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EXPRESSION OF HSP105 AND HSP60 DURING GERM CELL APOPTOSIS IN THE HEAT-TREATED TESTES OF ADULT CYNOMOLGUS MONKEYS (MACACA FASCICULARIS)

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1. ABSTRACT

To confirm that transient increase in temperature of the testis (43C for 30 minutes once daily for 2 consecutive days) could induce apoptosis of germ cells in non-human primates and to investigate the possible roles of Hsp105 and Hsp60 in regulation of germ cell loss, we conducted the study on eight cynomolgus monkeys. The sperm concentration on day 28 after heat shock decreased to 8.4% of pretreatment levels and recovered to baseline on day 144. Using the TUNEL assay, increased numbers of apoptotic spermatocytes and round spermatids were detected on days 3, 8, and 30 post heat treatment. Hsp105 and Hsp60 mRNA and protein levels were analyzed using *in situ* hybridization, RT-PCR, immunohistochemical and Western blot methods. Hsp105 was confined to nuclei of spermatids before treatment, decreased dramatically with the loss of spermatids on days 3, 8, and 30, before returning to baseline levels on days 84 and 144. The expression of Hsp60 was high on days 3, 8, 30 and was only detected in Sertoli cells and spermatogonia. These results suggested that exposure of the testis to heat resulted in selective, but reversible damage to the seminiferous epithelium via increased germ cell apoptosis. Temporal changes in the expression pattern of Hsp105 and Hsp60 in relation to germ cell death suggests they may be involved in key processes in regulation of germ cell apoptosis.

2. INTRODUCTION

Through the course of spermatogenesis, spermatogonia proliferate and differentiate into mature spermatozoa through a complex series of changes (1). This process is sensitive to modest elevation of testicular temperature. In most mammals, the testicular temperature is maintained constantly lower than the core body temperature to allow normal spermatogenesis (2). Exposure of the testis to body temperature or above by local testicular hyperthermia, cryptorchidism, or varicocele results in increased death of germ cells (3-7). Accumulated evidence indicates that a single exposure of the rat (43C for 15 min) or monkey (43C for 30 min) testes to heat resulted in selective and reversible damage to the seminiferous epithelium due to increased germ cell apoptosis (8-11). However, the underlying molecular mechanisms are not well understood.

Heat shock proteins (Hsps) comprise a highly conserved family identified in species from prokaryotic bacteria to mammals. Hsps act as molecular chaperones by assisting in the folding, assembly, and disassembly of other proteins (12, 13). Hsps are also induced in response to physiological and environmental stresses such as heat and protect cells from the cytotoxic effects of aggregated proteins produced by various types of stresses (14). Hsps also play a role in regulation of apoptosis by complex

mechanisms either by inhibiting the apoptotic response as chaperones of a key signaling protein or by directly promoting apoptosis (15, 16). According to their molecular weights, mammalian Hsps are classified into different families including Hsp105/110, Hsp90, Hsp70, Hsp60, and other small Hsps.

The 105kDa heat shock protein (Hsp105 alpha) is a member of the higher molecular mass heat shock protein family, can express constitutively but is also induced by various stresses in mammalian cells, whereas another 90kDa heat shock protein (Hsp105 beta, 42C-Hsp) is only synthesized in mammalian cells heated at 42C (17-21). Hsp105 alpha is composed of 858 amino acids and Hsp105 beta lacks 44 amino acids present in Hsp105 alpha probably due to alternative splicing (22). The predicted secondary structures of these two proteins are composed of amino-terminal ATP binding, beta-sheet, loop and carboxyl-terminal alpha-helical domains, homologous to those of Hsp70 proteins (22, 23). Both of them when complexed with Hsc70/Hsp70 (Hsc70 is the 70 kDa heat shock cognate protein, and is part of the Hsp70 family), can negatively regulate the Hsc70/Hsp70 chaperone activity (24, 25). Furthermore, depending on the cell type and the perturbation, Hsp105 alpha protects neuronal cells against apoptosis (26) while it enhances stress-induced apoptosis in embryonic cells (27).

Hsp60 is one of the best-characterized molecular chaperones in both eukaryotic and prokaryotic organisms (28). It is expressed constitutively and is moderately induced in response to environmental insults such as heat (29). 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 seven subunits (30, 31). It also localizes to other loci including the cell surface (32). The Hsp60/Hsp10 complex can provide a step-wise ATP dependent pathway for proper refolding of imported proteins (14, 33-36). Hsp60 has been detected in spermatogonia, early primary spermatocytes, Sertoli cells and Leydig cells in rat and human testes (37, 38). In infertile men, a decrease in the number of Hsp60-expressing spermatogonia paralleled low spermatogenic efficiency suggesting that a low level of Hsp60 expression in spermatogonia may indicate a decreased level of protection against stresses resulting in impairment of spermatogenesis (38).

In spermatogenesis, the role of Hsp70 family members have been studied extensively while Hsp105 and Hsp60 are poorly investigated. In the present study, we confirmed that heat exposure of the testis (43C for 30 minutes once daily for 2 consecutive days) induced germ cell apoptosis in cynomolgus monkeys. We also analyzed the spatio-temporal expression of Hsp105 and Hsp60 in the cynomolgus monkey testes as a first step to understand their possible roles in the process of germ cell loss.

3. MATERIALS AND METHODS

3.1. Animals and experimental protocol

Male adult (7 to 10 years old) cynomolgus monkeys *(Macaca fascicularis)* were obtained and housed at the Guangxi Hongfeng Primate Research Center, Institute of Zoology (IOZ), Chinese Academy of Sciences (CAS). Animal handling and experimentation were in accordance with the recommendation of the American Veterinary Medical Association and were approved by the Chinese academic committees of IOZ, CAS and the Harbor-University of California-Los Angeles Biomedical Research Institute Animal Care and Use Review Committee. These monkeys were housed in a standard animal facility under controlled temperature (22C) and photoperiod (12 hr of light and 12 hr of darkness), with free access to water and monkey chow. Heating of the scrota of the eight adult monkeys was performed as described previously by Lue *et al* (9). Briefly, under light sedation, testicular hyperthermia was induced by immersing the monkey scrota containing the testes into a thermostatically controlled water bath at 43C for 30 minutes once daily for 2 consecutive days. After heat treatment, animals were dried, examined for any redness or injury to the scrota, then returned to their cages and allowed to recover from the effect of the anesthesia. Inspection of the scrota after heat exposure showed no evidence of thermal injury to the scrotal skin after this short duration of modest increase in temperature. The animals were divided into to two groups, the three monkeys for semen evaluation and the other five monkeys for biopsies. Semen samples were collected on the day before (pretreatment phase, control) and on days 14, 28, 42, 56, 70, 84, 112, 144 after heat treatment. To ensure that at each time point of tissue collection we can get three different testis samples, we conducted biopsies in three monkeys on the day before (pretreatment phase, control) and choose one side testis of the one monkey for biopsy and the other two monkeys for side-castration on days 3, 8, 30, 84, and 144 after heat exposure.

