



Testosterone regulates granzyme K expression in rat testes

^{1,2}DUTTA Dibyendu, ¹PARK In, ¹GUILILAT Hiwot, ¹SANG Samuel, ¹TALAPATRA Arpita, ¹SINGHAL Barkha, ¹MILLS Nathaniel C

¹Department of Biology, Texas Woman's University, Denton, Texas, USA;

²Department of Professional Sciences, Middle Tennessee State University, Murfreesboro, Tennessee, USA

E-mail: NMills@twu.edu

Objective. Testosterone depletion induces increased germ cell apoptosis in testes. However, limited studies exist on genes that regulate the germ cell apoptosis. Granzymes (GZM) are serine proteases that induce apoptosis in various tissues. Multiple granzymes, including GZMA, GZMB and GZMN, are present in testes. Thus, we investigated which granzyme may be testosterone responsive and possibly may have a role in germ cell apoptosis after testosterone depletion.

Methods. Ethylene dimethane sulfonate (EDS), a toxicant that selectively ablates the Leydig cells, was injected into rats to withdraw the testosterone. The testosterone depletion effects after 7 days post-EDS were verified by replacing the testosterone exogenously into EDS-treated rats. Serum or testicular testosterone was measured by radioimmunoassay. Using qPCR, mRNAs of granzyme variants in testes were quantified. The germ cell apoptosis was identified by TUNEL assay and the localization of GZMK was by immunohistochemistry.

Results. EDS treatment eliminated the Leydig cells and depleted serum and testicular testosterone. At 7 days post-EDS, testis weights were reduced 18% with increased germ cell apoptosis plus elevation GZMK expression. GZMK was not associated with TUNEL-positive cells, but was localized to stripped cytoplasm of spermatids. In addition, apoptotic round spermatids were observed in the caput epididymis.

Conclusions. GZMK expression in testes is testosterone dependent. GZMK is located adjacent to germ cells in seminiferous tubules and the presence of apoptotic round spermatids in the epididymis suggest its role in the degradation of microtubules in ectoplasmic specializations. Thus, overexpression of GZMK may indirectly regulate germ cell apoptosis by premature release of round spermatids from seminiferous tubule lumen.

Key words: apoptosis, ethylene dimethane sulfonate, epididymis, blood-testis barrier, round spermatids, transition protein 1

Spermatogenesis is a continuous and sequential process by which haploid spermatozoa are produced in a strictly regulated cycle of germ cell renewal, proliferation, and differentiation (Eddy 2002). Through mitosis, the type A_{single}-spermatogonia either maintain a pool of the spermatogonial stem cells or become progenitor germ cells. From the progenitor germ cells, types A₁, A₂, A₃, A₄, intermediate and B-spermatogonia are formed and followed by meiotic

primary spermatocytes. Primary spermatocytes undergo meiosis I to become haploid secondary spermatocytes followed by meiosis II to form round spermatids. Finally, through specialized morphological differentiation (spermiogenesis), round spermatids become elongated spermatids and then spermatozoa (Perey et al. 1961). During the proliferative phase of normal spermatogenesis, apoptosis of many germ cells is a common phenomenon (Yin et al. 1997; Blan-

co-Rodriguez and Martinez-Garcia 1999; Print and Loveland 2000).

In the testis, haploid germ cells are sequestered in the adluminal compartment created by the Sertoli to Sertoli cell tight junctions. These junctions form the blood-testis barrier (BTB) on the periphery of seminiferous tubules. The maintenance of the BTB is essential to avoid autoimmune response to haploid germ cell specific surface antigens. Additional junctions between membranes of the Sertoli cells and germ cells (ectoplasmic specializations) are important to the positioning and morphological processing of the germ cells in the seminiferous tubule (Mruk and Cheng 2004). During the progression of spermatogenesis, the germ cells travel through the blood-testis barrier, undergo meiosis and transform by morphological changes to produce spermatozoa. Simultaneously, the ectoplasmic specializations in the adluminal compartment is used by the Sertoli cells and germ cells for spatial localization in the seminiferous tubule (Mruk and Cheng 2004). Thus, movement of the germ cells through the adluminal compartment and subsequent release of spermatozoa into the lumen of the seminiferous tubule requires orderly opening and closure of the Sertoli cell junctions. It is known that testosterone maintains germ cell viability and regulates the testicular junctions (Kerr et al. 2006). Through various models of testosterone deprivation, either by using GnRH antagonist and testosterone-estradiol treatment or with androgen receptor knockout mice, it has been demonstrated that absence of the testosterone leads to the disruption in testicular junctions and increased germ cell apoptosis (O'Donnell et al. 1994, 2006; Wang, et al. 2009; McCabe et al. 2010). The proteases are reported to be involved in the apoptosis of the germ cells and changes in the junctional architecture during spermiogenesis (Takano et al. 2004).

Granzymes are members of the serine protease family and are more extensively studied proteases in many tissues, especially in relation to apoptosis (Chowdhury and Lieberman 2008). They are secretory granule proteases, generally residing inside cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells that help in the elimination of the cancerous and virus infected cells by cleaving specific intracellular substrates (Chowdhury and Lieberman 2008; Chavez-Galan et al. 2009). Although granzymes located inside CTLs and NK cells are generally cytolytic, many granzyme variants in non-immune cells (granulosa cells, chondrocytes and Sertoli cells) have non-apoptotic extracellular functions such as remodeling of the extracellular matrix, inducing in-

flammation and signal transduction (Amsterdam et al. 2003; Buzza and Bird 2006; Chowdhury and Lieberman 2008; Romero and Andrade 2008; Anthony et al. 2010). In human and mouse testes, GZMB is expressed in the Sertoli cells and germ cells, and *Gzmn* is expressed only in the germ cells (Hirst et al. 2001; Takano et al. 2004). Both of these granzyme variants are proposed to possess non-apoptotic functions during spermatogenesis, such as modulation of the blood-testis barrier (Hirst et al. 2001; Takano et al. 2004). In *ob/ob* mouse testes, increased germ cell apoptosis has been reported along with an increase in the levels of mRNA of *Gzma* and *Gzmb* (Bhat et al. 2006). However, there is no report regarding the relative abundance and testosterone role in the regulation of different granzyme variants in testes.

