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Upregulation of heat shock protein 32 in Sertoli cells alleviates the impairments caused by heat shock-induced apoptosis in mouse testis

Lian Li · Zhao-Yu Han · Cheng-Min Li · Xiao-Qiang Jiang · Gen-Lin Wang

Abstract Heat stress results in apoptosis in testicular germ cells. A small heat shock protein (hsp), hsp32, is induced by heat stress in the testis, but little is known about its definitive function in physiological processes. To clarify the underlying role of hsp32, hsp32 expression and related signals in the heat shock pathway were analysed in mouse testes and Sertoli cells after heat stress in vivo and in vitro; meanwhile, expression of hsp32 was silenced only in the Sertoli cells using three different small interfering RNAs (siRNAs) to further verify the functional role of hsp32 in Sertoli cells, and hsp32-derived carbon monoxide (CO) contents in cultured media were analysed to clarify whether hsp32-derived CO involve in the apoptosis regulation mechanisms. The results from the in vivo experiment showed that the high expression levels of hsp32 ($P<0.05$) were observed whether chronic, moderate or acute, transient heat exposure. The in vitro experiment showed that acute, transient heat exposure resulted in increases in Sertoli cells apoptosis ($P<0.01$), the expression of hsp32 and caspase-3 activity; hsp32-siRNA knockdown of hsp32 expression resulted in upregulated apoptosis ($P<0.01$) and caspase-3 activity ($P<0.01$) in the Sertoli cells and hyperthermia increases CO ($P<0.01$) release by Sertoli cells. The results suggested that upregulating hsp32 in Sertoli cells inhibits caspase-3 activity and alleviates heat-induced impairments in mouse testis; hsp32-derived CO may involve in the regulation mechanism.

Keywords Heat shock protein 32 (hsp32) · Caspase-3 · Sertoli cells · Testis · Apoptosis

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Introduction

Physiological scrotal temperature is 2–8 °C lower than the core body temperature in most mammals (Kandell and Swerdloff 1988; Ivell 2007). The cooler temperature is required for normal spermatogenesis because a rise in the temperature of the testis caused by cryptorchidism, varicocele or high temperature conditions such as a transient heat stress will cause the apoptosis of male germ cells (Khan and Brown 2002). So far, the effects of heat stress on the testes of mammals, including the sperm number, fertility and testicular structure, have been intensively investigated. However, the mechanism of heat-induced germ cell apoptosis remains unclear.

Heat shock proteins (Hsps), synthesised in the cell in response to stress, are a conserved family of cytoprotective polypeptides (Grover 2002; Pei et al. 2012). A group of small 25–32 kDa Hsps is particularly important in protecting the cell under stress conditions. Heat shock protein 32 (hsp32), also known as heme oxygenase-1 (HO-1), is one member of this family with enzymatic activity and is induced by heat stress, heavy metals, inflammatory mediators and oxidised low-density lipoproteins (Goldbaum and Richter 2001; Morse and Choi 2002; Erdmann et al. 2005; Gabunia et al. 2012). It is inducible and normally expressed at low levels in almost all tissues including the testis, liver, kidney etc. (Maines and Ewing 1996; Bauer et al. 1998; Ashino et al. 2003; Barton et al. 2003; Turpeinen et al. 2007; Liu et al. 2011). It is suggested that hsp32 possesses cytoprotective and anti-apoptotic effects on cells (such as Jurkat T, hypothalamic neuronal primary human hepatocytes, cardiomyocyte cell etc.) subjected to stress (Borger and Essig 1998; Kiemer et al. 2003; Kondo et al. 2007; Burger et al. 2009). To date, few reports focused on the role of hsp32 in testes subjected to heat stress. Hsp32 activity in the male testis increased after heat shock; it was also found that hsp32

was present in the Sertoli cells and absent in the germ cells (Ewing and Maines 1995; Maines and Ewing 1996; Middendorff et al. 2000). However, few studies examined the expression changes of hsp32 in Sertoli cells and its role in alleviating heat-induced impairments after heat stress. Sertoli cells are the only somatic cells in the seminiferous epithelium; germ cells depend on Sertoli cells for structural and nutritional support (Cheng and Dolores 2002; Chalmel et al. 2007). Any factors that impair Sertoli cells may cause ill effects on spermatogenesis (Lee et al. 1999; Chen et al. 2008).

In mammalian systems, the three major pathways involved in the initiation of apoptosis, namely the "extrinsic" death receptor pathway, the "intrinsic" mitochondrial pathway, converge on a family of caspases and endoplasmic reticulum-mediated apoptosis pathway (Slee et al. 2004). In earlier studies using murine models of testicular hyperthermia, some researchers have provided evidence for the involvement of the mitochondria-dependent apoptotic pathway leading to the activation of the initiator caspase-9 and the executioner caspase-3 in heat-induced germ cell death (Sinha Hikim et al. 2003; Vera et al. 2004; Paul et al. 2009). Caspase 3 is activated by cleavage of its proenzyme (32 kDa) to yield two active subunits p18 and p12, which together form the active enzyme. The activation of caspase 3 was used as an improved index of apoptosis (Khan and Brown 2002). These investigations point to a significant involvement of caspases in heat cell damage in the testis. We analyse (a) the hsp32 and caspase-3 expression and caspase-3 activity of heated testis, the in vivo experiment, and then validated the gene expression in cultured Sertoli cells, the in vitro experiment. (b) To further clarify the functional role of hsp32 in Sertoli cells, hsp32 expression was silenced by small interfering RNA (siRNA). (c) To clarify whether hsp32-derived carbon monoxide (CO) involves in the apoptosis regulation mechanisms, the hsp32-derived CO content was measured in the cultured media. Our results suggested that hsp32 serves as a survival factor in the Sertoli cells and alleviates heat-induced impairments in the testis through hsp32-derived CO.

Materials and methods

In vivo experiment

Animals

Fifty male Kunming mice (18 days old, 12 ± 1 g) purchased from the Nanjing Qinglongshan Experimental

Animal Factory were used in this study. The mice were reared in separate cages under environmental conditions including free diet, 12–12 h light–dark cycles and a room temperature ranging from 23 to 25 °C. All experiments were carried out in accordance with the National Institutes of Health guidelines.

Animal heat treatment and tissue preparation

The chronic, moderate heat exposure (CMH group) consisted of 3-week-old mice exposed to a heat stress at 39 °C with 60 % relative humidity for 1.5 h/day until they were 9 weeks old. The transient, acute heat exposure (TAH group) consisted of 9-week-old mice exposed to a heat stress at 43 °C with 60 % relative humidity for 30 min. After heating, the mice were removed immediately and transferred to room temperature conditions. At various time points between 3 and 12 h after the completion of the heat treatment, the mice were anesthetised with pentobarbital sodium (4 mg/kg body weight, iv), then euthanised by cervical vertebra dislocation. Testis samples were collected and processed. At the end of the experiment, one testis from each animal was removed, divided into two halves and frozen in liquid N_2 for subsequent qualitative reverse transcription polymerase chain reaction (RT-PCR) and western blot analysis or fixed in 4 % paraformaldehyde and processed to be embedded into paraffin wax by a routine protocol. Next, 6-mm-thick sections were cut from tissues removed at different time points. The slides were processed for staining with haematoxylin and eosin (HE) by routine methods. The contralateral testis was used for the preparation of testicular cell suspension or testis homogenate.

Immunohistochemical analyses

Bouin-fixed, paraffin-embedded testicular sections from mouse were immunostained as described previously (Vera et al. 2004). Briefly, testicular sections were deparaffinized, hydrated by successive series of ethanols, rinsed in phosphate-buffered saline (PBS) and then incubated in 2 % H_2O_2 in PBS for 10 min to quench endogenous peroxidase. For the primate study, primary antibodies included rabbit polyclonal hsp32 (1:50) and active caspase 3 (Beyotime Biotechnologies, China; diluted 1:50). Immunoreactivity was detected using biotinylated goat anti-rabbit IgG secondary antibody followed by avidin-biotinylated horseradish peroxidase complex visualized with diaminobenzidine tetrahydrochloride per the manufacturer's instructions. Slides were counterstained with haematoxylin. Negative and positive controls were run for every assay. Except the primary

antibody was substituted with the rabbit IgG, the negative controls were processed in an identical manner.

In vitro experiment

Primary culture of mouse Sertoli cells and Sertoli cells heat treatment

Testes were obtained from 18-day-old mice that had been anesthetised with pentobarbital sodium (4 mg/kg body weight, IV). The isolation and purification of the Sertoli cells were completed within 2 to 3 h after castration with modifications to the previously described procedure (Mather et al. 1990). Briefly, the decapsulated testes were incubated with 1 mg/mL of collagenase (Sigma) and 75 U/mL of DNase I at 35 °C for 10 min with gentle oscillation. After discarding the tissue clumps, the suspensions were centrifuged, and the pellets were washed with DMEM/F12 two to three times. A second digestion was performed in a PBS solution containing 0.25 % trypsin and 75 U/mL DNase I for less than 5 min at room temperature, and then the suspensions were filtered through a 200-mesh stainless steel filter before they were centrifuged for 5 min at $180 \times g$. The cell suspensions were cultured in F12/DMEM supplemented with 10 % foetal bovine serum at 32 °C. After 24 h of culture, the medium was replaced to remove unattached germ cells, and 12–24 h later when the cells were confluent, the cells were hypotonically treated with 20 mm Tris (pH 7.4) for 3 min to lyse the residual germ cells. The medium was replenished every 3 days. Sertoli cells were identified by their distinct morphological characteristics, determined under an inverted phase contrast microscope.

For the heat treatment of cells, the dishes containing the cells were sealed with a paraffin membrane and put in a sterile 39 or 43 °C water bath for 1.5 h or 30 min. For the moderate heat stress condition, a 39 °C water bath was used for 1.5 h/day for 6 days, and then the cell cultures were terminated for analysis. For the acute heat exposure condition, a 43 °C water bath was used for 30 min, and then the dishes were immediately put back into the 37 °C CO_2 incubator and recovered for 3, 6 or 12 h. The cell density was $0.2 \times 10^6/cm^2$ for protein and RNA extractions.

Immunocytochemical analyses

Sertoli cells were fixed in 95 % ethanol. After treatment with 0.3 % hydrogen peroxide/methanol solution for 30 min to inhibit the intrinsic peroxidase reaction, the sections were vibrated in 0.01 M citrate buffer (pH 6.0) for 5 min in a microwave oven for better reaction of the antibody. Subsequently, the specimens were reacted with 1:100

polyclonal rabbit anti-Hsp32 and active caspase-3 antibody. The following processes were same as the immunohistochemical analyses.

