 30, 45, and 60. (**B, D–I**) Cryptorchid testis on d 5, 10, 15, 20, 30, 45, and 60, respectively. (**C**) Higher magnification of (B). ST, seminiferous tubule; Gc, germ cell. (A), (B), and (D–I): magnification is ×200; (C): magnification is ×400.

from 22 adult unilateral cryptorchid and normal monkeys. The strongest signals labeled appeared on d 5 after induction of cryptorchidism (Fig. 1B), whereas on d 10 the signals became very weak, with most of the germ cells depleted (Fig. 1D). Almost no labeled germ cell was observed on d 15, 20, 30, 45, and 60 (Fig. 1E–I). Moreover, these labeled cells were identified as primary spermatocytes (Fig. 1C). In contrast to the samples from cryptorchid testes, in contralateral scrotal testes at different times after operation (data not shown) and normal testes, only a few of speromatogenic cells showed DNA fragmentation (Fig. 1A).

Localization of HSP70-2 Protein and Its mRNA in Germ Cells of Cryptorchid Testis

High levels of immunoreactivity for HSP70-2 protein were detected, and there was no difference in the levels of HSP70-2 expression between the contralateral scrotal testis at different times after operation (data not shown) and normal testis (Fig. 2A). Within the tubules, the strongest signals were found in spermatocytes and spermatids (Fig. 2B). Sertoli cells, as well as Leydig cells and peritubular cells, were clearly negative for immunoreactive HSP70-2 protein.

In cryptorchid testis, as compared with that of the normal testis, which has normal spermatogenesis, no obvious change in the immunoreactivity of HSP70-2 protein was seen in germ cells of the cryptorchid testis on d 5 after operation (Fig. 2C). The immunoreactivity for HSP70-2 protein decreased dramatically on d 10 along with the loss of the apoptotic germ cells (Fig. 2D), and then dropped to the undetectable levels on d 15 and thereafter (Fig. 2E–I).

The expression patterns and cell specificity of *Hsp70-2* mRNA were almost consistent with those of its protein, except that the level of *Hsp70-2* mRNA expression decreased slightly on d 5 (Fig. 3).

Changes in Relative Content of Hsp70-2 mRNA in Cryptorchid Testis

Total RNA was isolated from the normal and cryptorchid testes. The RNA was subjected to Northern blot analysis. The relative amount of *Hsp70-2* mRNA in each fraction was estimated by densitometric scanning of the Northern blot. To normalize the results for the amount of total RNA loaded onto each lane of the gel used for the Northern blot, the blot was also probed with GAPDH probe. As compared