Ethylene dimethane sulfonate (EDS) is a non-volatile mild alkylating agent that selectively eliminates the mature Leydig cells (Dutta et al. 2010; 2011; Zhang et al. 2015) and depletes testosterone within 2 days post-EDS treatment (Woolveridge et al. 2001). Testosterone depletion due to EDS treatment results in increased germ cell apoptosis and testosterone replacement can reverse the Leydig cell elimination effects by maintaining the germ cell viability (Henriksen et al. 1995). Thus, EDS serves as a valuable tool to investigate the testosterone withdrawal and replacement effects on granzyme variants in the rat testis.

In this study, EDS was used to deplete testosterone and the expression pattern of different granzyme variants (*Gzma*, *Gzmb*, *Gzmk* and *Gzmn*) were evaluated in testes. Testosterone's role in the granzyme variants' expression pattern was confirmed by replacing the testosterone in EDS treated rats. Apart from *Gzmb*, the role of other granzyme variants such as *Gzma* and *Gzmk* in cytotoxicity and inflammation remain elusive (Plasman et al. 2014). In addition, the regulating factors of these granzymes in testes are not known. Therefore, it was hypothesized that expression of granzymes in testes may be testosterone dependent and may play a role in germ cell apoptosis.

Materials and Methods

Drugs and chemicals. Ethylene dimethane sulfonate (EDS) is not commercially available and was synthesized in our laboratory using a previously described method (Jackson and Jackson 1984). For injection, EDS was dissolved in 25% dimethylsulfoxide (DMSO)/75% water solution at 30 mg EDS/ml of solution. Testosterone propionate (TP) was purchased from Sigma-Aldrich and was dissolved in sesame seed oil (SSO) at a concentration of 20 mg TP/ml SSO.

Animals and treatment. Adult male Sprague-Dawley rats were purchased from Charles River Laboratories and acclimated to the University vivarium with water and rat chow *ad libitum*. All procedures for animal care and use were in accordance with the NIH guide for the Care and Use of Laboratory Animals and were approved by the IACUC at Texas Woman's University. Thirty rats (>90 days of age) were randomly assigned to five treatment groups with an n=6 per group. The groups were: [1] No treatment (NT); [2] Vehicle control (VEH) (DMSO+SSO); [3] Testosterone-supplemented (T) (DMSO+TP); [4] ethylene dimethane sulfonate (EDS) (EDS+SSO); and [5] testosterone-replaced (EDS+T).

For this study, a 7-day post-EDS treatment protocol was established because EDS-induced testosterone depletion and germ cell loss peaks at 7-day post-EDS treatment (Henriksen et al. 1995; Zhang et al. 2015). Rats in EDS and EDS+T groups received a single intraperitoneal (i.p.) dose of EDS at 75 mg/kg of body weight. For rats receiving testosterone supplementation or replacement, 10 mg TP/kg of body weight were injected subcutaneously (s.c.) on days 0 and the 2nd, 4th and 6th day post-EDS. Rats in vehicle control received 2.5 ml of 25% DMSO/75% water solution/kg body weight via i.p. injection, and SSO at 0.5 ml/kg body weight via s.c. injection.

On day 7, rats were sacrificed following anesthesia with Isoflurane. After decapitation, trunk blood was collected, allowed to coagulate for 30 min on ice and then centrifuged at 8300×g for 10 min at 4°C to measure serum testosterone levels. For intratesticular testosterone (ITT) measurement, tes-

ticular tissues were decapsulated, and approximately 200 mg of the testis was homogenized using an Ultra Turrax for one min at 2300 rpm in 5.0 ml of 20 mM NaC₂H₃O₂ (sodium acetate), pH 5.0, to collect the testicular homogenate. Since testosterone in the testis remains bound to androgen binding proteins (ABP)/sex-hormone binding globulin (SHBG), testicular tissue was homogenized in a buffer having a pH of 5.0 to allow testosterone to separate from ABP (O'Donnell et al. 1994). Homogenates were centrifuged at 8000×g for 10 min at 4°C and supernatants were collected.

Radioimmunoassay. Testosterone in serum and testes was quantified using a radioimmunoassay (RIA) kit [Testosterone RIA DSL-4100 (Diagnostic Systems Laboratories, Webster, TX)] following the manufacturer's instructions. The detection limit of the assay was 0.05 ng/ml.

RNA extraction and reverse transcription. Two hundred milligrams (200 mg) of the testis and one whole epididymis from each rat was used to extract total RNA using TRIzol (Invitrogen) according to the manufacturer's protocol. The total RNA was dissolved in 2.0 mM Tris, 0.2 mM EDTA (pH 7.2) and spectrophotometrically analyzed for purity (A_{260nm}/A_{280nm} , >1.9) and concentration (25 A_{260nm} /mg RNA). From each extract, 3.0 µg of total RNA were subjected to 1.5% agarose gel electrophoresis, stained with 0.5 µg/ml of ethidium bromide and quantified using Alpha Inno-Tech software to verify RNA intactness with 28S/18S >1.0 giving consistent RT and qPCR results.

For reverse transcription of mRNA, 2.0 µg of total RNA was used to synthesize complementary DNA (cDNA) using Invitrogen's SuperScript III First-

Table 1
List of genes with accession number and sequences of gene specific primers used for qPCR

Gene	GenBank Accession Number	Primer Sequences	Product Length
Inls3	NM_053680	Sense: TGCAGTGGCTGGAGCAACGACATC Antisense: TTCATTGGCACAGCTGTNAGGTGGG	265 bp
Gzma	NM_153468	Sense: GAAATCTGAGGTCATTCTTGGGGCTCA Antisense: TGGTTCCTGGTTTCACATCATCCCC	215 bp
Gzmb	NM_138517	Sense: AGTGATCAACCCTGTCTTGGCAGATG Antisense: AAAGAGCACACCAGTGACTTGGGTC	235 bp
Gzmk	NM_017119	Sense: AAGCTTCGCACGGCTGCAGAACTG Antisense: GCGATCCCCTGCACAGATCATGTC	250 bp
Gzmn	NM_001191116	Sense: CGCAAAGACCACGTAATCCAGGG Antisense: CGAGTCAACCCTTGGGAAGGAGCCTC	222 bp
Tnp1	NM_017056	Sense: CATGGCATGAGGAGAGGCAAGAACC Antisense: TGGCAGTCCCCCTCTGATGTCTTG	215 bp
CD8	NM_031539	Sense: GCCTGAACTGCTGCAAGTCCTGTG Antisense: GACGCCAAAAAGCCAGTCATGCTG	231 bp
Gapdh	NM_017008	Sense: TGAACGGGAAGCTCACTGGCATGG Antisense: CAATGCCAGCCCCAGCATCAAAG	234 bp

Strand Synthesis kit with oligo (dT)₂₀ as primer. The final reaction products were diluted 25-fold with 25 µg/ml acetylated-bovine serum albumin (BSA) (Invitrogen) for quantifiable polymerase chain reaction (Katuru et al. 2011).