Design and application of small interfering RNAs

Three siRNAs against hsp32 were designed according to guidelines published by Reynolds et al. (2004) (sequence 1: 5′-CCG AGA AUG CUG AGU UCA UTT-3′, sequence 2: 5′-CCA CAC AGC ACU AUG UAA ATT-3′ and sequence 3: 5′-GCU GAC AGA GGA ACA CAA ATT-3′). In addition, a negative control siRNA with FAM (5′-UUC UCC GAA CGU GUC ACG UTT-3′) was synthesised. SiRNAs (25–100 nM) were transfected into the Sertoli cells using lipofection (Invitrogen) according to the manufacturers' instructions. Twenty-four hours after transfection, expression of hsp32 was determined by real-time PCR. After 48 h, the cells were used to analyse the apoptotic rate and gene expression.

Cell cycle and apoptosis analysis by flow cytometry

Single cell suspensions of testicular tissue were prepared by enzymatic digestion. Briefly, decapsulated testes were incubated with 0.5 mg/mL of collagenase (Sigma) at room temperature for 15 min with gentle oscillation and then filtered through 200 mm copper meshes to collect cells. Primary cultures of Sertoli cells were prepared by enzymatic digestion. The testicular cells and Sertoli cells were then fixed with 70 % ice-cold ethanol and incubated with RNase (100 μg/mL) and propidium iodide (4 μg/mL) in PBS. Testicular cell cycle phases were analysed by propidium iodide staining using flow cytometry (FCM). Apoptosis of testicular cells and Sertoli cells was assessed by measuring fluorescein isothiocyanate-annexin V staining, and then the cells were analysed by flow cytometry.

RNA extraction and qualitative real-time PCR analysis

The level of hsp32 and caspase-3 mRNA in the testis and Sertoli cells was evaluated by qualitative real-time PCR analysis which was performed on the ABI Prism Sequence Detection System (Applied Biosystems). Total RNA was extracted from testis and Sertoli cells using the Total RNA isolation Kit (Roche) according to the manufacturer's instructions. An aliquot of the total RNA was treated with DNase I, and then it was reverse-transcribed into cDNA with random hexamer primers (Takara, Dalian, China). The resulting cDNA was used for SYBR Green (Applied Biosystems) real-time PCR amplification of hsp32 and caspase-3. PCR reactions were performed with the following primers: 5′-TTT

Fig. 1 Histological evaluation of the testes from mice stressed at 39 °C, chronic, moderate heat exposure. The testes were harvested at 1, 3 and 6 weeks of moderate heat exposure. Morphological examination of the testes revealed substantial morphologic differences (**d**, **b**, **f**) from the normal control testis (**a**–**c**). In control testis, all stages of spermatogenesis were observed, including round spermatids, elongated spermatids and sperms (**a**–**c**). After 1 week of heat exposure, an increase in the disorder of the spermatogenic cell array was detected (**d**). Aberrant vacuoles (indicated by *asterisks*) were observed at 3 weeks (**e**). Intermittent heat exposure for 6 weeks did not affect the morphology of the testis tissue (**f**). *Scale bar*= 30 μm
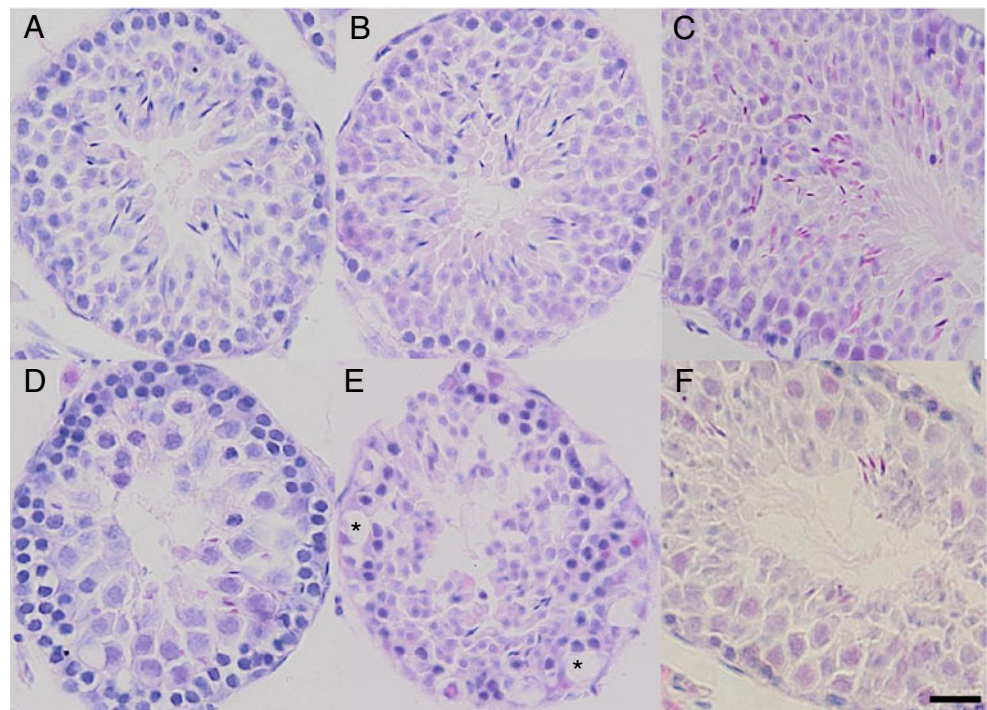
TCC ACG GCG ACT CAG-3′ and 5′-CAA CGG TAA ACA ACA CGA TC-3′ for mouse hsp32; 5′-CTG GAC TGT GGC ATT GAG AC-3′ and 5′-GCA AAG GGA CTG GAT GAA CC-3′ for mouse caspase-3. All mRNA analyses were performed in triplicate, expression levels of hsp32 and caspase-3 were corrected using an endogenous control (mouse GADPH, primers 5′-GGA TTT CCC TGG GTC TTC-3′ and 5′-TAA GAA AGG CAA ACC AGA A-3′) and the fold differences in mRNA expression of samples were relative to the internal control sample, which was included in all runs.

Western blot analysis

The expression levels of hsp32 in the testis and Sertoli cells and the expression levels of caspase-3 protein in testis were evaluated using western blot analysis. Proteins from the testicular tissue and Sertoli cells were extracted by homogenisation in ice-cold extraction buffer (pH 7.5) containing 20 mmol/L HEPES, 15 mmol/L MgCl$_2$, 50 mmol/L NaF, 1 mmol/L dithiothreitol, 3 mg/mL leupeptin, 80 mmol/L *b*-glycerophosphate and 50 mmol/L phenylmethylsulphonyl fluoride. The crude homogenates were centrifuged at 12,000×g for 20 min at 4 °C, and the resultant supernatant contained the soluble protein fraction. The total protein content of the supernatant was determined using the Bradford (1976) method. After diluting 1:5 with sample buffer (pH 6.8, 60 mmol/L Tris–Cl, 200 mmol/L dithiothreitol,
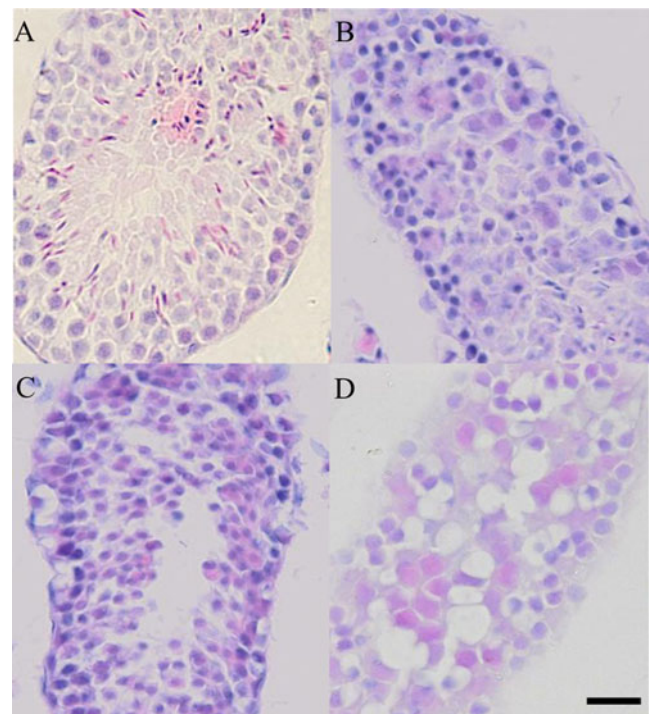

Fig. 2 Histology of the testes from mice stressed at 43 °C transient heat exposure for 30 min. The testes were harvested at 3, 6 and 12 h after transient, acute heat exposure. Morphological examination of the testes revealed substantial morphologic differences at 3–12 h in the transient, acute heat exposure group (**c**–**d**) from the normal testis (**a**). Morphological observation revealed no differences at 3 h (**b**) from the normal testis (**a**). At the 6- and 12-h time point, vacuoles were observed (**c**, **d**), an increase in the disorder of the spermatogenic cell array was detected and more vacuoles were observed at 12 h (**d**). *Scale bar* = 30 μm

2 % sodium dodecyl sulphate (SDS), 0.1 % bromophenol blue and 25 % glycerol) and heating to 95 °C for 10 min, the samples, containing equal amounts of protein (60 µg), were separated by SDS–polyacrylamide gel electrophoresis in gels containing 10 % acrylamide. The proteins were electrically transferred to an immunoblot polyvinylidene difluoride membrane (Bio-Rad, Shanghai, China) using the same Bio-Rad system according to the method described previously (Kong et al. 2000). The membranes were blocked with 10 % nonfat dry milk in 25 mmol/L Tris-buffered saline, pH 7.2, plus 0.1 % Tween 20 (TBST) for 1 h at room temperature and then incubated with an polyclonal rabbit anti-hsp32 (Boster Biotechnologies, China, diluted 1:1,000) and caspase-3 antibody (Beyotime Biotechnologies, China, diluted 1:1,000) or anti-mouse β-actin monoclonal antibody (HaiGen, China, diluted 1:1,000) for an additional 1 h at room temperature with constant shaking. The membranes were then washed in TBST for 6–10 min and incubated with a secondary antibody, rabbit anti-mouse IgG-AP (Santa Cruz Biotechnologies, diluted 1:1,000), for 1 h at room temperature. After the membranes were thoroughly washed in TBST, the signal was visualised after incubation with ECL for 2–5 min.