3.2. Semen analysis

Monkeys were restrained in a primate chair under light anesthesia with ketamine (4mg/kg). After recovered from light anesthesia, they were electroejaculated as described previously by Lue *et al* (6). The volume of each ejaculate including both fluid and coagulum fractions was recorded, and the sperm number was determined from the fluid fraction using a hematocytometer and expressed as spermatozoa$\times 10^7$/ml.

3.3. Testicular tissues

Open testicular biopsies or castrations were performed under general anesthesia with ketamine (10mg/kg) and atropine (0.05mg/kg) as premedication. Post-operatively, the animals were given oxymorphone (0.1mg/kg) for analgesia. The operation was performed under aseptic conditions and only one testis from each monkey was used for tissue collection. One portion of the tissue were immersion-fixed in Bouin's solution and embedded in paraffin, subsequently sectioned for histological observation, immunohistochemistry, *in situ* detection of apoptosis and *in situ* hybridization, and the other portion snap-frozen in liquid nitrogen for RNA and protein isolation.

3.4. *In situ* analysis of testicular cell DNA fragmentation

Apoptotic cells were identified by the TdT-mediated ddUTP end-labelling (TUNEL) method. The

Table 1. Specific primer pairs of PCR

Gene	Primer	Sequence	Product (bp)
Hsp105	sense	5′-GATGAAGCAGTAGCCAGAG-3′	391
	antisense	5′-CCACCATAGATGCCGTAG-3′	391
Hsp60	sense	5′-GGATGCCTATGTTCTG-3′	226
	antisense	5′-ATCTTTAAGCTGGTTCT-3′	226
GAPDH	sense	5′-ACCACAGTCCATGCCATCAC-3′	452
	antisense	5'- TCCACCACCCTGTTGCTGTA-3'	452

testis sections (5μm) were deparaffinized, rehydrated, and pretreated with Proteinase K (10μg/ml) at 37C for 15 min and washed in phosphate-buffered saline (PBS). After incubation in TdT buffer (200mM potassium cacodylate; 25mM Tris; 0.25mg/mL bovine serum albumin (BSA); and 5mM $CoCl_2$, pH6.6) at room temperature for 10 min, the slides was then subjected to DNA 3'-end labeling with 1μM ddUTP (Boehringer Mannheim, Germany) and 1U/μl TdT (Promega, Madison, WI) at 37C for 1 hour. The slides were washed for three times in Tris buffer (100mM Tris; 150mM NaCl, pH7.5), and incubated with 1% blocking buffer (Boehringer Mannheim) at room temperature for 30 min before incubation with AP-conjugated anti-DIG antibody (1:5000, Boehringer Mannheim) at 4C overnight. The color was developed by nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP, Boehringer-Mannheim) in alkaline phosphatase buffer (100mM Tris; 100mM NaCl; 50 mM $MgCl_2$, pH9.5). To remove nonspecific staining, slides were rinsed in 95% ethanol for 30 min and then mounted. The negative controls were similarly processed with the omission of TdT. For statistical analysis, more than 5000 germ cells per section were counted by three persons, and the apoptotic index (AI) was expressed by the number of TUNEL-positive germ cells per1000 germ cells (39).

3.5. *In situ* hybridization analysis

The Dig-labeled antisense and sense cRNA probes of Hsp105 and Hsp60 was synthesized according to manufacture's instructions (Boehringer Mannheim). Deparaffinized and rehydrated tissue sections (5μm) were pretreated with 10μg/ml proteinase K at 37C for 15 min, and then subjected to fixation in 4% paraformaldehyde to preserve mRNA and terminate proteinase K digestion. Before hybridization, the sections were dehydrated through a graded ethanol series and allowed to air dry. After prehybridization at 50°C for 3 hrs in prehybridization buffer containing 50% deionized formamide, 2×saline sodium citrate (SSC) without probe, hybridization was carried out at 55°C overnight with DIG-labeled antisense or sense probe in hybridization buffer (10mM Tris-Cl ,pH 7.5; 2×SSC; 50% deionized formamide; 1× Denhardt; 2.5mM DTT; 5%dextran sulfate; 250μg/ml yeast tRNA; and 0.5% SDS). After hybridization, the sections were thoroughly washed in 2x SSC,1x SSC, and 0.1x SSC twice for 15 min each at 42°C, rinsed in DIG-buffer I (0.1M maleic acid; 150mM NaCl, pH7.5) for 5 min, blocked with DIG-buffer II (1% blocking reagent in DIG-buffer I) at room temperature for 1 hr , incubated with AP-conjugated anti-DIG antibody at 4°C overnight (1:5000), and washed in DIG buffer I for three times. The hybridization signals were visualized with NBT/BCIP in alkaline phosphatase buffer. The slides were rinsed in 95% ethanol for 30 min and mounted. Sense probe hybridizations were performed as in the negative controls.

3.6. RT-PCR analysis

Total RNA was isolated from the testis tissue using Trizol reagent (Gibco-BRL, Grand Island, NY) according to the manufacturer's instructions. The first strand complementary DNA was synthesized from reverse transcription (RT) of 2μg of total RNA using oligo dT primer and Superscript II reverse transcriptase (Gibco-BRL). After the RT reaction, 1μl of the incubation mixture was used as the template for the subsequent PCR amplification. Specific PCR primer pairs from human genes used in this study are summarized in Table 1. The PCR products corresponding to nucleotides 1450-1840bp of human Hsp105 alpha mRNA (Genebank accession number: AB003334), nucleotides 744-969bp of human Hsp60 mRNA (M34664) and nucleotides 601-1052bp of human GAPDH mRNA (NM_002046) were collected between 23 to 38 cycles, and the exponential increase in PCR products was confirmed. PCR reactions of Hsp105, Hsp60 and GAPDH were performed for 28, 30, and 28 cycles with an annealing temperature of 52-55C, respectively. Amplification of GAPDH gene transcripts was used to confirm RNA integrity and efficiency. The PCR products of three separately PCR reactions were separated on 1% agarose gel. Intensities of autoradiographic bands were estimated by Densitometric scanning using the BioImage scanner (Cheshire, U.K).