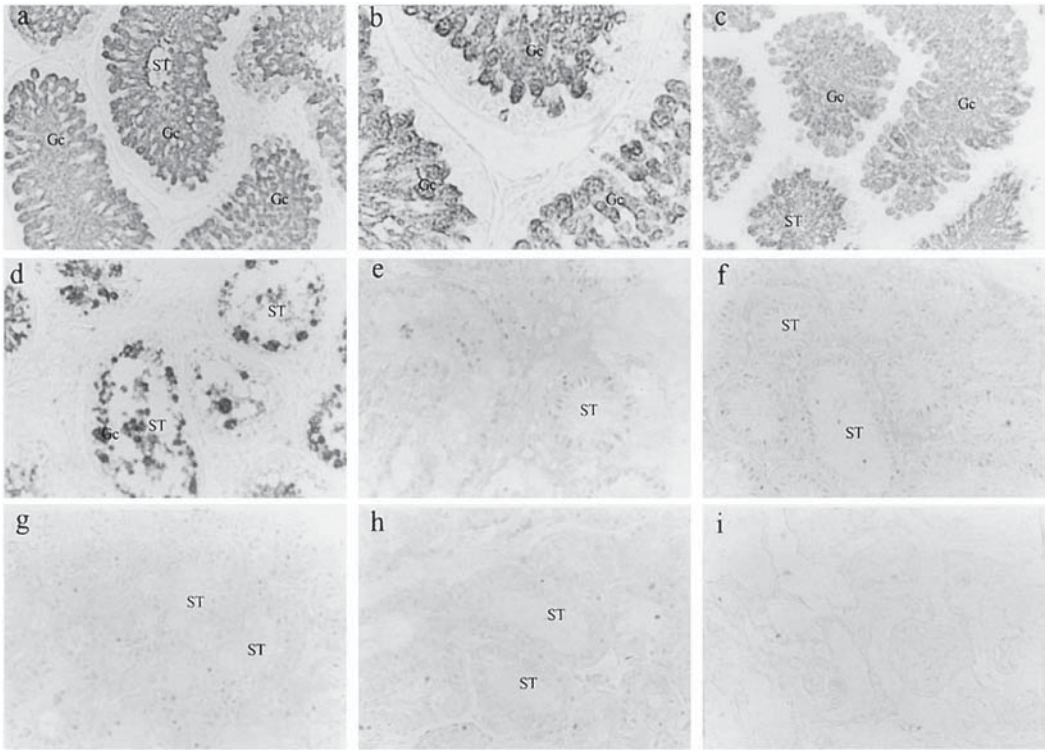

Fig. 2. Immunohistochemical localization of HSP70-2 protein in normal and cryptorchid testis. (**A**) Normal testis; (**B**) higher magnification of (A); (**C–I**) cryptorchid testis on d 5, 10, 15, 20, 30, 45, and 60, respectively. Compared with the expression of HSP70 protein in the normal testis, the level of HSP70-2 expression was almost unchanged in the cryptorchid testis on d 5. It decreased markedly along with the loss of the apoptotic germ cells on d 10. No HSP70-2 protein could be detected on d 15, 20, 30, 45, and 60 after the induction of cryptorchidism. ST, seminiferous tubule; Gc, germ cell. (A) and (C–I): magnification is ×200; (B): magnification is ×400.

with its expression in normal testis, the level of *Hsp70-2* mRNA decreased slightly in the cryptorchid testis on d 5 after operation (Fig. 4); however, it dropped dramatically in the cryptorchid testis on d 10. The estimated level of *Hsp70-2* transcript was five times lower than that observed in the normal testis. Almost no *Hsp70-2* mRNA was detected in the cryptorchid testis on d 15 and thereafter (Fig. 4A).

Discussion

Although the linkage of germ cell apoptosis to cryptorchidism, the failure of the testis to descend into the scrotum, has been documented, the detailed molecular mechanism has remained unclear. We have reported that the expression of TR2, which modulates many signal pathways, was completely repressed in the surgery-induced cryptorchidism of rats and rhesus monkeys *(12,13)*. Further studies have shown that repression of TR2 in cryptorchid testis was induced by the p53 → p21 → CDK → Rb → E2F signal pathway. In return, TR2 could also control the expression of p53 and Rb through the regulation of human papillomavirus 16 E6/E7 genes *(14)*. These data suggest that a feedback control mechanism between TR2 and p53/Rb tumor suppressor might play an important role in germ cell apoptosis in cryptorchid testis.

Here we demonstrated the changes in *Hsp70-2* expression and its possible role in germ cell apoptosis in the unilateral cryptorchid monkey testis. Its expression pattern in normal monkey testis was similar to that in mice *(15,16)* and rat *(17)*. Expression of *Hsp70-2* gene was restricted to spermatocytes and spermatids and regulated developmentally in the normal monkey testis (Figs. 2A,B, 3A,B, 4). The results provided new evidence for the hypothesis that the *Hsp70-2* is a relatively conserved gene from bacteria to human and may play an important role in spermatogenesis in mammals *(16,18)*.

HSP70-2 protein, as a molecular chaperone, exists in complexes with other proteins that regulate their adenosine triphosphatase activity and may determine which proteins they assist *(19–21)*. Data from gene knockout mice further demonstrated that HSP70-2 is a chaperone for CDC2, whose kinase activity has a key role in triggering the G/M transition during spermatogenesis *(16)*. In *Hsp70-2* gene knockout mice (*Hsp70-2*$^{-/-}$), disruption of CDC2/cyclin B1 assembly into