Caspase-3 activity assay of testis tissue and Sertoli cells

After centrifugation of the crude homogenates of testis tissue and treated cells as previously described, caspase-3 enzymatic activity was determined using the CasPASE Apoptosis Assay Kit (Geno Technology Inc., St. Louis, MO, USA). Caspase kit reagents were prepared prior to use on treated cells lysed and tissue homogenised according to a modified manufacturer's protocol. In this study, the tissue were homogenised and the cells were lysed with a sonicator, and the caspase-3 enzymatic activity in the supernatant and lysates were determined as described. Briefly, microtiter wells were set up in duplicate for controls, blank and test cells (lysates). Then, 10 µL of the tissue lysate was incubated in a 96-well plate with 90 µL of CasPACE assay buffer containing 5 µL of the caspase substrate, Ac-DEVD. Fifty microlitres of 2× CasPACE assay buffer was transferred into each well and then followed by the addition

Fig. 3 Testicular cell cycle and apoptotic rate after chronic, moderate heat exposure by FCM. **a** The cell cycle was determined after propidium iodide staining and FCM analysis; **b** the percentage of apoptotic cells was determined by combined annexin V/propidium iodide staining and FCM analysis. *White bar* control group, *gray bar* CMH group. The results are represented as the mean (±SE) of five mice. *$P<0.05$ and *$P<0.01$ compared to the control
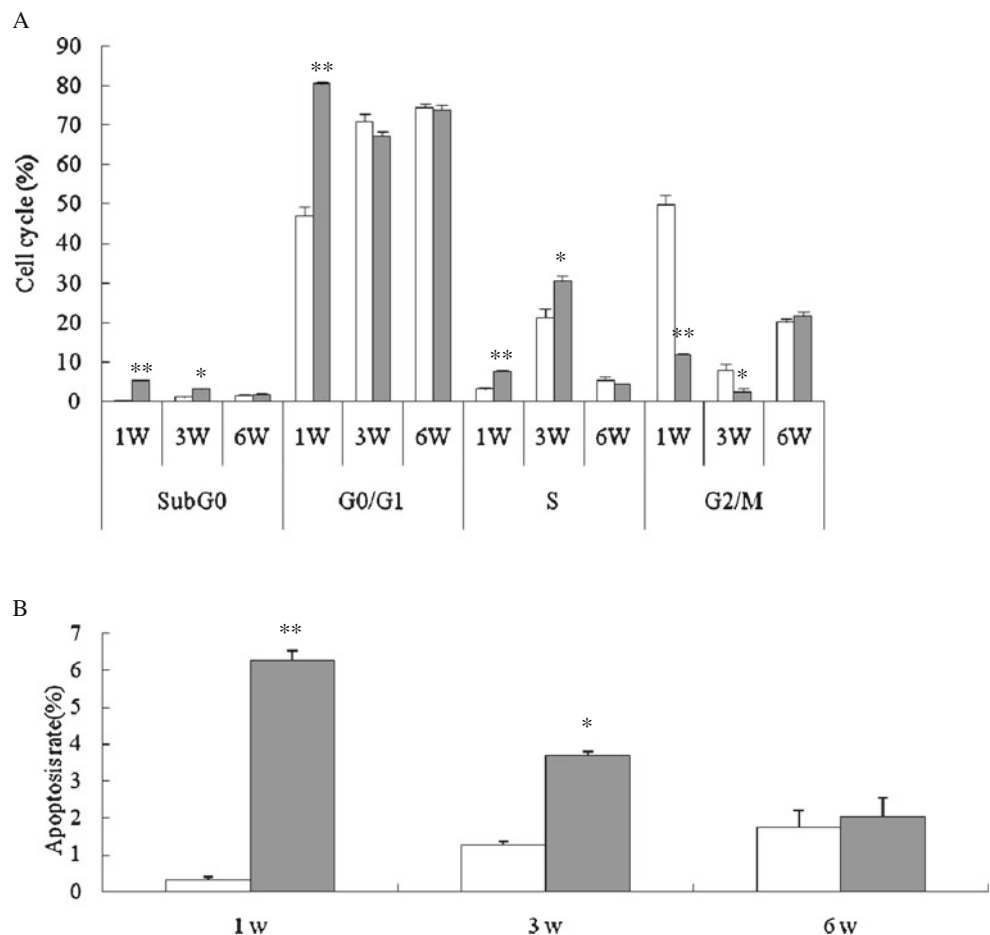
of 50 μL of the cell lysate to the wells and the addition of 5 μL of the Ac-DEVD. The plate was incubated at 35 °C for 2 h and then read on an ELISA microplate reader at 405 nm wavelength. The level of caspase-3 (CPP32) enzymatic activity in the lysates was directly proportional to the colour reaction. Therefore, to quantify CPP32 expression in the lysates, the fold increase of caspase-3 protease activity was determined by comparing absorbance from the treated samples with the non-treated controls.
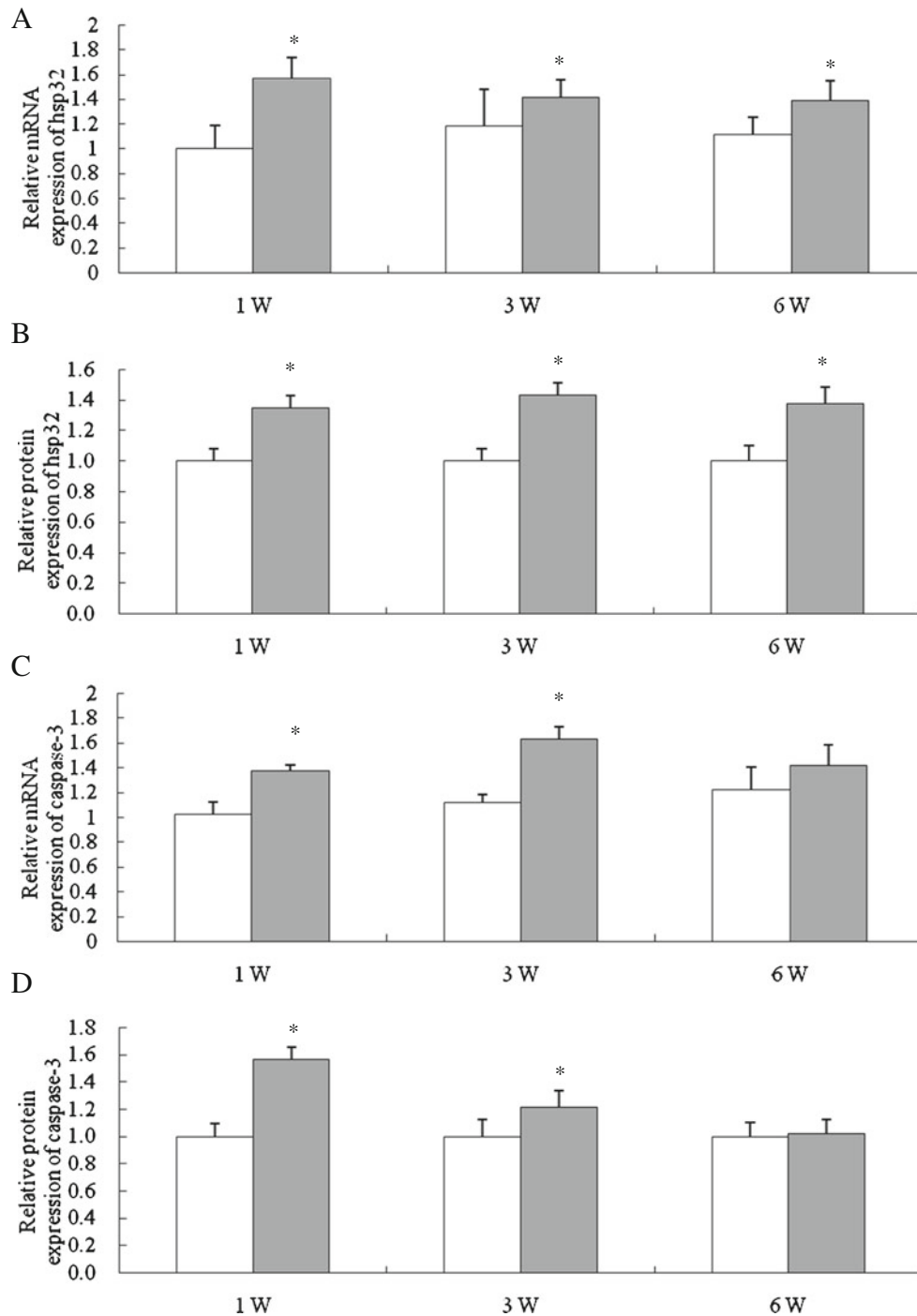

Fig. 4 a, c The expression of hsp32 and caspase-3 mRNA in the testis of control and chronic, moderate heat-exposed mice. The results are represented as the mean (±SE) of five mice. **b, d** Quantitative analysis of the expression of hsp32 and caspase-3 protein; **b, d** data from the testis samples. **e** Western blot results of the control and the heated testis samples are shown (β-actin is shown as a loading control). **f** Relative caspase-3 activity in the testis after moderate heat-exposed mice. *White bar* control group, *gray bar* CMH group. *M* marker. *$P<0.05$ and **$P<0.01$ compared to the control
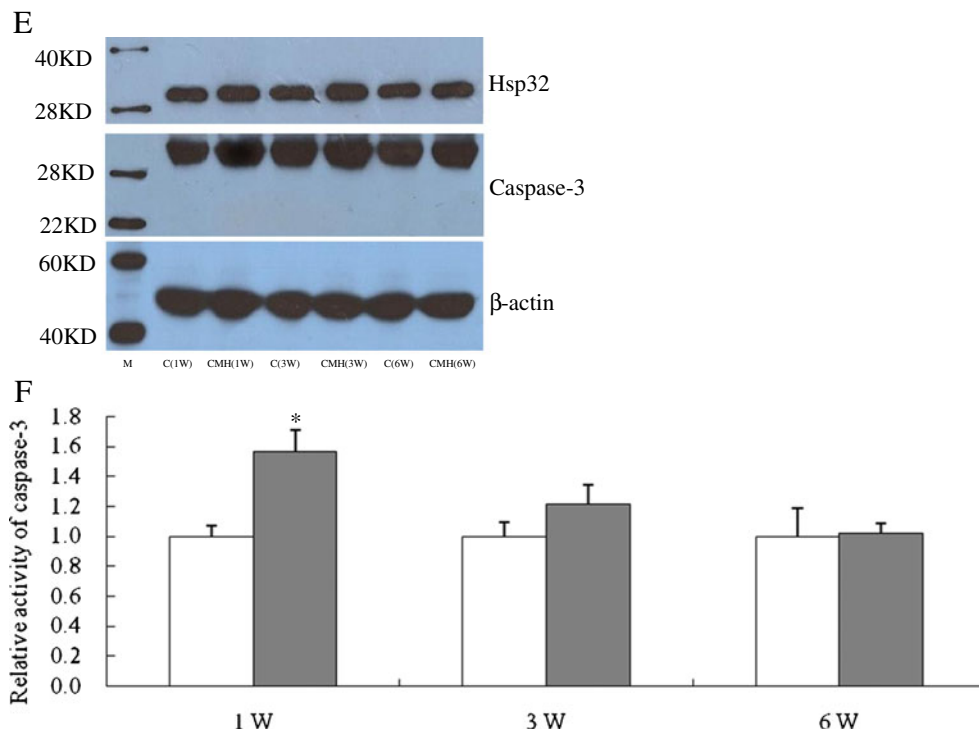
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F