Quantifiable real-time polymerase chain reaction (qPCR). Roche's FastStart SYBR Green Master Mix kit was used to assess the relative mRNA abundance by qPCR using Opticon 2 thermal cycler and Opticon Monitor 3.1 software (Bio-Rad). Gene specific primers were designed using Invitrogen's Vector NTI[®] Advance 11 software (Table 1). Verification of qPCR products were done by melting curve analysis, agarose gel (1.5%) electrophoresis and DNA sequencing (Genewiz, NJ). Reactions without the template cDNA were run concurrently as negative controls and glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) was used as the reference gene (Sutton et al. 1998). Difference in quantity of the mRNAs were calculated by $(2)^{-\Delta\Delta Cq}$ method where Cq is quantification cycle (Livak and Schmittgen 2001).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. From each rat, one testis and one epididymis were fixed by immersing them in modified Davidson's fixative (mDF) for 24 h at 4°C. Tissues were paraffin embedded and 6 µm thick cross-sections were mounted on slides subbed with a chromated-collagen mixture. TUNEL assay was performed by following the previously described method (Dutta et al. 2012). Briefly, the tissue sections were deparaffinized, rehydrated and treated with proteinase K (3.0 µg/ml) (Amresco) for 1.0 min at room temperature. Apoptotic cells were detected using ApopTag[®] Plus Peroxidase *In Situ* Apoptosis Kit (Millipore). Normal nuclei were counterstained with Immunomaster's hematoxylin diluted 1:5 with water and stained for 3 min (American MasterTech). As a negative control, sections containing no recombinant terminal deoxynucleotidyl transferase (TdT) were processed in parallel.

Immunohistochemistry (IHC). For IHC, sections were deparaffinized by three 15 min xylene washes followed by five 15 min washes in 100% ethanol to remove xylene and reduce autofluorescence generated by remnant xylene. Between every wash, slides were air-dried for 15 min for better section adherence. After complete xylene removal, slides were rehydrated in descending concentration (90% and 70%) of ethanol for 15 min and then maintained in deionized water at room temperature until antigen retrieval. Rehydrated sections were boiled in 1.0 L of 1.0 mM EDTA (pH 7.5) for 15 min in an 800-Watt microwave oven for antigen retrieval (McCabe et al. 2010). Sections

were incubated in 10% normal donkey serum (NDS) (Jackson ImmunoResearch) diluted in phosphate buffer saline (PBS) for 1.0 h in a 37°C humidified chamber to block non-specific antigenic sites. Primary antibodies [goat anti-GZMK (sc-49021; Santa Cruz Biotechnology) and rabbit anti-CD8b (sc-9147; Santa Cruz Biotechnology)] were diluted (1:15) with NDS in PBS. In a humidified chamber, sections were covered with primary antibodies and incubated at room temperature (23°C) for 18 h. For negative controls, primary antibody was replaced with NDS. Primary antibodies were detected with either donkey anti-goat DyLight 594 (705-515-147; Jackson ImmunoResearch) or donkey anti-rabbit DyLight 649 (711-495-152; Jackson ImmunoResearch) diluted 1:500 with NDS. Sections were mounted in Vectashield mounting media with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories).

Imaging and image analysis. Images of TUNEL assay were evaluated with Nikon's Eclipse 90i digital microscope, and photographs were captured and analyzed using Nikon's NIS-Elements Basic Research version 3.1 software. A total of ten random fields per cross-section from each rat (n=6) in each treatment group were counted to quantify the number of apoptotic cells in the testis (Dutta et al. 2012). Immunofluorescent images were visualized with Nikon's Eclipse Ti confocal microscope and photographs were captured with Nikon's NIS-Elements Advanced Research version 3.1 software.

Statistical analysis. Levels of mRNA expression data were normalized to the untreated (NT) group and compared by 2-way analysis of variance (ANOVA) using SPSS version 15 software. Post-hoc comparisons were performed using Newman-Keuls test, and p-value ≤0.05 was considered significant (Zar 1999). All data are reported as mean ± standard error of mean (SEM), and graphs were generated using GraphPad Prism, version 5.04 software.

Results

Elimination of Leydig cells and depletion of testosterone. EDS-induced elimination of Leydig cells was confirmed by quantifying the mRNA levels of insulin-like peptide 3 (*Insl3*), a Leydig cells specific marker in the testis (Mendis-Handagama et al. 2007; Koeva et al. 2008). *Insl3* declined to <2% of control (NT and VEH) in EDS-treated rats (Figure 1). Testosterone replacement in EDS+T rats did not improve the level of *Insl3* and was similar to EDS-treated rats. Testosterone supplementation reduced *Insl3* to 30% of controls levels.

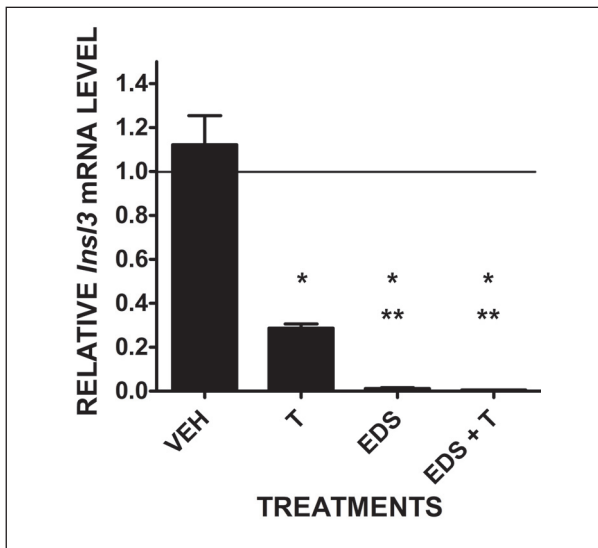


Figure 1. Relative level of Insulin-like 3 (*Insl3*) mRNA, a Leydig cell marker, in the testis after EDS treatment. *Gapdh* was used as the reference gene to calculate relative fold difference using $(2)^{-\Delta\Delta Cq}$ method. Error bars represent SEM with n=6. The horizontal line represents the value of *Insl3* for untreated (NT) rats normalized to 1.0. Single asterisk (*) indicates significant difference from VEH. Double asterisk (**) indicates significant difference from T.