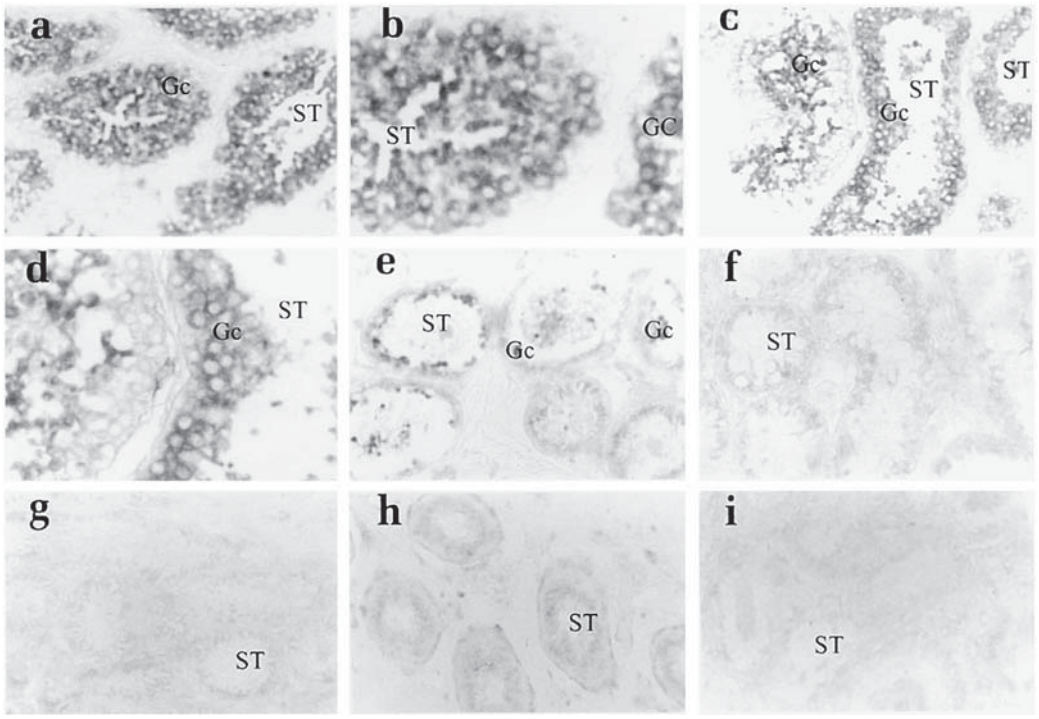

Fig. 3. ISH of *Hsp70-2* mRNA in normal and cryptorchid monkey testis. (**A**) Normal testis; (**C, E–I**) cryptorchid testis on d 5, 10, 15, 20, 30, and 45, respectively. Compared with the expression of Hsp70 mRNA in the normal testis, the level of *Hsp70-2* mRNA expression decreased slightly on d 5; however, it decreased dramatically along with the loss of the apoptotic germ cells on d 10. No *Hsp70-2* mRNA could be noted on d 15, 20, 30, and 45 after the induction of cryptorchidism. (**B, D**) Higher magnification of (A) and (C), respectively. ST, seminiferous tubule; Gc, germ cell. (A), (C), and (E–I): magnification is ×200; (B) and (D): magnification is ×400.

a heterodimer complex in pachytene spermatocytes, thus prevents CDC2 phosphorylation and the development of kinase activity necessary for G2/M phase transition, and the spermatocytes undergo apoptosis *(22)*. In addition, as a molecular chaperone, HSP70-2 protein can help proteins involved in DNA repair or recombination to fold or assemble into complexes, preventing apoptosis of pachytene spermatocytes *(16)*. However, the precise mechanisms by which HSP70-2 protein functions as chaperone to prevent apoptosis of germ cells are not well understood.

Increasing evidence has shown that heat stress in cryptorchid testis causes loss of germ cells by apoptosis *(10,11,13)*. Our TUNEL result is consistent with previous reports.