Fig. 4 (continued)

Fig. 5 Testicular cell cycle and apoptotic rate after transient, acute heat exposure by FCM. **a** The cell cycle was determined after propidium iodide staining and FCM analysis; **b** the percentage of apoptotic cells was determined by combined annexin V/propidium iodide staining and FCM analysis. *White bar* control group, *gray bar* TAH group. The results are represented the mean (±SE) of five mice. *$P<0.05$ and **$P<0.01$ compared to the control
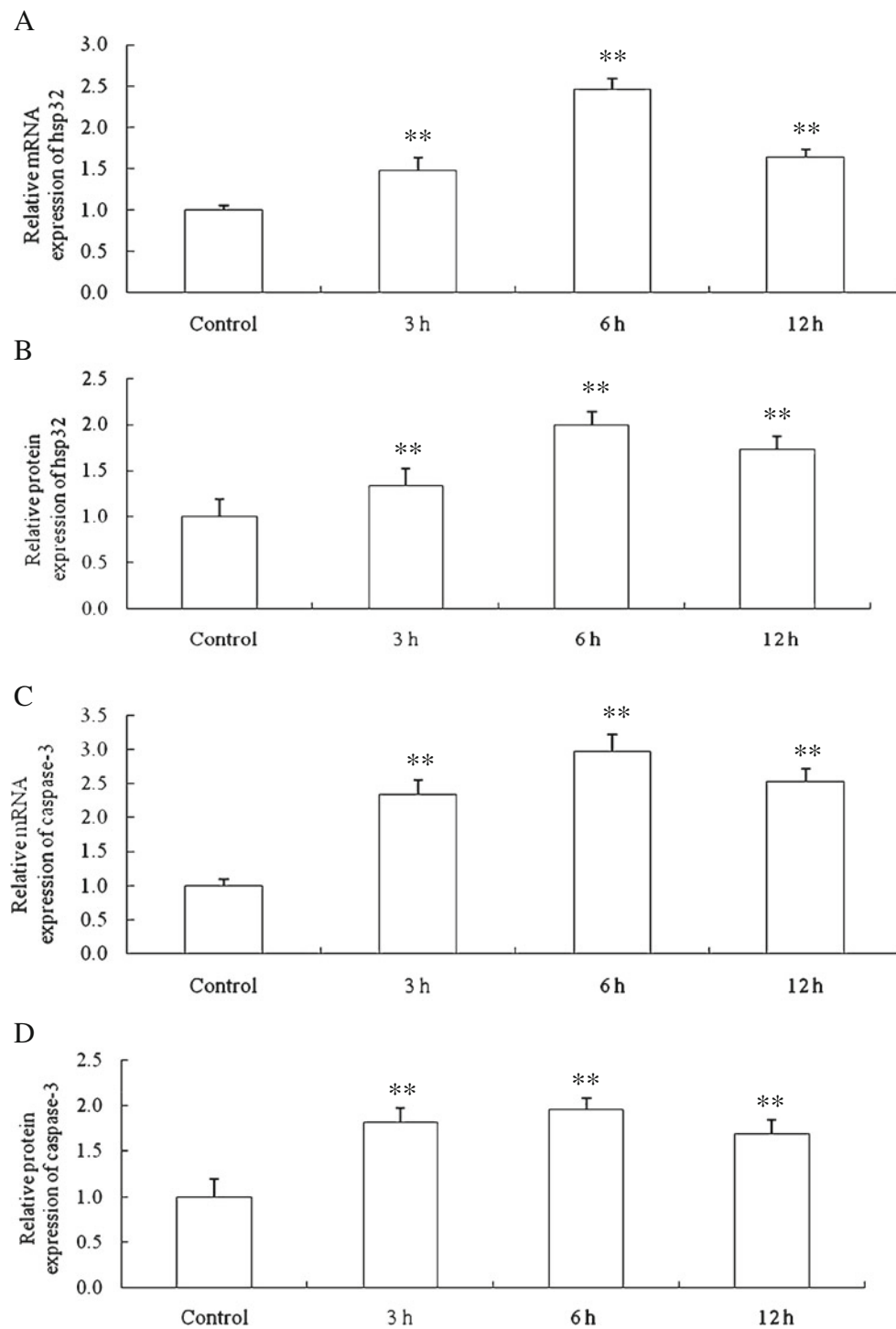
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B
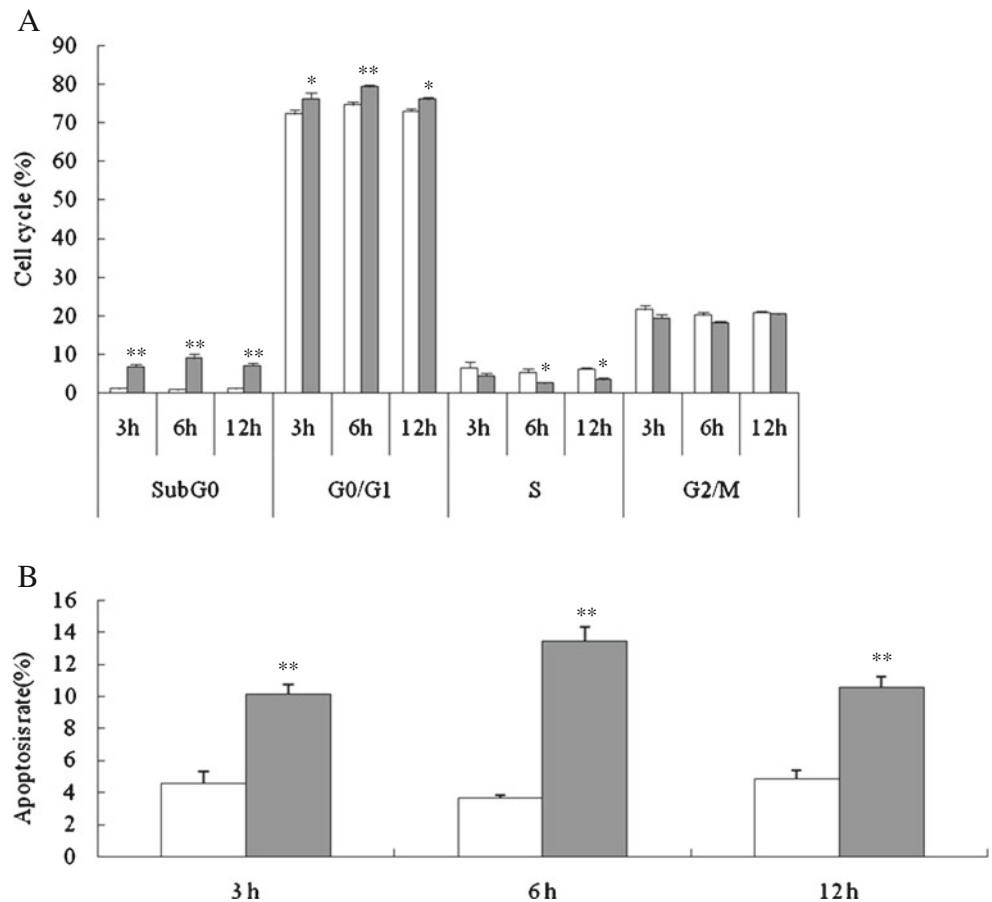
Fig. 6 **a**, **c** The expression of Hsp32 and caspase-3 mRNA in the testis of the control and transient, acute heat-exposed mice. The results are represented as the mean (±SE) of five mice. **b**, **d** Quantitative analysis of the expression of Hsp32 and caspase-3 protein; **b**, **d** the data from the testis samples. **e** Western blot results of the control and heated testis samples are shown (β-actin is shown as a loading control). **f** Relative caspase-3 activity in the testis after acute heat-exposed mice. *M* marker. *P<0.05 and **P<0.01 compared to the control. **g**–**j** Hsp32 and caspase-3 immunoreactivity in control and TAH group testis. Higher magnification of a seminiferous tubule demonstrates that hsp32 is present exclusively in the adluminal (*arrow*) parts of Sertoli cells (**h**); no staining was visible after pre-adsorption of anti-hsp32 to an excess of the corresponding antigen (**g**); mice show a marked increase (*arrow*) in the number of active caspase 3-positive germ cells 6 h (**j**) after heat treatment compared with controls (**i**), in which no such staining was detected. *Scale bar=* 30 μm
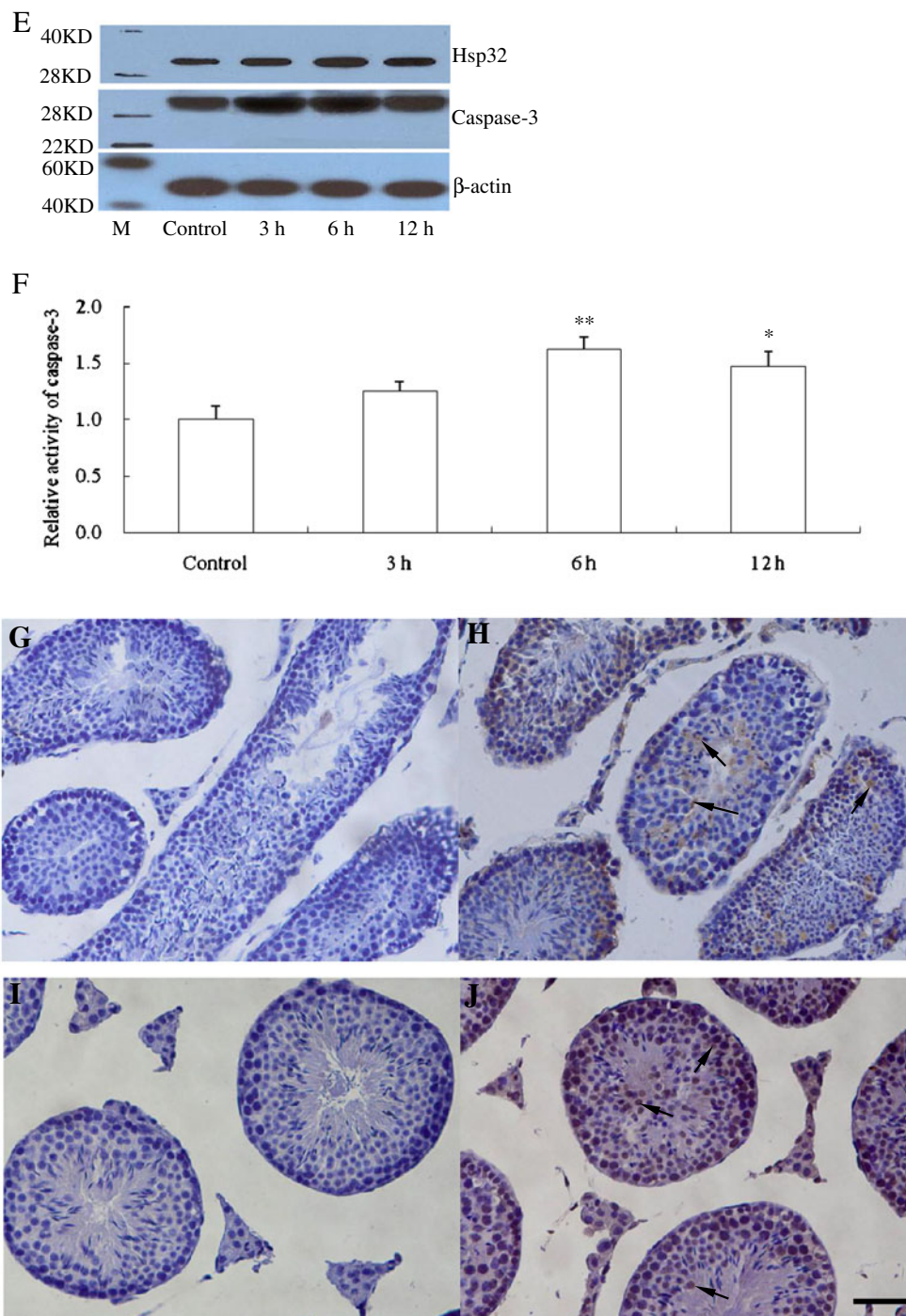
Fig. 6 (continued)