3.7. Immunohistochemical analysis

Following deparaffinization and rehydration, 5μm sections were subjected to antigen retrieval using EDTA buffer (10mM, pH8.0) at 98C for 15 min and then cooled naturally to room temperature. After two washes in PBS, the sections were sequentially incubated with 10% normal blocking serum for 30 min to suppress non-specific bindings, primary antibodies including rabbit anti-Hsp105(sc-6241, 1:200) and goat anti-Hsp60 (sc-1052, 1:200) at 4C overnight (Santa Cruz Biotechnology, Inc), biotinylated secondary antibodies(1:200) for 30 min, avidin-alkaline phosphatase complex and Vector Red according to the manufacturer's protocol (Vectastain ABC-AP kit, Vector Laboratories, Burlingame, CA). Endogenous alkaline phosphatase activity was inhibited with levamisole (Sigma Corp., St. Louis, MO). Intervening PBS washes were performed after incubation when necessary. Sections were counterstained with hematoxylin, dehrdrated through a graded series of ethanol, cleared in xylene and then mounted. As a negative control, sections were incubated with normal rabbit or goat IgG instead of the primary antibodies.

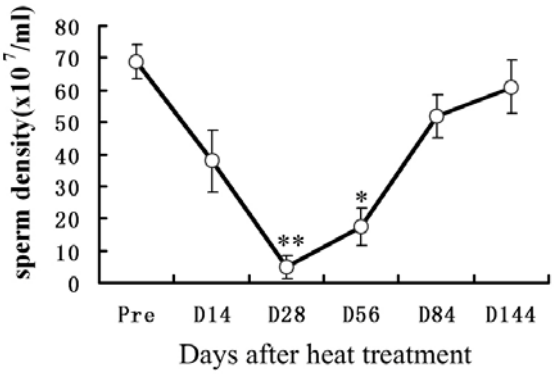

Figure 1. Effect of mild testicular hyperthermia on sperm density of the monkeys. Effect of mild testicular hyperthermia on sperm density of the monkeys on the day before (Pre) and days 14 (D14), 28 (D28), 56 (D56), 84 (D84) and 144 (D144) after heat shock. Data are presented as mean±SEM (n=3). Value with * is significantly different (P<0.05) or ** (P<0.01).

3.8. Western blot analysis

The snap-frozen testis in liquid nitrogen was homogenized in lysis buffer (5mM phosphate buffer, pH 7.2, containing 0.1% Triton X-100, 1mM phenylmethylsulfonylfluoride, 1 mg/L chymostatin) and the protein content of the supernatant from centrifugation was determined by spectrophotometer, using bovine serum albumin as a standard. 40μg total protein per lane was separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and was electrophoretic transferred to the nitrocellulose membranes (Bio-Rad, Hercules, CA). After blocked in 5% non-fat milk in TBST (0.09%NaCl, 0.1%Tween-20, 100mM Tris-HCl, pH7.5), the membranes were incubated with the primary antibodies including rabbit-anti Hsp105 (1:1000) and goat anti-Hsp60 (1:1000) at 4C overnight. beta-actin (Sigma Corp., St. Louis, MO) was used as a loading control. Following three washes in TBST, membranes were incubated with HRP-conjugated secondary antibodies (1:2500) at room temperature for 1 hr. Reactive bands were visualized by SuperSignal® West Pico Chemiluminescent substrate (Pierce, Rockford, IL) and the membranes were then subjected to x-ray autoradiography. Band intensities were determined by Quantity one software (Bio-Rad, Hercules, CA).

3.9. Statistical analysis

Samples from three individual animals at each tissue collection time point were analyzed. Statistical analysis was performed using Statistical Package for Social Science (SPSS for Windows package release 10.0; SPSS Inc., Chicago, IL). Statistical significance was determined by oneway AVONA. Post-Hoc comparisons between treatment group means were made using Fisher's protected least-significance-difference test. Differences were considered significant if P<0.05. Values shown in all the figures were given as the mean±SEM. For TUNEL, RT-PCR, Western blot, immunocytochemistry and *in situ* hybridization, data are representative from one of at least three separate experiments.

4. RESULTS

4.1. Sperm concentration changes

The mean sperm concentrations decreased to 8.4% of pretreatment levels (P<0.01) on day 28 after heat treatment. This heat-induced oligozoospermia persisted until day 56 before returning to pretreatment levels on days 84 and 144 (Figure 1).

4.2. Morphological changes of seminiferous tubules after heat stress

We examined histology of the monkey testes before and after heat stress. As shown in Figure 2, on day 3 after heat shock, some germ cells were detached from the epithelium in some seminiferous tubules. Obvious degeneration of the seminiferous epithelium characterized by epithelial disorganization, vacuolization and formation of multinucleated giant cells was observed on days 8 and 30. Most of the seminiferous epithelium became thinner and the lumen of the tubules was devoid of the majority of spermatocytes and round spermatids. Some of the seminiferous tubules contained spermatozoa even though spermatocytes and round spermatids were absent on days 8, and 30. The epithelium recovered with the re-appearance of spermatids, spermatocytes and spermatozoa in the tubules on day 84 and fully restored to normal appearance on day 144. The spermatogonia were not affected in most of seminiferous tubules throughout the period after heat exposure. Only a few seminiferous tubules exhibited Sertoli cell on days 8 and 30.

4.3. Germ cell apoptosis induced by heat stress

TUNEL detection of DNA fragmentation was performed to test whether the heat-induced loss of germ cells occurred via apoptosis (Figure 3A). In untreated testes (Figure 3A, Pre), spontaneous apoptosis of very few germ cells were observed in some of the seminiferous tubules. Based on their localization and morphology, the majority of spontaneous apoptotic germ cells were spermatocytes. Heat stress resulted in a marked increase in the incidence of germ cell apoptosis on day 3. The strongest TUNEL signals were observed in spermatocytes and round spermatids from day 8 to day 30 after heat treatment which decreased by day 84 and day 144. The increase in TUNEL-positive cells correlated with the degeneration of seminiferous epithelium shown in Figure 2. Quantitative analysis of the number of TUNEL-positive cells (Figure 3B) revealed that the apoptotic index increased significantly on day 3 (P<0.05), peaked on day 8 (P<0.01) and then decreased by day 84 and day 144 which became not significantly different from those of the pretreatment samples.