The pattern of *Hsp70-2* expression in monkey cryptorchid testis is interesting compared with that in normal testis. The level of *Hsp70-2* mRNA decreased slightly (Fig. 3) while the HSP70-2 protein level was almost unchanged on d 5 after the induction of cryptorchidism (Fig. 2). Levels of both *Hsp70-2* mRNA and protein dropped dramatically along with the loss of the apoptotic germ cells in the cryptorchid testis on d 10; almost no *Hsp70-2* mRNA and protein were detected in monkey cryptorchid testis on d 15 and thereafter. These changes were also confirmed by Northern blot analysis (Fig. 4). It is therefore suggested that *Hsp70-2* does not inhibit germ cell apoptosis induced by heat stress at the early stage during surgery-induced cryptorchidism. From these results, we proposed that although the expression level of *Hsp70-2* was highest in temperature-sensitive pachytene spermatocytes, the immediate early genes responsible for germ cell apoptosis in response to heat stress in the abdomen may be other molecules, such as p53 *(23)*. Regarding the expression of *Hsp70-2* in a single germ cell, however, the levels of *Hsp70-2* mRNA and protein increased in cryptorchid testis on d 10, compared with the expression of *Hsp70-2* in normal testis and cryptorchid testis on d 5 after operation. It is therefore suggested that *Hsp70-2* may inhibit germ cell apoptosis induced by heat stress at the later stage during surgery-induced cryptorchidism, but the action of this inhibition was canceled out by those of the apoptosis-induced genes, such as p53, so that almost all germ cells except for spermatogonia underwent apoptosis and were lost.

Materials and Methods

Animals and Tissue Preparation

Twenty-one male adult rhesus monkeys ages 5–7 yr were obtained from Kunming Institute of Zoology, Chinese Academy of Sciences (CAS) and approved for study by academic committees of both the Institute of Zoology and the Kunming Institute Primate Research Center, CAS. To induce

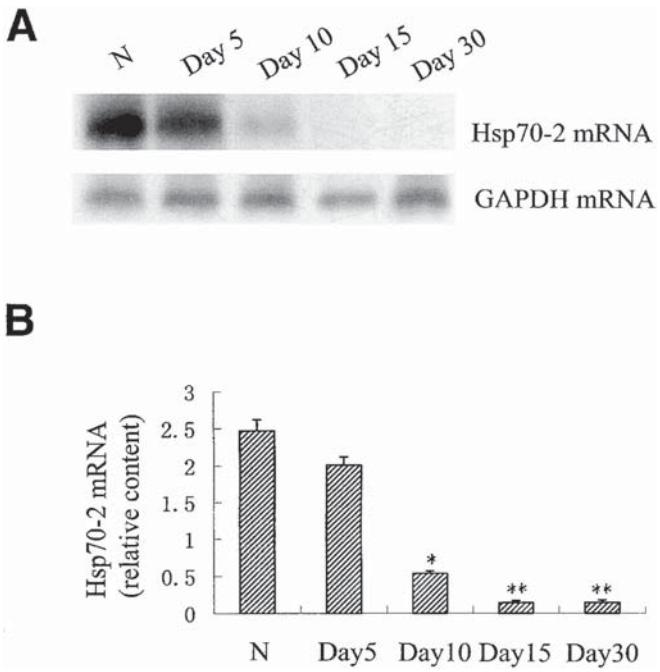

Fig. 4. Expression of *Hsp70-2* mRNA in normal and cryptorchid monkey testis. (**A**) Northern blot hybridization analysis using DIG-labeled *Hsp70-2* RNA probe. N, normal testis; Day 5, Day 10, Day 15, and Day 30 represent cryptorchid testis on d 5, 10, 15, and 30, respectively. GAPDH is used as a control. (**B**) The relative contents of *Hsp70-2* mRNA were normalized by the amount of GAPDH in the same samples. Values are the means ± SEM of three separate experiments. The level of *Hsp70-2* mRNA dropped slightly in the cryptorchid testis on d 5 after operation and decreased dramatically on d 10 and thereafter. Statistical significance was determined between the control (N) and cryptorchid groups at various times after operation for the relative content of *Hsp70-2* mRNA. $*p < 0.05$; $**p < 0.01$.

unilateral cryptorchidism, the animals were anesthetized by injection of pentobarbital sodium, and a small incision was made in the abdomen. The gubernaculum was cut on the right side to displace the testes into the abdomen. Descent of the testes was prevented by closure of the inguinal canal on the right side by suturing. The testes were removed at different intervals after surgery, decapsulated, and divided into quarters. One quarter was fixed in Bouin's solution and embedded in paraffin prior to sectioning (6 μm) for TUNEL, ISH, or immunohistochemistry. The other quarters were snap-frozen in liquid nitrogen and stored at −70ºC for RNA analysis.