Since the Leydig cells produce testosterone, EDS-induced elimination of Leydig cells should also result in an ablation of testosterone. Indeed, testosterone was undetectable (ND <0.05 ng/g) in serum or testicular tissue in 7-day post-EDS treated rats (Figure 2). Normal serum testosterone levels (1-10 ng/ml) were observed in untreated (NT) and vehicle-treated (VEH) rats. Exogenous administration of testosterone propionate increased the serum testosterone levels by 7- to 10-fold above normal in the T and EDS+T treatment groups. The concentration of intratesticular testosterone (ITT) has been reported to be between 60–100 ng/gm of the testis (Sharpe 1994). Consistent with the literature, the average levels of ITT in this study were 68 and 85 ng/g of the testis in NT- and VEH-treated rats, respectively. However, even with a 7- to 10-fold elevation in serum testosterone levels, ITT in T and EDS+T groups was 6- to 10-fold lower, compared to the NT and VEH treatment groups, respectively. Although complete restoration of ITT was not observed, the average concentration of ITT in the EDS+T group was comparable to the testosterone-supplemented (T) group.

Increased germ cell apoptosis and decline in testicular weight. With elimination of adult Leydig cells and depletion of testosterone in EDS treated

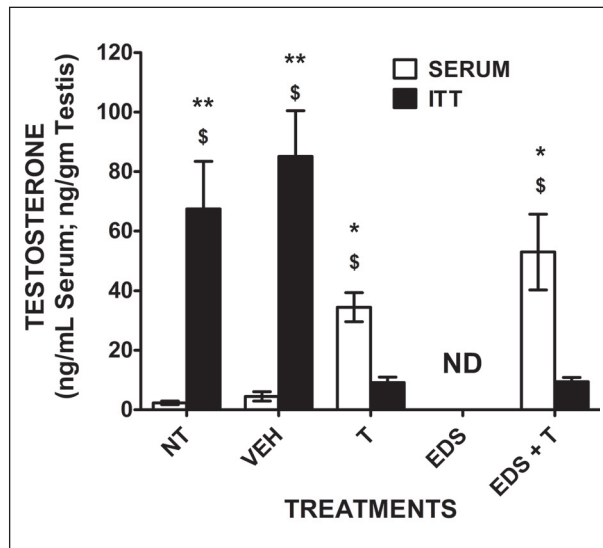


Figure 2. Mean serum testosterone (ng/ml) and intratesticular testosterone (ITT) (ng/gm testis). Error bars represent SEM with n=6. In EDS-treated rats, serum testosterone and ITT were below the detection level (0.05 ng/ml) of the assay (ND). Single asterisk (*) indicates significant difference in serum testosterone concentration from other treatment groups, double asterisk (**) indicates significant difference in ITT concentration from other treatment groups, and dollar (\$) indicates significant difference between serum testosterone and ITT concentrations within the same treatment group.

rats, there was an 18% reduction in testicular weight compared to other treatment groups (Figure 3). Since the Leydig cells constitute 2.7% of total testicular volume (Mori and Christensen 1980), 18% decline in testicular weight would represent not only loss of Leydig cells and ITT, but also germ cells. Indeed, in EDS-treated rats, there was 120–140 apoptotic cells/mm² of testicular cross section, which was significantly higher than the numbers observed in the NT, VEH, T and EDS+T groups (Figure 4). By using both, cell morphology and localization in the seminiferous tubule, the germ cells undergoing apoptosis in EDS-treated rats were identified as primary spermatocytes and earlier stages of round spermatids (Figure 5C). This is in sharp contrast to the occasional apoptotic spermatogonium observed in VEH, T and EDS+T (Figure 5A, B, D). Thus, testosterone depletion following EDS treatment resulted in increased germ cell apoptosis and lends support to the data that exogenous testosterone replacement maintains testicular weight by preventing germ cell loss in the absence of endogenous testosterone production.

Expression profile of granzyme variants. In rat testes, *Gzmk* was the least and *Gzmn* was the more abundant granzyme variant. The average qPCR quantification cycle (Cq) values of *Gzmk*, *Gzma*, *Gzmb*

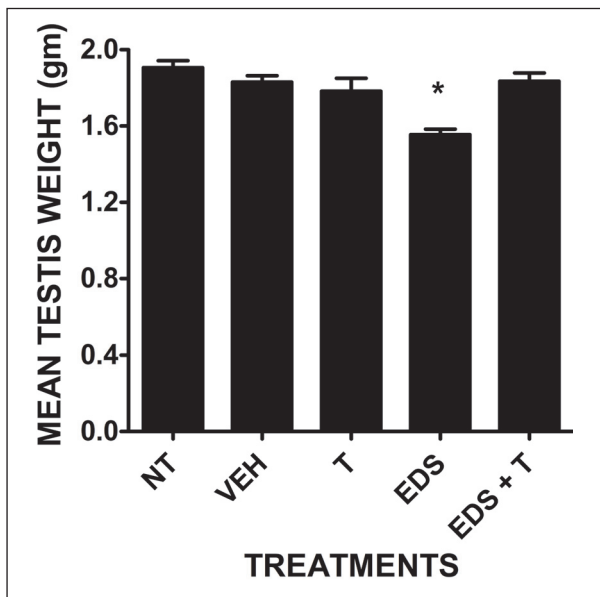


Figure 3. Average testis weights of 7 days post EDS treated rats. Error bars represent SEM with n=6. Asterisk (*) indicates significant difference from all other treatment groups.