4.4. Hsp105 expression in cynomolgus monkey testes after heat shock

To show that Hsp105 might play a role in heat induced germ cell apoptosis, we examined the Hsp105 mRNA expression pattern in the heat-treated monkey testes (Figure 4A). Within the seminiferous tubules, the strongest signal was confined to spermatids, while no signal was detected in other cells such as spermatogonia, spermatocytes, Sertoli cells, and Leydig cells. Compared with its expression in pretreatment testes, Hsp105 mRNA

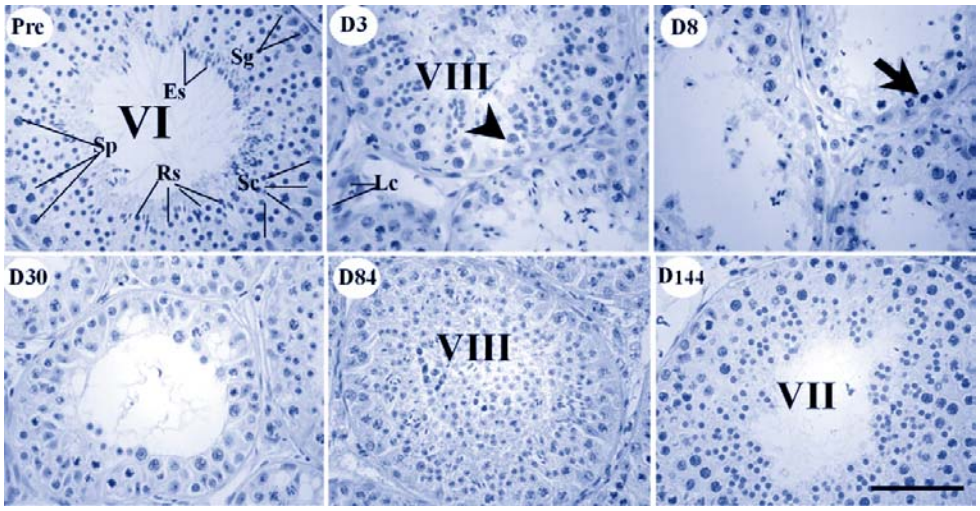

Figure 2. Seminiferous tubule morphology of heat-treated monkey testis. Testes were fixed in Bouin's solution and embedded in paraffin. Sections were stained by hematoxylin-eosin. Tubules before heat stress (Pre) contained spermatogonia (Sg) at the periphery of seminiferous tubules, spermatocytes (Sp), round spermatids (Rs) and elongating spermatids (Es) soon to be released into the tubule lumen. From day 3 to day 30 upon heat stress (D3, D8, D30), tubules became disorganized and exhibited varying degrees of damages, including giant cell formation (black arrow head) and marked loss of germ cells (black arrow). On days 84 and 144 upon heat stress (D84, D144), tubules were of normal morphology. Sc: Sertoli cell; Lc:Leydig cell. VI, VII and VIII represent different stages of the seminiferous tubules. Bar=100μm.

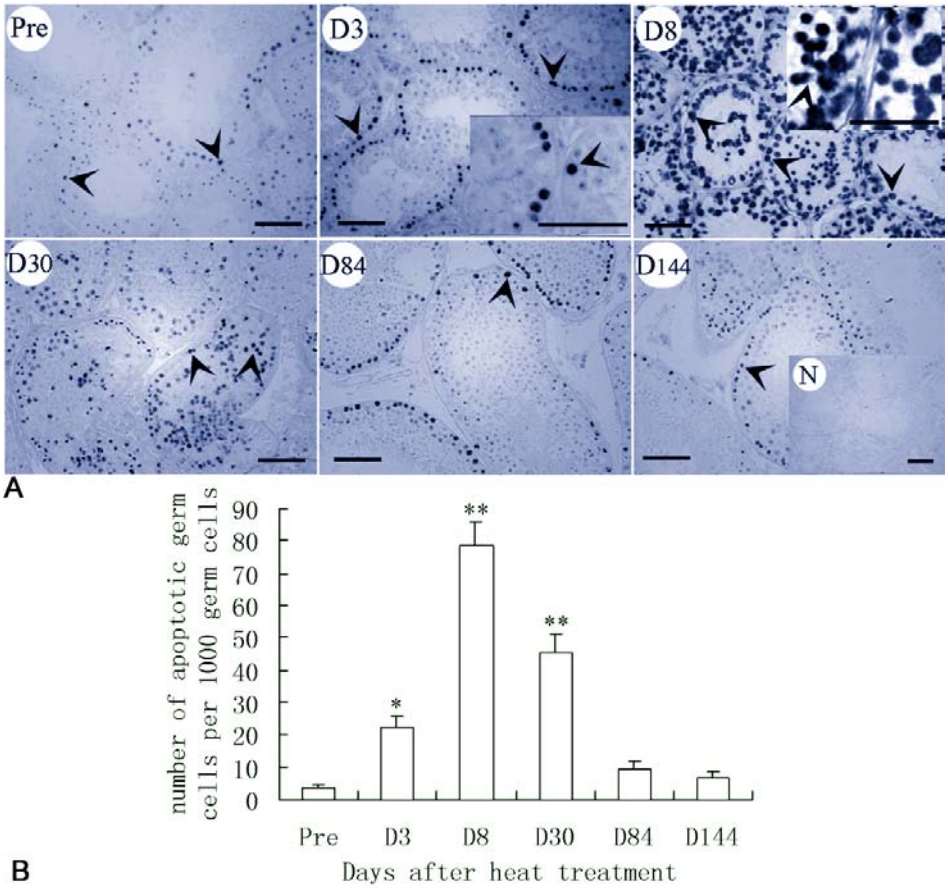

Figure 3. TUNEL detection of apoptotic germ cells before (Pre) and after heat stress (D3, D8, D30, D84, D144). (A) Apoptotic nuclei were stained dark (arrow head). The insets in D3 and D8 pictures were of higher magnification. N: a negative control with TdT left out. Bar= 100μm. (B) Statistical analysis of apoptotic index. AI, the number of apoptotic germ cells per 1000 testicular germ cells. Data are presented as mean±SEM (n=3). Number with * is significantly different ($P<0.05$) or ** ($P<0.01$).