Materials and Reagents

The plasmid containing *Hsp70-2* cDNA and the primary antibody to mouse HSP70-2 protein were kindly provided by Dr. E. M. Eddy, National Institute of Environmental Health Sciences, Research Triangle Park, NC. RNA labeling mixture, *in situ* cell death detection kit, alkaline phosphatase (AP)–conjugated anti-digoxigenin (DIG) IgG, and CDP-Star™ chemiluminescence reagent were purchased from Boehringer Mannheim GmbH (Mannheim, Germany). T3 and T7 RNA polymerase, *Eco*R1 and *Hind*III, and the substrates 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) were obtained from Prom (Madison, WI). Levamisole and paraformaldehyde were from Sigma (St. Louis, MO). Zeta-Probe nylon membrane and Gene Linker UV chamber were purchased from Bio-Rad (Richmond, CA). Acid guanidine thiocyanate and agarose gel were from Gibco-BRL (Life Technologies, Gaithersburg, MD). Formamide and APES were obtained from Beijing Zhongshan Biotechnology.

In Situ Analysis of Testicular Cell DNA Fragmentation

DNA fragmentation in histologic sections was done using a nonradioactive detection method *(24)* with slight modification. Sections (6 μm) were mounted on 3-aminopropyltyzethoxysilane (APES)-coated slides, deparaffinized, and hydrated. The slides were incubated with proteinase (20 μg/mL) for 15 min at 37ºC and washed in phosphate-buffered saline (PBS). Then DNA 3'-end labeling with ddUTP was preformed after incubating the slides for 10 min in terminal transferase buffer (200 m*M* potassium cacodylate; 25 m*M* Tris; 0.25 mg/mL of bovine serum albumin (BSA); and 5 m*M* $CoCl_2$, pH 6.6) at room temperature. Terminal transferase (1 U/μL), DIG-ddUTP (1 μ*M*), and dideoxy adenosine triphosphate (49 μ*M*) were added in fresh buffer and incubated at 37ºC in a humidified chamber for 1 h. After three washes in Tris buffer, the slides were incubated with a blocking buffer (100 m*M* Tris; 150 m*M* NaCl, pH 7.5; and 2% [w/v] blocking reagent) for 30 min at room temperature before adding anti-DIG antibody conjugated to AP. After incubation with the antibody (1:10,000 in 2% [w/v] blocking reagent; 100 m*M* Tris; and 150 m*M* NaCl, pH 7.5) at room temperature for 2 h in a humidified chamber, the slides were washed three times in Tris buffer and finally equilibrated in alkaline phosphatase buffer (100 m*M* Tris; 100 m*M* NaCl; and 50 m*M* $MgCl_2$, pH 9.5) before adding enzyme sustrates (337.5 μg/mL of NBT and 175 μg/mL of BCIP) for AP. After 60 min, the color reaction was terminated with 10 m*M* Tris and 1 m*M* EDTA (pH 8.0) and counterstained with eosin. Blue staining represented a positive reaction.

Immunohistochemistry

Immunohistochemistry was carried out with a Vectastain ABC Kit (Vector), as recommended by the manufacturer. Deparaffinized sections were incubated with 10% normal goat serum (NGS) in PBS for 30 min. The primary antibody to mouse HSP70-2 protein raised in rabbits was diluted in PBS containing 10% NGS and incubated with the sections for 1 h (the control groups were incubated with 10% NGS in PBS instead of primary antibodies). Then the sections were washed in PBS three times for 5 min each, incubated with biotinylated second antibody for 1 h, and washed in PBS three times for 5 min each. After incubation with avidin-biotin-peroxidase complex in PBS for 1 h and washing in

PBS three times for 5 min each, the sections were incubated in diaminobenzidine tetrahydrochloride in 0.05 *M* Tris-HCl (pH 7.2) with 0.01% H_2O_2 for 2–7 min. The sections were dehydrated through a graded series of ethanol, cleared in xylene, and then mounted.