Measurement of carboxyhaemoglobin in conditioned medium

To examine whether Sertoli cells released CO into the cultured medium after heat treatment (43 °C for 30 min and then recovery for 6 h) or hsp32 siRNA interference (48 h after transfection), we quantified the relative amounts of CO produced by adding Hb to the medium and measuring carboxyhaemoglobin (HbCO) levels. Hb (50 μM) was added to the cells for the last hour of incubation, and HbCO was measured spectrophotometrically at 570 nm wavelength.

Statistical analysis

The data were expressed as the mean±SE and analysed using one-way ANOVAs in the statistical software package SPSS12.0 (SPSS Inc., Chicago, IL, USA). The data were transformed to ensure homogeneity of variance. Least significant difference's multiple comparisons were used to identify differences between the groups at the 5 and 1 % significance levels.

Results

Morphology of heat exposure testis

Pathological lesions were observed by histopathological examination. In the HE-stained sections of testes from the control mice (Fig. 1a–c), all stages of spermatogenesis were observed, including round spermatids, elongated spermatids and sperms. Compared with the control group, the germ cells (including spermatocytes and spermatids) decreased in the CMH mice after 1 week of intermittent heat exposure (Fig. 1d). After 3 weeks of intermittent heat exposure, the seminiferous tubules atrophied, the number of spermatogenic cells decreased, the seminiferous epithelial cells disintegrated and vacuolation occurred in the CMH group (Fig. 1e). Intermittent heat exposure for 6 weeks did not affect the morphology of testis tissue (Fig. 1f).

Compared with the control group, there was no difference in the morphology of testis 3 h after acute transient treatment (Fig. 2b). However, disorder of the spermatogenic cell array in the seminiferous tubules was observed at 6 h (Fig. 2c). Twelve hours after the acute, transient heat treatment, some tubules contained abundant vacuoles and loss of spermatogenic cells was detected (Fig. 2d).

Cell cycle and cell apoptosis after chronic moderate heat exposure in mouse testis

The effects of heat exposure on the cell cycle and apoptosis of testicular cells were examined by using flow cytometry. After intermittent heat exposure for 1 week, the populations of sub-G_0, G_0/G_1 and S phase cells in the group increased significantly ($P<0.01$) compared to the control group. After intermittent heat exposure for 3 weeks, the population of sub-G_0 and S phase cells in the CMH group increased significantly ($P<0.05$) and G_2/M phase cells decreased significantly ($P<0.05$) (Fig. 3a). Apoptosis was found in the CMH group after heat exposure for 1 and 3 weeks at a rate significantly higher than the control group ($P<0.01$). However, after 6 weeks of heat exposure, the cell cycle and the apoptotic rate showed no significant changes ($P>0.05$) compared to the control group (Fig. 3b).

Effects of chronic, moderate heat exposure on hsp32 and caspase-3 expression in mouse testis

Chronic, moderate heat stress at 39 °C caused upregulation of hsp32 and caspase-3 mRNA expression (Fig. 4). Hsp32 mRNA levels significantly increased after intermittent heat exposure for 1, 3 and 6 weeks compared to the control group ($P<0.05$). Caspase-3 mRNA levels significantly increased after intermittent heat exposure for 1 and 3 weeks, but no significant difference was observed in caspase-3 mRNA

Fig. 7 The effects of moderate and acute heat treatment on Sertoli cell apoptosis. The results are represented as the mean (±SE) of three independent experiments. *P <0.05 and **P<0.01 compared to the control
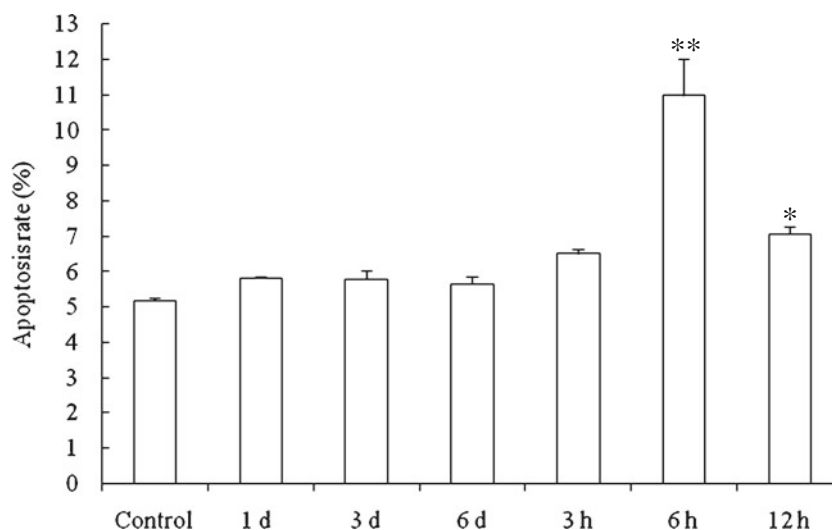
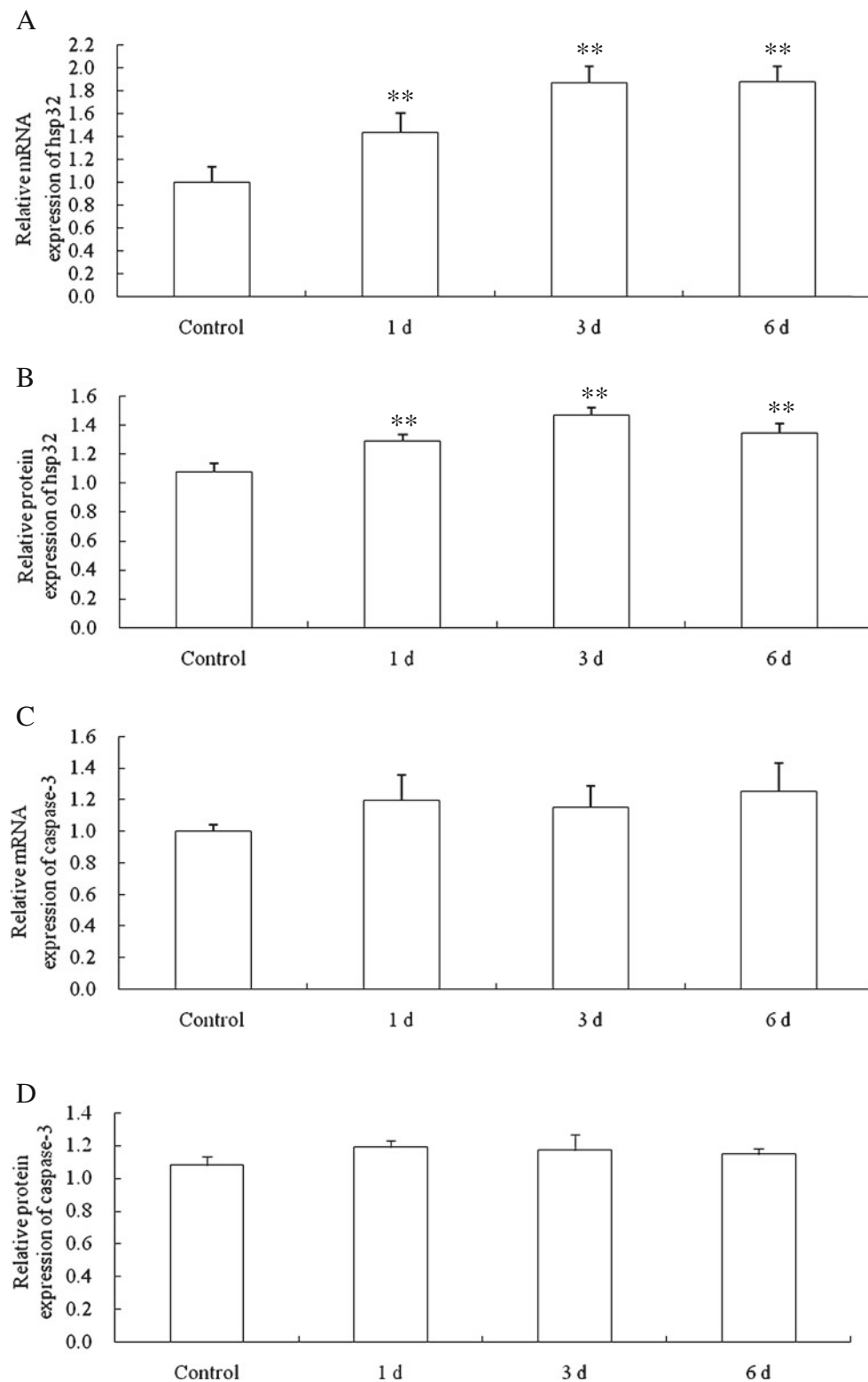**Fig. 8** **a**, **c** The expression of hsp32 and caspase-3 mRNA in the Sertoli cells after moderate heat exposure. **b**, **d** Quantitative analysis of the expression of hsp32 and caspase-3 protein; **b**, **d** data from the heat-exposed Sertoli cells. **e** Western blot results after moderate heat exposure. Protein from whole cell lysates was used in western blotting to detect hsp32 and caspase-3. **f** Relative caspase-3 activity in the Sertoli cells after moderate heat exposure. The results are represented as the mean (±SE) of three independent experiments. *$P<0.05$ and **$P<0.01$ compared to the control
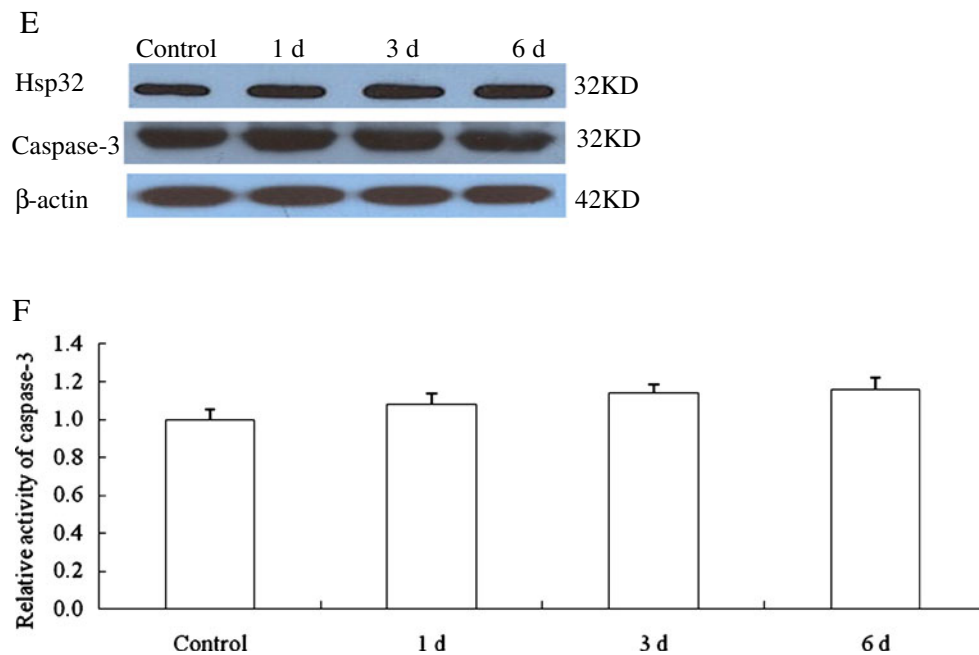
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Fig. 8 (continued)