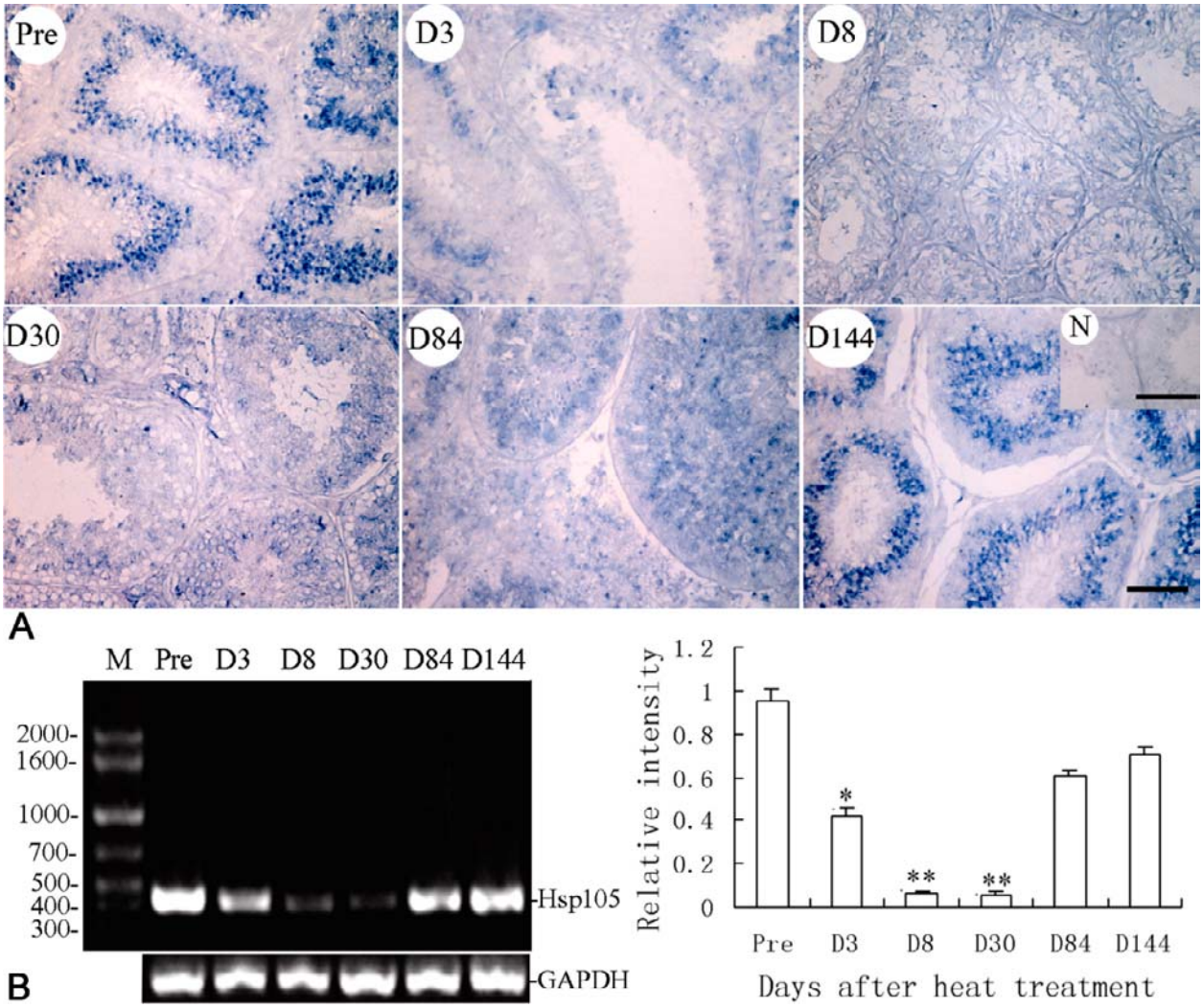

Figure 4. Expression of Hsp105 mRNA in monkey testes. (A) *In situ* hybridization analysis of Hsp105 mRNA expression in testis of monkeys before (Pre) and after heat stress (D3, D8, D30, D84, D144). N: negative control with sense probe on D144 sections. Bar=100μm. (B) RT-PCR analysis of Hsp105 mRNA expression in the heat-treated monkey testis. mRNA levels were determined by densitometry. GAPDH was used as the internal control. The relative intensity was the ratio of Hsp105 mRNA to GAPDH. Data are presented as mean±SEM (n=3). Bar with * is significantly different ($P<0.05$) or ** ($P<0.01$). M: 1kb plus DNA marker (bp).

started to drop perceptively on day 3 and continued to drop through days 8 and 30 after heat shock. Consistent with spermatogenic recovery, Hsp105 mRNA levels returned to pretreatment levels on day 144. *In situ* changes in Hsp 105 mRNA expression were further confirmed by RT-PCR analysis (Figure 4B). To determine the exact sites where Hsp105 proteins were produced, immunohistochemistry staining was performed. As expected, the expression patterns of Hsp105 protein were similar to those of its mRNA. As shown in Figure 5A, the positive signal was present in the nuclei of spermatids before heat treatment. It was not detected in spermatids on day 3 after treatment. Interestingly, the signal was detected in the cytoplasm of a few spermatogonia located near the basement membrane of tubules on days 8 and 30. On days 84 and 144, the positive signal was again detected in the nuclei of spermatids similar to baseline. Quantitative analysis of Hsp105 protein is shown in Figure 5B. The expression levels on days 3, 8, and 30 were only 20-40% of the pretreatment values ($P<0.01$), while on day 84 and thereafter they were comparable with the pretreatment samples.

4.5. Hsp60 expression in heat-treated monkey testes

Unlike Hsp 105, Hsp60 mRNA expression, detected by *in situ* hybridization and RT-PCR, was much higher than that of before treatment (Figure 6A and B). From days 3 to 30, the expression level of Hsp60 mRNA in the Sertoli cells and some spermatogonia was the most