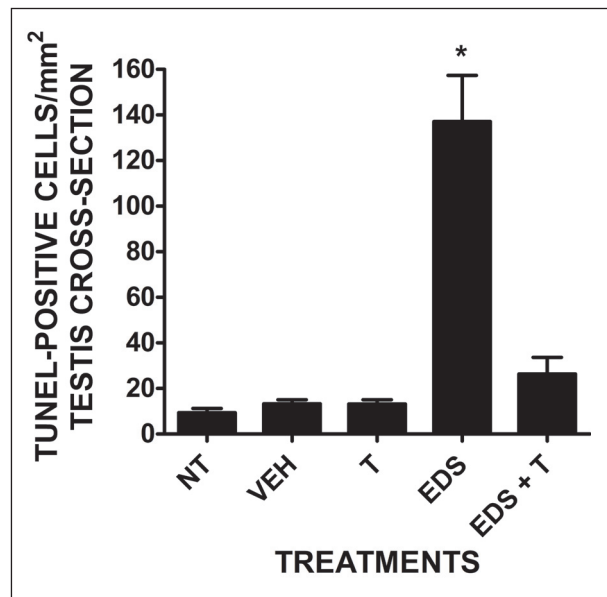


Figure 4. Average number of TUNEL-positive cells/mm² testis cross-section. Error bars represent SEM with n=6. Asterisk (*) indicates significant difference from other treatments.

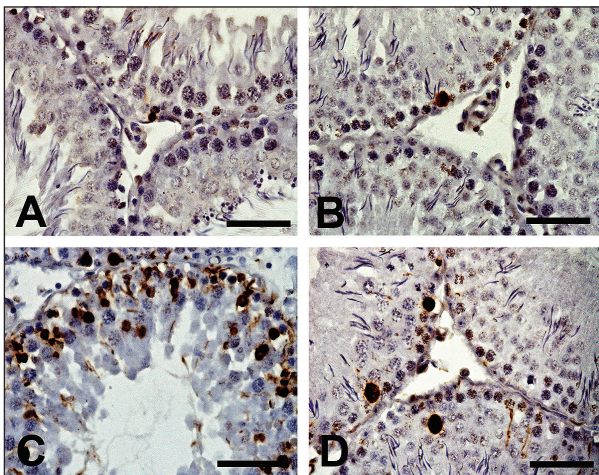


Figure 5. TUNEL-positive (apoptotic) cells (brown) in the rat seminiferous tubule cross-sections. Normal nuclei were counter-stained with hematoxylin. (A) VEH; (B) T; (C) EDS; and (D) EDS+T. Scale bars are 50 µm.

and *Gzmn* were 28.5, 27.5, 26.5 and 21, respectively. Hence, assigning the value of mRNA level of *Gzmk* 1.0, the relative abundance of *Gzma*, *Gzmb* and *Gzmn* mRNA were calculated to be 2-, 4-, and 181-fold higher, respectively, compared to the level of *Gzmk*.

Out of all the granzyme variants assayed (*Gzma*, *Gzmb*, *Gzmk* and *Gzmn*), only the level of *Gzmk* was significantly elevated in EDS-treated rat testes compared to other treatment groups. Testosterone

replacement in EDS+T group restored *Gzmk* to the control (VEH and T) level (Figure 6C). Neither testosterone withdrawal via EDS treatment, nor supplementation or replacement of testosterone after EDS treatment had any significant effect on *Gzma* or *Gzmb* levels (Figure 6A, B). Although, *Gzmn* was slightly higher after EDS treatment, the value was statistically similar to NT, VEH, T and EDS+T groups (Figure 6D). This demonstrates that *Gzmk* is a testosterone-responsive gene in testes. Since, there was a significant increase in both the germ cell apoptosis, and the level of *Gzmk* post-EDS, further investigation was carried out to determine whether *Gzmk* was localized to the same cell populations exhibiting positive TUNEL staining (apoptosis).

Using immunohistochemistry, strong GZMK staining was detected inside the seminiferous tubules in the testis of EDS treated rats. GZMK staining was closely associated with stripped cytoplasm of elongated spermatids (Figure 7C). Little GZMK staining was found in association with primary spermatocytes and round spermatids – the cell types with increased apoptosis in EDS treated rats. In control testes, more punctate and reduced staining of GZMK was observed in similar stages of germ cells, corroborating the qPCR data (Figure 7A, C and Figure 5C). GZMK staining in EDS-treated animals with testosterone replacement (EDS+T) also had a punctate appearance similar to the controls (Figure 7A, D). Staining in the interstitium of the testis in the negative control where