levels after 6 weeks of intermittent heat exposure. Similar to the expression of mRNA, hsp32 protein levels in the CMH group also increased significantly after heat exposure for 1, 3 and 6 weeks, and caspase-3 protein levels increased significantly after heat exposure for 1 and 3 weeks ($P<0.05$). Caspase-3 activity was assessed after heat exposure (Fig. 4f). After the 1-week heat exposure, we detected an increase in caspase-3 activity compared with control ($P<0.05$). The 3-week heat exposure time point caspase-3 activity also increased, but there are no significant different. The 6-week heat exposure did not alter caspase-3 activity.

Cell cycle and cell apoptosis after transient, acute heat exposure in mouse testis

As shown in Fig. 5a, the percentage of sub-G_0 and G_0/G_1 cells in the TAH group was significantly higher than that in the control group, while the percentage of S cells was significantly lower ($P<0.05$). No significant change in the percentage of G_2 cells was found between the TAH group and the control group. The apoptotic rate in the TAH group was also significantly higher ($P<0.01$) compared to the control group (Fig. 5b).

Effects of transient, acute heat exposure on hsp32 and caspase-3 expression in mouse testis

The data showed that the mRNA levels of hsp32 and caspase-3 increased 3 h after acute heat exposure and maintained at

relatively high levels at 6 and 12 h ($P<0.01$) in the TAH group (Fig. 6a, b). Moreover, the total hsp32 and caspase-3 protein expression in the testes were also increased significantly at 3, 6 and 12 h ($P<0.01$) in the TAH group (Fig. 6c, d) compared to the control groups. Caspase-3 activity was assessed after acute heat exposure (Fig. 6f). Caspase-3 activity followed the same trend with regard to caspase-3 protein expression.

Localization of hsp32 and active caspase-3 expressions was determined in testis by immunohistostaining. Hsp32 expression in Sertoli cells of testis was strong 6 h after acute heat exposure compared with controls, in which no such staining was detected. Moreover, active caspase-3 expression was positive in the testis 6 h after heat exposure (Fig. 6g–j).

Apoptosis of cultured Sertoli cells after moderate or acute heat exposure

We examined the effects of heat exposure on Sertoli cell apoptosis (Fig. 7). Moderate heat exposure did not affect the apoptotic rate of Sertoli cells. However, the apoptotic ratio of Sertoli cells was significantly higher ($P<0.01$) at 6 and 12 h after acute heat exposure compared to the control group.

Effects of moderate and transient, acute heat exposure on hsp32 and caspase-3 expression in cultured Sertoli cells

As shown in Fig. 8, there was no significant difference in caspase-3 protein expression and caspase-3 activity after
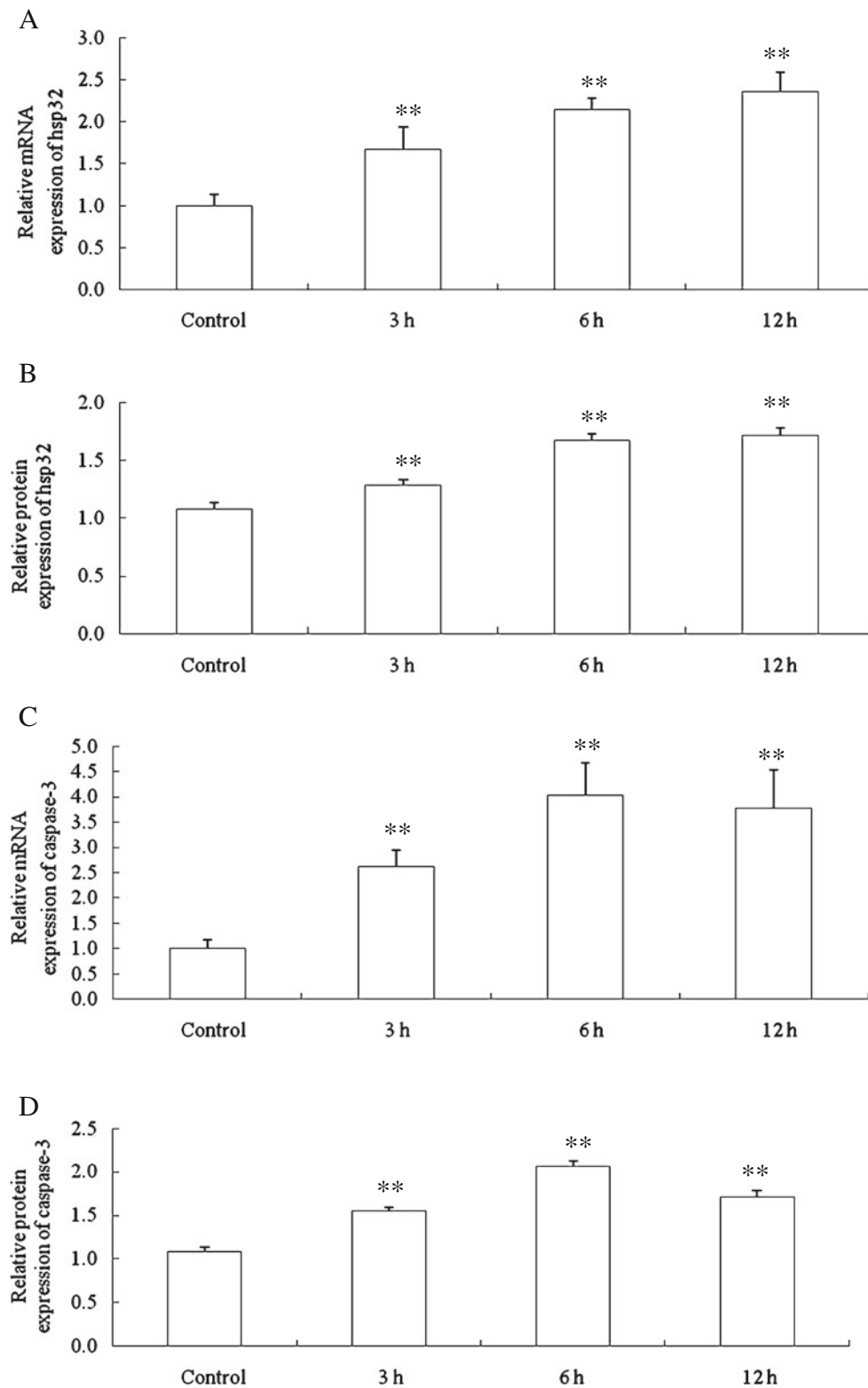
Fig. 9 **a**, **c** The expression of hsp32 and caspase-3 mRNA in the Sertoli cells after acute heat exposure. **b**, **d** Quantitative analysis of the expression of Hsp32 and caspase-3 protein; **b**, **d** data on the heat-exposed Sertoli cells. **e** Western blot results after acute heat exposure. Protein from whole cell lysates was used in western blotting to detect hsp32 and caspase-3. **f** Relative caspase-3 activity in the Sertoli cells after acute heat exposure. The results are represented as the mean (±SE) of three independent experiments. *$P<0.05$ and **$P<0.01$ compared to the control. **g–j** Hsp32 and caspase-3 localization in Sertoli cells in control and heat-treated groups by immunocytochemical staining. Sertoli cells exposed to 43 for 30 min and then recovery for 6 h. **g**, **i** Control; **h**, **j** Hsp32 and active caspase-3 localization in heat-treated Sertoli cells (indicated by *arrow*). *Scale bar*=30 μm
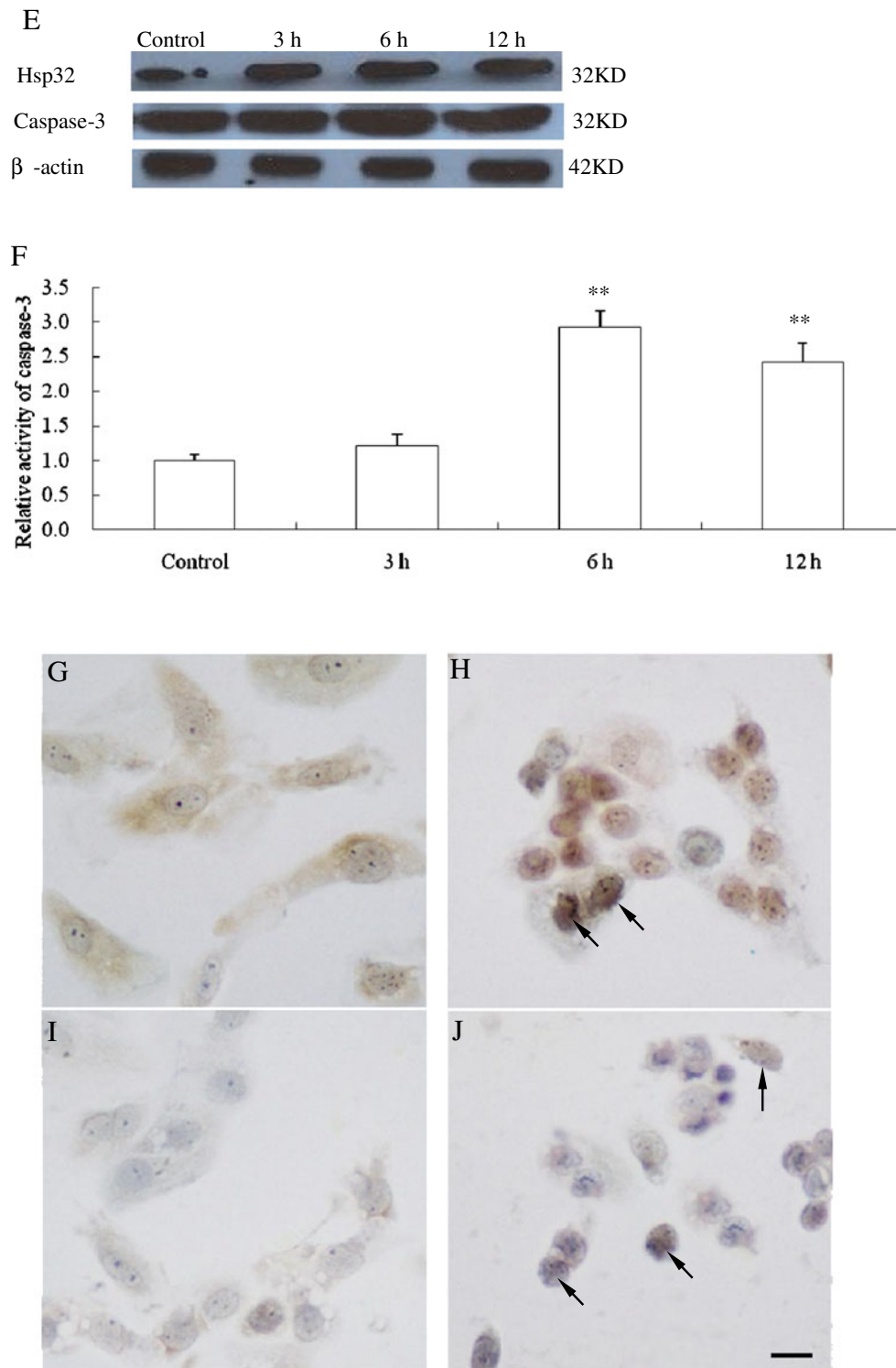
Fig. 9 (continued)