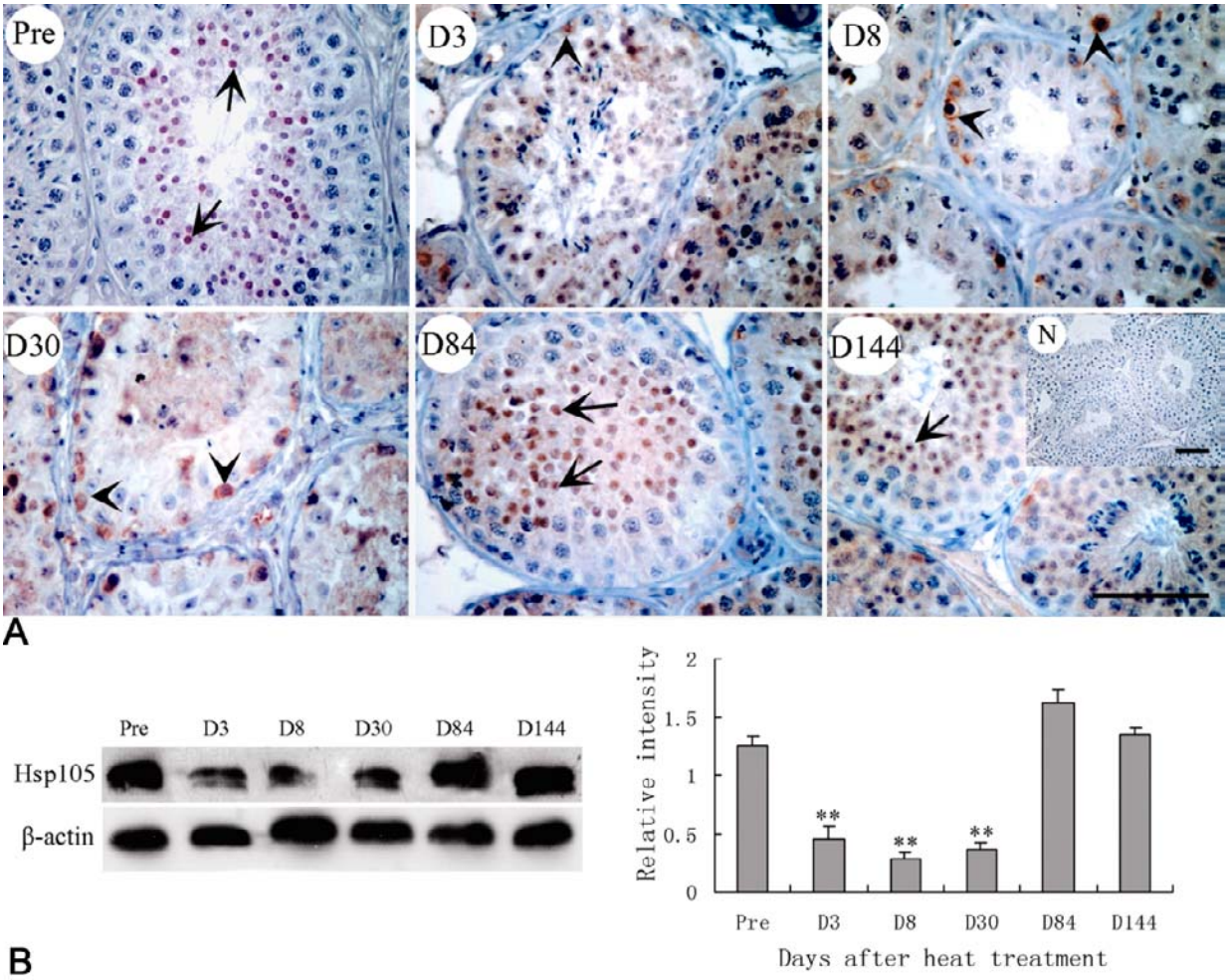

Figure 5. Expression of Hsp105 protein in monkey testes. (A) Immunohistochemcal localization of Hsp105 protein in testis of monkeys before (Pre) and after heat stress (D3, D8, D30, D84, D144). Arrows and arrow heads indicate positive staining in spermatids and spermatogonia, respectively. N: control section without primary antibody. Bar=100μm. (B) Western blot analysis of Hsp105 protein in the heat-treated money testes. beta-actin was used as an internal control. The relative intensity was determined by the ratio of Hsp105 protein to beta-actin protein as measured by densitometry. Data are presented as mean±SEM (n=3). Bar with ** is significantly different (P<0.01).

abundant (P<0.01)(Figure 6B). The result was also confirmed by immunohistochemical and Western blot analysis (Figure 7A and B). Hsp60 was localized predominantly in Sertoli cells and spermatogonia. As shown in Figure 7B, Hsp60 levels were significantly elevated (P<0.01) above pretreatment levels on days 3, 8, and 30 after heat treatment before declining to pretreatment levels on days 84 and 144.

5. DISCUSSION

The lower scrotal temperature than the core body temperature is an important prerequisite for optimal spermatogenesis (2). It has been reported that elevation of testicular temperature by 1°C suppresses spermatogenesis by 14% and thus decreases sperm output (40). Observations made in the infertile men with oligozoospermia or azoospermia suggest that some of the men may have higher testicular temperature due to an intrinsic defect in scrotal thermoregulation, varicocele, or occupational exposure to high temperatures (7, 10, 41, 42). Earlier studies using nonhuman primates as a model to test the effect of heat on spermatogenesis showed that the higher the temperature and the longer the duration of heat exposure, the more severe the germinal epithelium damage (43). While in our study, we found that a single testicular hyperthermia at 43C for 30 minutes once daily for 2 consecutive days in the cynomolgus monkeys induced oligozoospermia on day 28 which persisted until day 56 after heat treatment. The sperm concentration returned to pretreatment level 84 days after heat shock. This is in good agreement with the report of Lue *et al.* (6), who reported that local testicular heat treatment at 43C for 30 min once daily for 6 consecutive days induced azoospermia in the 2 of 3 monkeys by 6 weeks, and spermatogenesis recovered to pretreatment levels by 12 weeks after the first heat exposure. Thus,