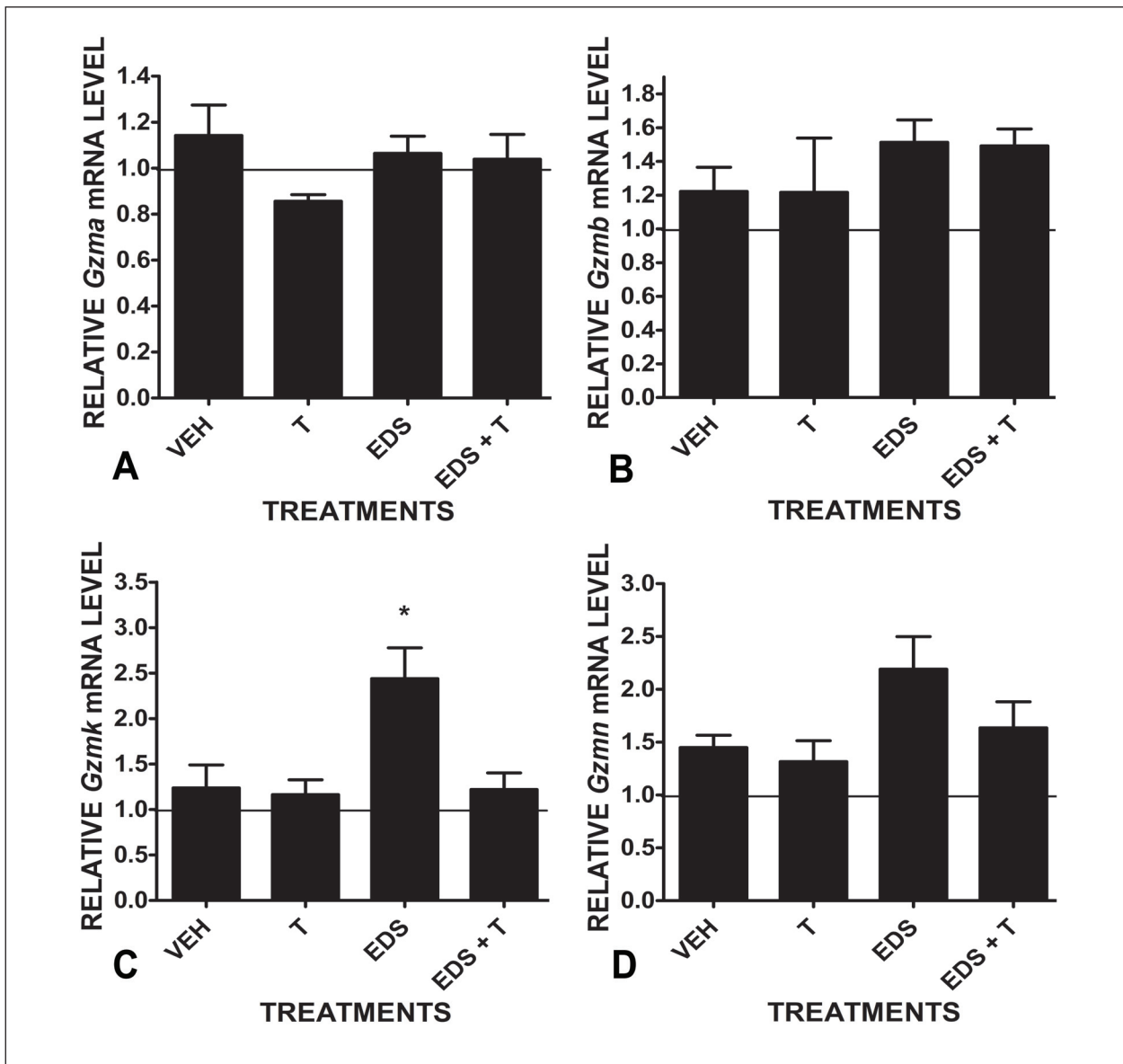


Figure 6. Relative mRNA levels of (A) *Gzma*; (B) *Gzmb*; (C) *Gzmk*; and (D) *Gzmn* in the testis. *Gapdh* was used as the reference gene to calculate relative fold difference using $(2)^{-\Delta\Delta Cq}$ method. Error bars represent SEM with n=6. The horizontal line at 1.0 represents the normalized level of granzymes for untreated (NT) rats. Error bars represent SEM with n=6. Asterisk (*) indicates significant difference from other treatment groups.

primary antibody was omitted (Figure 7B) is due to the autofluorescence generated from the red blood cells in the blood vessels.

Presence of apoptotic round spermatids in the epididymis. Since proteases are proposed to modulate testicular barriers and in this study GZMK expression was not localized to the nuclei of apoptotic cells, it was hypothesized that GZMK may regulate the junctional architecture of ectoplasmic specializations. Thus, an increased GZMK expression in EDS

treated rats would indicate perturbation of junctional complexes and premature release of spermatids from Sertoli cell attachments. Indeed, while apoptotic round spermatids were detected inside the lumen of the caput epididymis of EDS-treated rats (Figure 8C), few apoptotic cells were observed in caput epididymis of VEH, T, and EDS+T rats. The mRNA of transition protein 1 (*Tnp1*) is prevalent in round spermatids (Mali et al. 1989; Meistrich et al. 2003) and very low in mature spermatozoa and thus, mRNA for *Tnp1*

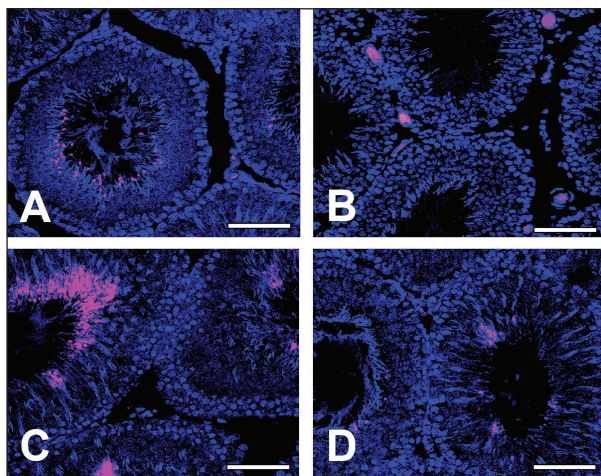


Figure 7. Immuno-localization of GZMK (red) in rat seminiferous tubule cross-sections of (A) VEH; (B) Negative control; (C) EDS; and (D) EDS+T. Polyclonal goat anti-GZMK (rat) was detected using DyLight 594-conjugated donkey anti-goat polyclonal antibody. Nuclei were stained with DAPI. Scale bars are 50 μ m.

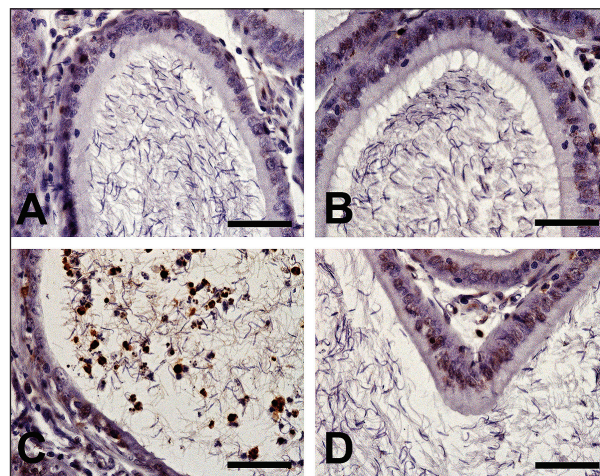


Figure 8. TUNEL-positive (apoptotic) cells (brown) in the caput epididymis of (A) VEH; (B) T; (C) EDS; and (D) EDS+T. Cross-sections were counterstained with hematoxylin for nuclear morphology identification. Scale bars are 50 μ m.

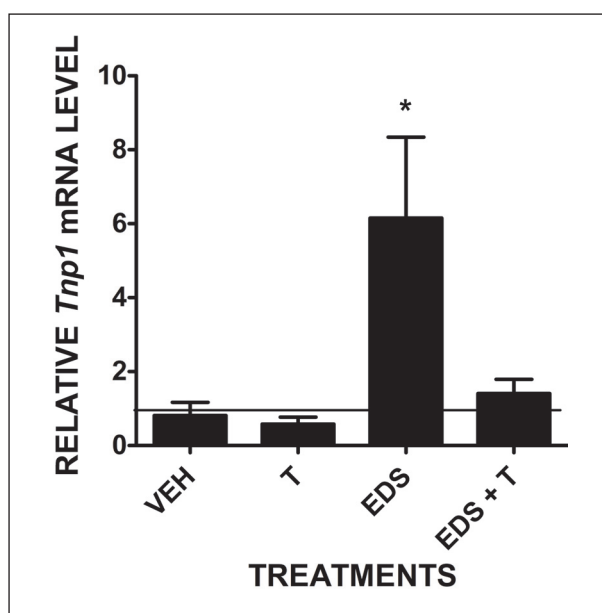


Figure 9. Relative level of Transition protein 1 (*Tnp1*) mRNA, a round spermatid-specific marker, in the testis after EDS treatment. *Gapdh* was used as the reference gene to calculate relative fold difference using $(2)^{-\Delta\Delta C_q}$ method. Error bars represent SEM with n=6. The horizontal line represents the value of *Tnp1* for untreated (NT) rats normalized to 1.0. Single asterisk (*) indicates significant difference from other treatment groups.