moderate heat exposure; however, the mRNA and protein levels of hsp32 were significantly higher ($P < 0.01$) compared to the control group. As shown in Fig. 9, transient,

acute heat exposure significantly affected the mRNA and protein expression of hsp32 and caspase-3 in the Sertoli cells. However, caspase-3 activity was significantly higher
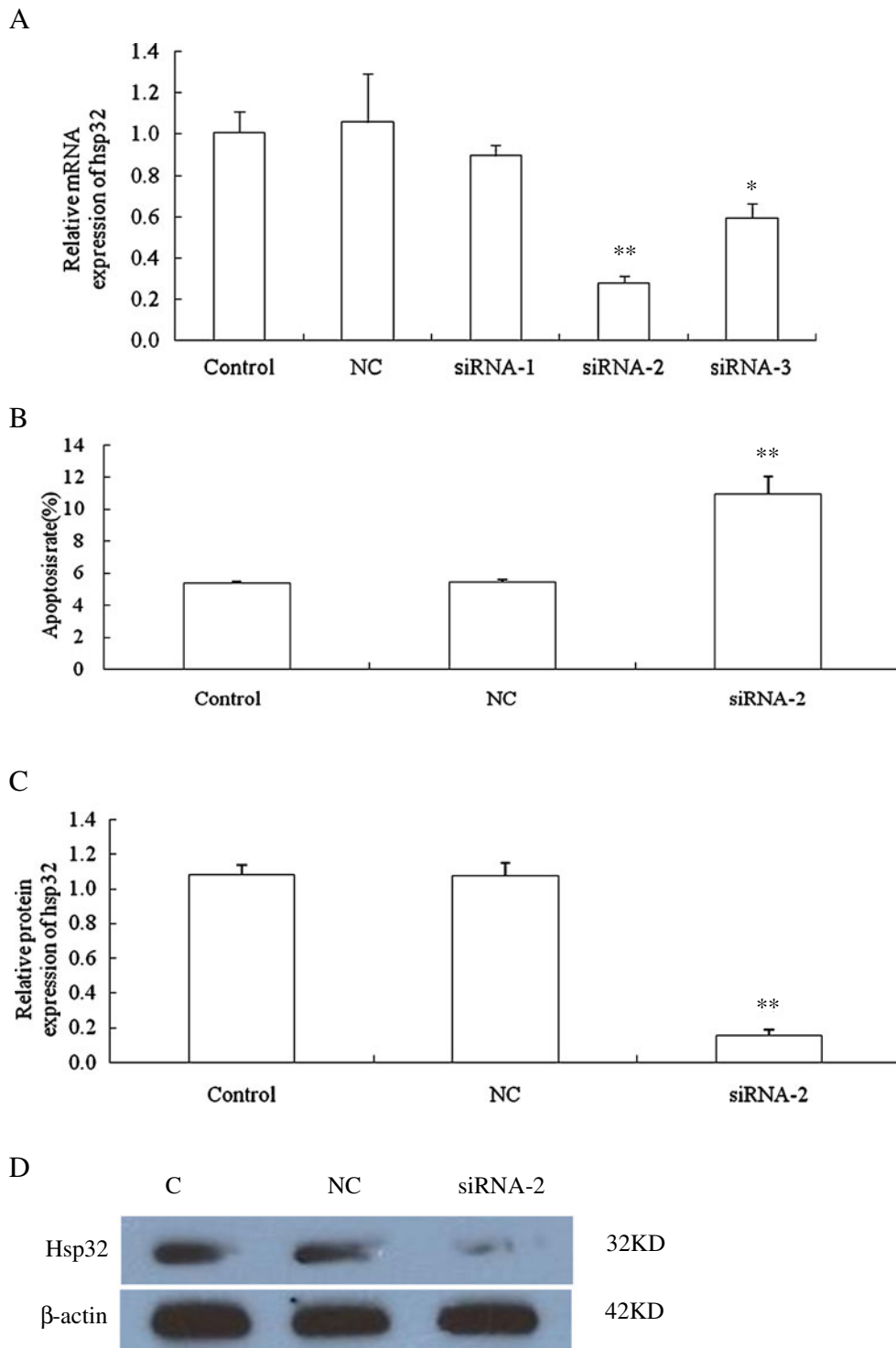
A

B

C

D

Fig. 10 Knockdown of hsp32 expression in the Sertoli cells is followed by apoptosis. **a** The effects of three different siRNAs (1–3) specific for Hsp32 on hsp32 mRNA expression in the Sertoli cells compared with the untransfected cells (**c**) or the cells transfected with a control siRNA (NC) are shown. **b** The effects of siRNA-2 specific for hsp32 on the apoptotic rate of Sertoli cells compared with the untransfected cells (**c**) or the cells transfected with a control siRNA (NC) are shown. **c**, **d** The Sertoli cells were incubated in a control medium (**c**) or were transfected with a negative control siRNA or hsp32-specific siRNA-2. At 48 h after transfection, Hsp32 expression was determined by western blotting. Expression of β-actin is shown as a loading control. **e** Relative expression of caspase-3 mRNA in Sertoli cells. **f** Relative caspase-3 activity in Sertoli cells
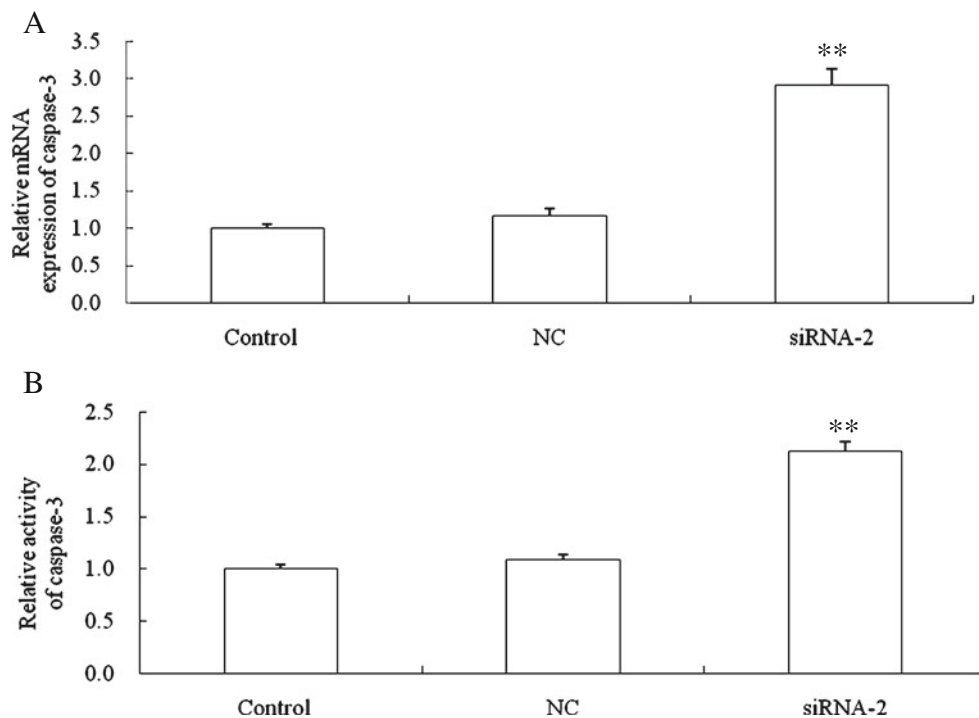
A

B

Fig. 10 (continued)

($P<0.01$) at 6 and 12 h after acute heat exposure compared to the control group (Fig. 9f).