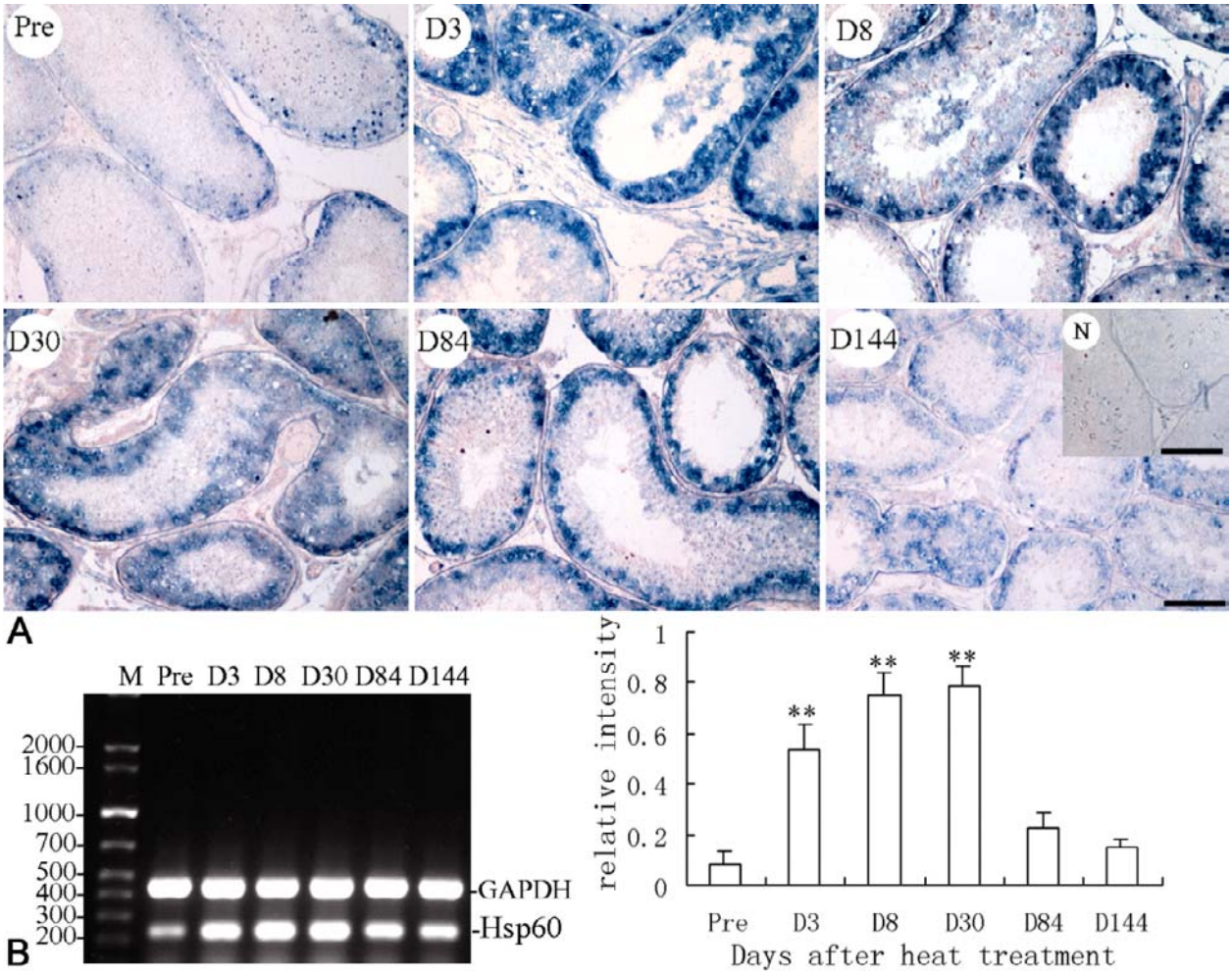

Figure 6. Hsp60 mRNA expression in monkey testes. (A) *In situ* hybridization analysis of Hsp60 mRNA expression in testis of monkeys before (Pre) and after heat stress (D3, D8, D30, D84, D144). N: control section hybridized with sense probe on D30. Bar=100μm. (B) RT-PCR analysis of Hsp60 mRNA expression in the heat-treated monkey testis. GAPDH was used as an internal control. The relative intensity was determined by the ratio of Hsp60 mRNA to GAPDH mRNA as measured by densitometry. Data are presented as mean±SEM (n=3). Bar with ** is significantly different (P<0.01). M: 1kb plus DNA marker (bp).

short-term mild testicular hyperthermia may cause reversible damages to the seminiferous tubules in monkeys.

Many studies have shown that apoptotic germ cells can be detected in the testis of adult mice 1 day after experimental cryptorchidism (44) and 8 hrs after heat shock (45). In adult rat testis, germ cell apoptosis was markedly increased within 1 day after heat shock (9) and in adult monkey testis, apoptosis was seen 3 days after immersion of their scrota in a water bath at 43°C for 30 min once daily for 6 consecutive days (6). Based on our prior finding, we shortened the heat exposure to only two days to induce apoptosis. Using TUNEL assay in the present study, we found that apoptosis was apparently increased in germ cells as early as day 3 after heat shock and the incidence of germ cell apoptosis returned to the pretreatment control levels by 84 and 144 days after treatment, the time at which spermatogenesis was fully recovered. The apoptotic germ cells were mainly primary spermatocytes and round spermatids, which is similar with our previous report that a single transient local testicular heat exposure induced germ cell apoptosis in a cell-specific fashion (4, 9). Consistent with the occurrence of apoptosis, most seminiferous tubules showed massive degeneration with only Sertoli cells and spermatogonia that survived the stress. The degeneration was temporary and recovered fully with the reappearance of spermatids and spermatozoa in the seminiferous tubules by 144 days after heat shock.

While we know the pathways for germ cell apoptosis (46, 47), the precise molecular mechanisms related to heat-affected spermatogenesis and germ cell apoptosis are still being unravelled. In the present study, we provide the first evidence in the spatio-temporal expression