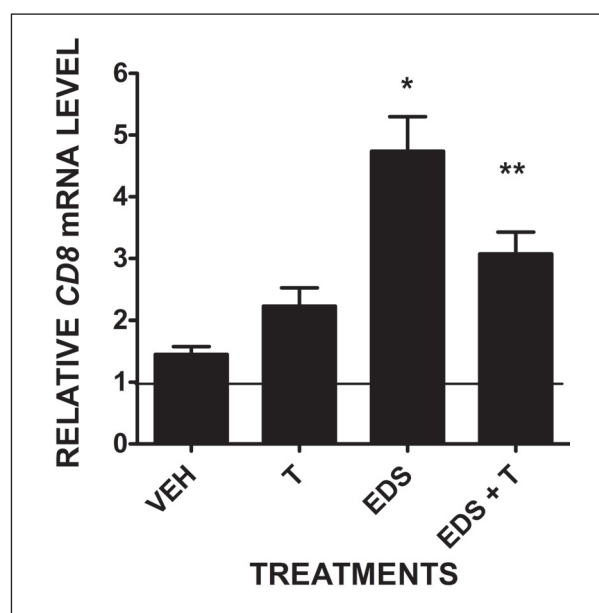


Figure 10. Relative level of Cluster of differentiation 8 (*CD8*) mRNA, a marker for cytotoxic T cells, in the testis. *Gapdh* was used as the reference gene to calculate relative fold difference using $(2)^{-\Delta\Delta C_q}$ method. Error bars represent SEM with n=6. The horizontal line represents the value of *Tnp1* for untreated (NT) rats normalized to 1.0. Single asterisk (*) indicates significant difference from other treatment groups. Double asterisk (**) indicates significant difference from VEH.

is negligible in normal epididymis. Therefore, the presence of round spermatids in the EDS-treated rat epididymis was confirmed by quantifying the levels of *Tnp1* in the epididymis. A significant (6-fold) in-

crease in *Tnp1* mRNA level was observed in the EDS-treated rat epididymis (Figure 9). In contrast, *Tnp1* in testosterone replaced (EDS+T) group was similar to the levels observed in controls. Thus, GZMK in testes

may have a role in maintaining ectoplasmic specialization during spermatogenesis.

Abundance and localization of CD8⁺ T cells.

Cytotoxic T cells (CD8⁺ T cells) generally release granzymes (Andersen et al. 2006). Therefore, it was hypothesized that the increased presence of GZMK in testosterone depleted testes may be due to the presence of CD8⁺ T cells inside the seminiferous tubule. The mRNA level of *CD8* was significantly higher (5-fold) in EDS-treated rat testes compared to controls, and testosterone replacement (EDS+T) partially reversed it (Figure 10). Although increased, the levels for *CD8* in VEH and T were not significantly different from the NT group. This observation is in agreement with an earlier report that CD8⁺ T cell population increases post-EDS treatment, and DMSO also influences proliferation of CD8⁺ T cells (Hedger et al. 1998). With immunohistochemistry, CD8 staining was detected only in the interstitium, and not inside the seminiferous tubules of the testis (Figure 11A, B), which is consistent with the fact that CD8⁺ T cells reside in the interstitium and do not cross the blood-testis barrier to enter the seminiferous tubule (Hedger et al. 1998). Therefore, CD8⁺ T cells may not contribute to the increased GMZK expression observed in the seminiferous tubule adluminal areas in EDS-treated rat testes.

Discussion

Here, we are showing the GZMK-mediated disruption of the testicular junctions and increased germ cell apoptosis following EDS-induced testosterone depletion in the rat testes. The loss of the *Insl3* in the testis post-EDS treatment confirms elimination of the mature Leydig cells, resulting in testosterone depletion to an undetectable level. With the loss of testosterone, there was a significant reduction in the testicular weight and a substantial increase in the germ cell apoptosis, consistent with the previous reports (Morris et al. 1997; Taylor et al. 1998; Nandi et al. 1999; Taylor et al. 1999; Woolveridge et al. 1999). Although the testosterone replacement had no effect on the *Insl3* level, the testicular weights and viability of germ cells were maintained at the control levels with replacement of testosterone (EDS+T). Interestingly, the level for *Insl3* was significantly lower in T compared to VEH. The expression of *Insl3* is directly regulated by luteinizing hormone (LH) in the testis (Ferlin et al. 2006). Therefore, exogenous supplemental testosterone treatment (T) resulted in an elevated circulating testosterone that may have down-regulated the mRNA expression of *Insl3* by the negative feedback