Localization of hsp32 and active caspase-3 expressions was determined in Sertoli cells by immunocytostaining. After heat exposure, hsp32 expression in Sertoli cells was stronger than in controls, in which weakly positive staining was detected. Moreover, active caspase-3 was positive in Sertoli cells at 6 h after heat exposure (Fig. 9g–j).

Knocking down hsp32 expression by siRNA leads to increases in the apoptotic rate and caspase-3 expression in the Sertoli cells

To further investigate the functional role of hsp32 in the Sertoli cells, the expression of hsp32 was specifically silenced in the Sertoli cells using three different siRNAs.

Sequences 2 and 3 showed significant effects on the expression of hsp32 mRNA (Fig. 10a). The siRNA-induced knockdown of hsp32 was found to be associated with a significant increase in apoptosis in the Sertoli cells (Fig. 10b). The knockdown of Hsp32 expression was further confirmed by western blot analysis (Fig. 10c, d). Overexpression of caspase-3 in these cells was observed by qualitative real-time PCR and caspase-3 activity analysis (Fig. 10e, f).

Hyperthermia increases CO release by Sertoli cells

To confirm that whether Sertoli cells exposed to hyperthermia release more CO than Sertoli cells in normal or hsp32 knockdown condition, we quantified the relative amounts of CO produced. Hyperthermia Sertoli cells generated 1.6-fold

Fig. 11 Measurement of HbCO in conditioned medium of Sertoli cells under hyperthermia, conditioned media obtained from Sertoli cells knocking down hsp32 expression and normal cultured conditions. Values are represented as percentages of control HbCO levels in conditioned medium of Sertoli cells for 6 h. *$P<0.05$; **$P<0.01$ versus control ($n=3$)
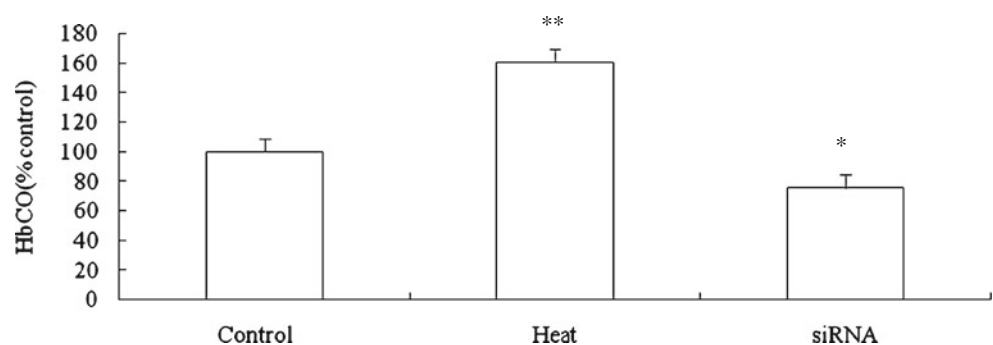
greater CO than cells incubated in normal environments, and the CO produced could be decreased by inhibiting HO-1 expression with siRNA (Fig. 11).

Discussion

Hsp32 is a stress-related, cytoprotective molecule that possesses anti-apoptotic activity in various cells (Borger and Essig 1998; Kiemer et al. 2003; Kondo et al. 2007; Burger et al. 2009). The testis is a major organ that abundantly expresses hsp32 under stress conditions. In the present study, we investigated the change in hsp32 expression after mouse testis tissue was exposed to heat. Our data showed that moderate and acute heat exposure could induce overexpression of hsp32. Furthermore, this result was also confirmed in cultured Sertoli cells. These findings suggest a functional role for hsp32 when mouse testis is exposed to heat, demonstrating for the first time a dynamic trend in hsp32 expression.

Contrary to other small Hsps, such as hsp27 can act as a molecular chaperone that prevents the aggregation of distorted proteins and promotes functional refolding under permissive conditions (Doshi et al. 2009). Hsp32 has no known chaperoning activity, but it does mitigate cellular injury by its antioxidant, anti-apoptotic and anti-inflammatory effects (Novo et al. 2011; Brouard et al.

2000). Some studies reported that the by-product of hsp32/HO-1 metabolism, CO, is produced at a relatively high level in injured liver and lung tissues via the induction of hsp32 and contributes to the suppression of pro-apoptotic mediators (i.e. caspase-3) and/or the induction of anti-apoptotic mediators (Bcl-x, Bcl-2 and Bag-1) (Ke et al. 2002; Sass et al. 2003; Ryter and Choi 2009). In heat-induced apoptosis, mitochondria-dependent apoptotic pathway is the key apoptotic pathway which leading to the activation of the initiator caspase-9 and the executioner caspase-3 (Sinha Hikim et al. 2003; Vera et al. 2004; Paul et al. 2009). In the present study, moderate heat exposure increased expression levels of caspase-3 and caspase-3 activity in mouse testis at 1 and 3 weeks after heat exposure, but no significant difference was found in caspase-3 mRNA, protein level and active caspase-3 after 6 weeks of intermittent heat exposure. Surprisingly, we found hsp32 mRNA and protein levels significantly increased during the whole heat exposure stage (Fig. 4). Under transient acute heat exposure, we located active caspase-3 protein by an antibody that recognized the active form of caspase-3. The intense caspase-3 immunoreactivity is mainly localized to germ cells in testis. Consistent with the immunohistochemical findings, caspase-3 activity analysis revealed a significant increase after acute heat exposure. These results suggest that overexpression of hsp32 could alleviate the apoptosis of testicular cells via attenuating the caspase-3 expression on the transcriptional level.

Fig. 12 Proposed regulation mechanism of hsp32 in heat-induced testes. Heat-induced apoptosis of the germ cells may be alleviated by the upregulation of hsp32 in the Sertoli cells via the inhibition of caspase-3 or caspase-9 activity in mouse testis, hsp32-derived CO may involve in the regulation mechanism. *Rightwards arrow* activation, *left tack* inhibition
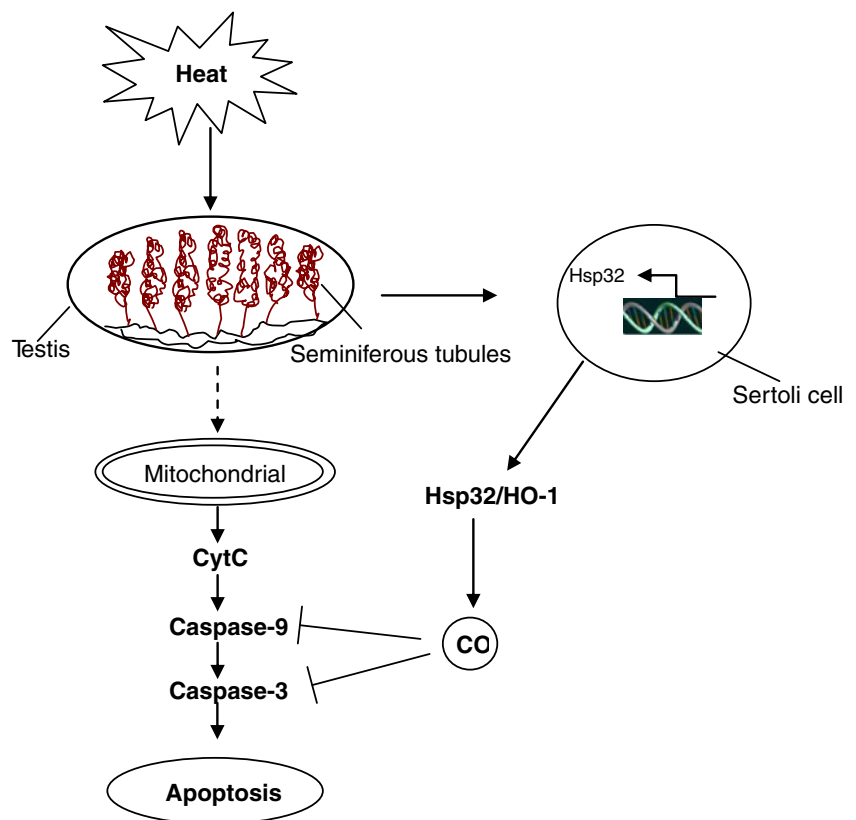
Meanwhile, the changes in testicular morphology and cell cycle indirectly support a protective role for hsp32 during heat stress in mouse testis (Figs. 1 and 3).

In our in vitro experiment, caspase-3 activity and the apoptotic rate of the Sertoli cells were affected only under acute heat exposure. Therefore, the expression of hsp32 was significantly higher than control under both heat exposure types. These results are consistent with in vivo experiment and confirm the protective role of hsp32. To further confirm the anti-apoptotic role of hsp32 in the Sertoli cells, three different hsp32-specific siRNAs were used. In these experiments, we were able to show that the siRNA-induced knockdown of hsp32 is associated with the induction of apoptosis in the Sertoli cells (Fig. 10b). Meanwhile, caspase-3 sharply increased and reached overexpressed levels (Fig. 10e, f). These data first provide clear evidence for the functional role of hsp32 as an important survival factor in cultured Sertoli cells.

Hsp32 has no known chaperoning activity, but CO which is metabolism by-product of hsp32 may play a role in suppressing apoptotic signals. Recently reports showed that CO has an anti-apoptotic role in different types of cell, such as endothelial cells, DAOY cells and vascular smooth muscle cells (Wang et al. 2011; Al-Owais et al. 2012; Liu et al. 2003). In present study, we determined whether the change of hsp32-derived CO can be found in cultured Sertoli cells or not after treatment. We found that significantly higher CO levels were released into the medium of hyperthermia cells, and significantly lower CO levels were measured in the medium of hsp32-specific siRNAs knockdown Sertoli cells. CO is a gaseous modulator of gene expression (Middendorff et al. 2000; Dulak and Jozkowicz 2003); CO can freely diffuse across membranes. The CO generated from the activity of hsp32/HO-1 could have its effects in an autocrine or paracrine manner in the same or neighbouring cells. Therefore, hsp32-derived CO in Sertoli cells may inhibit apoptosis signals in testis. Taken together, the data demonstrate the level of CO generated from hsp32 in Sertoli cells after different treatments and provided initial evidence for a functional role of CO derived hsp32 as part of the testicular protective mechanism.

In summary, our results indicated the hsp32 expression and roles in testis and cultured Sertoli cells response to heat stress and suggested that hsp32 could alleviate the apoptosis of testicular cells via attenuating the expression of caspase-3 and caspase-3 activity. Meanwhile, Hsp32-derived CO may involve in the apoptosis mediated by caspase-3 (Fig. 12). Clearly, further investigations are needed to investigate the possible anti-apoptotic mechanisms of hsp32 in heat stressed testis.

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