Preparation of DIG Hsp70-2 cRNA Probes and InSH

Antisense *Hsp70-2* cRNA was transcribed with T7 polymerase after the plasmid was linearized by digestion of *Eco*R1, and sense *Hsp70-2* cRNA was transcribed with T3 polymerase after the plasmid was linearized by digestion of *Hind*III. The testis sections were deparaffinized, rehydrated, and pretreated with proteinase K (20 µg/mL) for 15 min and washed in PBS for 5 min. Sections were then fixed in 4% paraformaldehyde in PBS for 5 min and washed in PBS for 10 min. Before hybridization, the sections were dehydrated through a graded ethanol series and allowed to air-dry. The sections were prehybridized with 50% formamide and 2X saline sodium citrate (SSC) for 2 h at room temperature, then hybridized overnight with DIG-labeled *Hsp70-2* RNA probe in hybridization buffer (10 m*M* Tris-HCl, pH 7.5; 2X SSC; 50% deionized formamide; 1X Denhardt's; 2.5 m*M* dithiothreitol; 5% dextran sulfate; 250 µg/mL of yeast tRNA; 0.5% sodium dodecyl sulfate [SDS]) at 48ºC. After hybridization, the sections were thoroughly washed in 2X, 1X, and 0.1X SSC at 42ºC, each twice for 15 min each time. The sections were then rinsed in DIG Buffer I (0.1 *M* maleic acid; 150 m*M* NaCl, pH 7.5) for 5 min, blocked with 1% blocking reagent in DIG Buffer I for 1 h, incubated with AP-conjugated anti-DIG IgG diluted 1:500 in DIG Buffer I containing 1% blocking reagent for 1 h, and washed in DIG buffer I for three times for 5 min each. The bound antibody was detected by a standard immuno-AP reaction, using BCIP and NBT as substrates (containing 1 m*M* levamisole) for 2–6 h. The sections were dehydrated and mounted as described for immunohistochemistry. For control hybridization, the sections were hybridized with *Hsp70-2* sense RNA probe.

RNA Isolation and Northern Analysis

Total RNA was extracted from the normal and cryptorchid testes by a single-step acid guanidine thiocyanate–phenol–chloroform procedure *(25)*. Twenty micrograms of total RNA was electrophoresed on a formaldehyde denatured 1% agarose gel, vacuum blotted to a piece of Zeta-Probe nylon membrane at 45 mbar for 3 h, and crosslinked at 100 mJ by a GS Gene Linker UV chamber. The membrane was prehybridized in 50% deionized formamide, 5X SSC, 0.1% *N*-lauroylsarcosine, 0.02% SDS, and 2% blocking reagent at 68ºC for 2 h. It was then hybridized overnight to DIG-labeled RNA probe (about 400 ng of probe in 10 mL of prehybridization buffer). After hybridization, the membrane was washed with 2X SSC twice for 10 min each at room temperature and 0.1X SSC twice for 15 min each at 68ºC. The membrane was then rinsed in DIG Buffer I for 5 min, blocked with 1% blocking reagent in DIG Buffer I for 30 min, incubated with AP-conjugated anti-DIG IgG diluted 1:10,000 in DIG Buffer I containing 1% blocking reagent for 30 min, and washed in DIG buffer I three times for 10 min each. The membrane was next rinsed for 5 min in 0.1 *M* Tris-HCl/0.15 *M* NaCl (pH 9.5), incubated with CDP-Star chemiluminescence reagent, and exposed with a piece of FUJI medical X-ray film for 2–10 min. The relative contents of *Hsp70-2* mRNA were obtained by densitometric analysis using a Personal Densitometer SI (Molecular Dynamics) corrected for the amount of GAPDH and averaged for each replicate.

Data Analysis

Testes from three different individual monkeys were analyzed for each treatment group. Experiments were repeated at least three times, and one representative result is shown from at least three similar results. Quantitative data represent the mean ± SEM of at least three individual experiments.

Acknowledgments

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