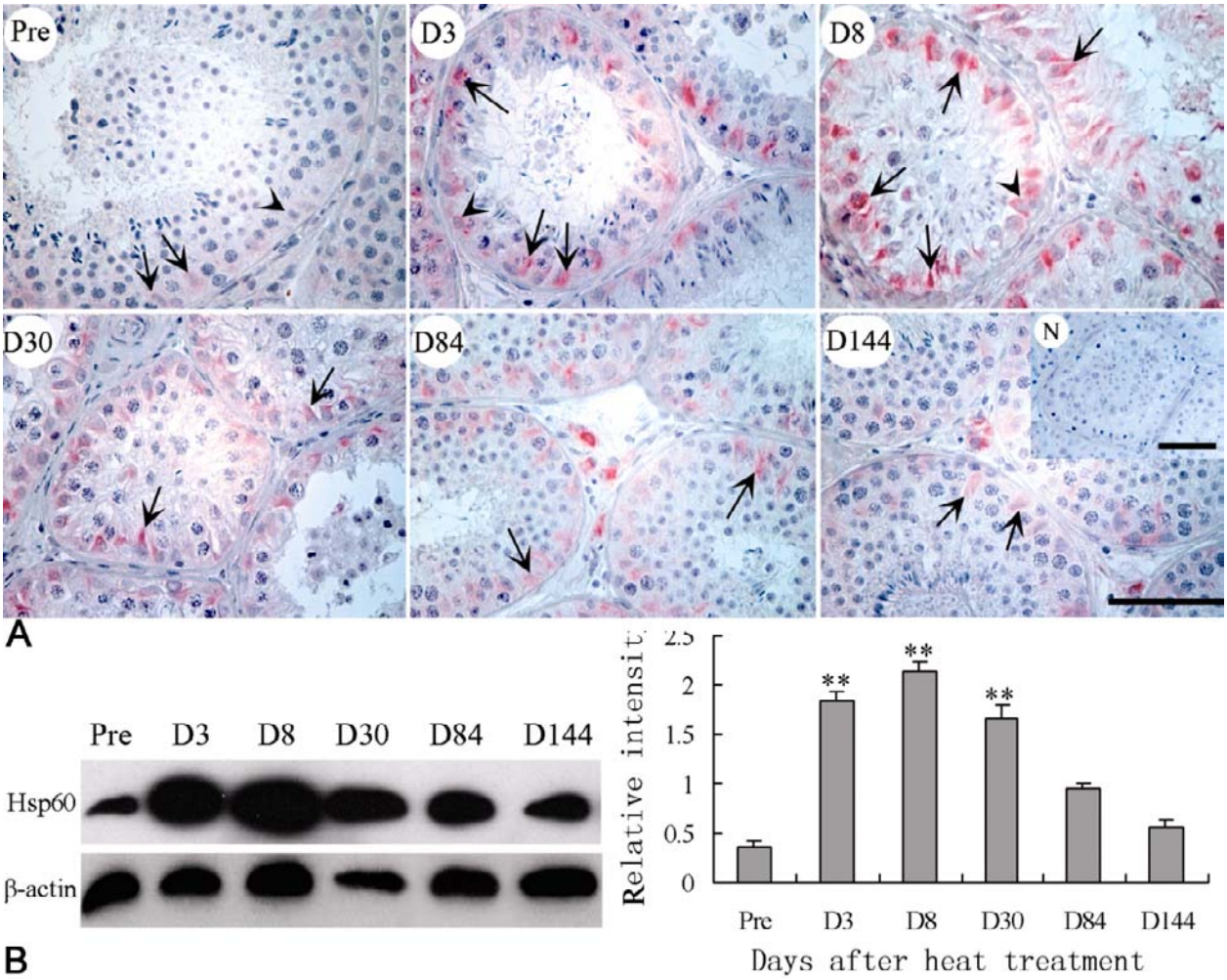

Figure 7. Hsp60 protein expression in monkey testes. (A) Immunohistochemical localization of Hsp60 protein in testis of monkeys before (Pre) and after heat stress (D3, D8, D30, D84, D144). Arrows and arrow heads indicate positive staining in Sertoli cells and spermatogonia respectively. N: the negative control without primary antibody. Bar=100μm. (B) Western blot analysis of Hsp60 protein. To normalize protein amounts loaded, blots were re-probed with beta-actin monoclonal antibody. The relative intensity was determined by the ratio of Hsp60 protein to beta-actin protein as measured by densitometry. Data are presented as mean±SEM (n=3). Bar with ** is significantly different ($P<0.01$).

patterns of Hsp105 and Hsp60 in the heat-treated cynomolgus monkey testis, and explore the possibility that Hsp105 and Hsp60 might be involved in the process. Before treatment, Hsp105 was mainly confined to the nuclei of spermatids, and the positive signals markedly dropped in spermatids from day 3 to day 30 after acute heat shock and shifted to cytoplasm of spermatogonia close to the basement membrane. When the cells recovered from thermal stress, concomitant with the decrease in germ cell death, the expression pattern of Hsp105 recovered to that of pretreatment phase. That a shift in Hsp105 immunoactivity from spermatids to cytoplasm of spermatogonia occurred while Hsp105 mRNA was not apparent in the spermatogonial cytoplasm is probably due to the difference of transcriptional or translational regulation. Our experiments cannot address the question of causality, whether germ cell apoptosis after exposure to heat resulted in reduced expression of Hsp105 or the decreased protective effect of Hsp105 in spermatids triggered the death of germ cells. Similar to our prior studies in rats and monkeys, the spermatogonia seemed less susceptible to heat shock than more advanced grem cells (6, 9). The temporal changes in expression of Hsp105 suggest that Hsp105 might be involved in the process. Increased scrotal temperature has been reported to cause Hsp105 to form a complex with p53, thus trapping p53 in the cytoplasm and away from its functional nuclear site. This stabilization of p53 may prevent the induction of apoptosis by p53 (48). High scrotal temperatures cause condensation of nuclear chromatin, which activates and induces p53 to translocate to the nucleoplasm that leads to cell cycle arrest or apoptosis (49, 50). Taken together, we conclude that that Hsp105 might be involved in the heat induced germ cell apoptosis in monkeys.

Hsp60 was reported to be expressed in Leydig cells during neonatal and prepubertal period in rat testis, not only in spermatogonia, but primary spermatocytes after puberty (51). In the present study, Hsp60 was only detected in Sertoli cells and a few of the spermatogonia before heat treatment. Hsp60 signals were elevated after heat shock. Despite the expression of Hsp60 in Sertoli cells and spermatogonia, apoptosis was rarely detected. Recent studies have suggested an anti-apoptotic role for cytosolic Hsp60 and Hsp10, in various types of cells, and these two proteins can be induced when cells are under stress (52, 53). By overexpressing Hsp60 and Hsp10, Shan *et al.* (54) found that both of them can independently modulate post-translational modification of Bcl-2 protein family. Hsp60 overexpression was associated with better induction of Bcl-xl, additional suppression of Bax and more profound inhibition on Caspase 3. Moreover, Hsp60 also down-regulated Bad in the doxorubicin-treated cells. These observations suggest Hsp60 has stronger anti-apoptosis properties in cells (54). Perhaps in our study, the increased expression levels of Hsp60 in Sertoli cells and spermatogonia suggest that the increase in Hsp60 could exert a protective role in Sertoli cells and spermatogonia after heat shock.

In conclusion, using nonhuman primates as a model for the study of human spermatogenesis to examine the effects and possible mechanisms of mild testicular hyperthermia inducing apoptosis of male germ cells, we identified spatio-temporal changes in the expression of Hsp105 and Hsp60 in testis. Our results suggest that heat treatment of the monkey testis (43C for 30 minutes once daily for 2 consecutive days) results in selective, but reversible damage to the seminiferous epithelium through increased germ cell apoptosis. Hsp105 and Hsp60 might be involved in heat-induced germ cell apoptosis in monkeys.

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