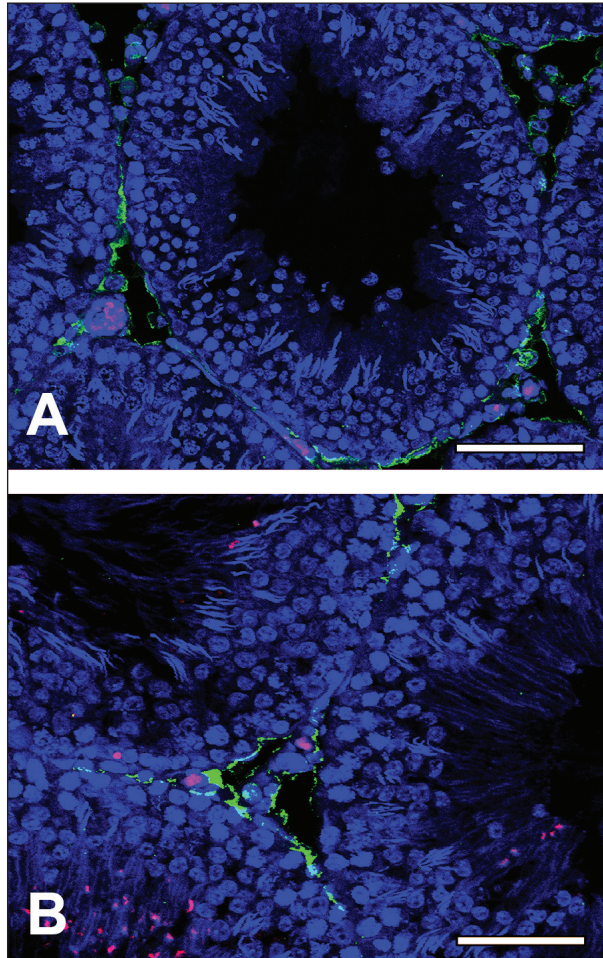


Figure 11. Immuno-localization of CD8 (green) and GZMK (red) in cross-sections of seminiferous tubules using confocal microscopy: (A) VEH; and (B) EDS. Polyclonal rabbit anti-CD8b (rat) was detected by DyLight 649-conjugated donkey anti-rabbit polyclonal antibody. Polyclonal goat anti-GZMK (rat) was detected by DyLight 594-conjugated donkey anti-goat polyclonal antibody. Nuclei were stained by DAPI. Scale bars are 50 μ m.

via the hypothalamic-pituitary-gonad axis for release of the LH from the anterior pituitary. Although normal germ cell viability and testicular weights were maintained with testosterone replacement after EDS, the testosterone did not have any protective effect on EDS-induced elimination of the Leydig cells.

Depletion of testosterone resulted in an increased germ cell apoptosis. Since, proteases play a significant role in apoptosis, the mRNA expression levels of multiple granzyme variants were evaluated in the testis. There are five identified granzyme variants in human, eleven in mouse, and seven in rat (Grossman et al. 2003). Among them, *Gzma*, *Gzmb* and *Gzmk* are common between human and rat. Since *Gzma*,

Gzmb, and *Gzmn* are present in testes (Hirst et al. 2001; Takano et al. 2004; Bhat et al. 2006), comparative analysis of mRNA expression levels for *Gzma*, *Gzmb*, *Gzmk*, and *Gzmn* was performed. The relative abundance of *Gzmn* was highest and *Gzmk* lowest in the normal testes. However, out of all the assayed granzyme variants, only *Gzmk* was responsive to testosterone. It is known that GZMB is expressed in the Sertoli and germ cells and is not associated with the germ cell apoptosis (Hirst et al. 2001). Similarly, GZMK may also be expressed in the Sertoli cells. In this study, GZMK was detected in association with the stripped cytoplasm of the elongated spermatids. Thus, GZMK may play a role in modifying the ectoplasmic specialization and movement of germ cells in the adluminal compartment. Alternately, GZMK may also initiate germ cell apoptosis and have a role in the degradation of the apoptotic bodies in testes. GZMK induce apoptosis independently of the caspase-mediated pathways through the nuclear morphological changes and single-strand DNA breaks (Bovenschen et al. 2009). Even though, GZMK staining was absent in the nuclei of the same germ cell stages in the seminiferous tubule that showed apoptosis after EDS treatment. The presence of TUNEL-positive (demonstrating DNA fragmentation) germ cells in the testes of EDS-treated rats suggest a possible role of GZMK in germ cell apoptosis.

Besides being present in CTLs and NK cells for apoptosis, GZMK is also expressed in other cell types that have non-apoptotic functions. Expression of *Gzmk* has been reported in the cerebral cortex, hippocampus, and diencephalon in mouse brain (Sue-moto et al. 1999), where it may process or degrade neuropeptides and β -amyloid precursor protein in the brain (Wiegand et al. 1993). Similarly, GZMK in testes may also have proteolytic activity to regulate translocation of spermatids.

During spermiation, spermatozoa disengage from the ectoplasmic specializations associated with Sertoli cells while progressively moving towards the lumen. In addition, the Sertoli cells selectively retain excess cytoplasm of spermatids as residual bodies as spermatozoa are released (Kerr et al. 2006). However, the exact mechanism of spermatozoa release from ectoplasmic specialization remain elusive. It has been reported that when spermatids begin to lose

their relationship to the ectoplasmic specialization, structures rich in filaments and microtubules, called tubulobule, starts to appear (Vogl et al. 2000). These structures are retained until the sperms are released (Kerr et al. 2006).

Tubulobular structures help in germ cell translocation and indirectly regulate the formation of the residual bodies by pulling the excess cytoplasm from the lumen to the apical trunk of the Sertoli cell (Kerr et al. 2006). Thus, tubulobular complexes are responsible for the internalization and disassembly of ectoplasmic specializations at the time of spermiation (Guttman et al. 2004). Testosterone is known to be essential in sustaining the Sertoli cell integrity (Mills 1990) and maintaining the ectoplasmic specializations (O'Donnell et al. 1994, 2006; Mruk and Cheng 2004; Wang et al. 2009; McCabe et al. 2010). Considering the above mentioning and results of this study, GZMK may play a crucial role in facilitating the degradation of tubulobular structures and translocation of spermatids. The tubulobular structures in testes are made by microtubules (Vogl et al. 2000), and GZMK, a tryptase, specifically targets of β -tubulin (Bovenschen et al. 2009). Moreover, microtubules are abundant in the Sertoli cell cytoplasm, are oriented in the direction of the spermatid translocation and surround the cisterns where spermatids remain embedded (Redenbach et al. 1992; Vogl et al. 2000). Thus, GZMK may modulate the structural rearrangement of ectoplasmic specializations and help in the timed movement and release of spermatids during spermiogenesis. Consistent with this hypothesis, detection of GZMK staining in association with the stripped cytoplasm of the elongated spermatids and overexpression of *Gzmk* in the absence of testosterone indicates a premature degradation of the tubulobular structures and ectoplasmic specializations. Perturbation of the integrity of the tubulobular structures may lead to the detachment and release of the round spermatids from the Sertoli cells. The round spermatids prematurely detached from their position in the adluminal compartment flush into the caput of the epididymis and undergo apoptosis. GZMK, in response to testosterone, may play a crucial role in modulating the ectoplasmic specialization in testes, and may indirectly regulate the germ cell apoptosis.

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