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Enhancement of germ cell apoptosis induced by ethanol in transgenic mice overexpressing Fas Ligand

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ABSTRACT

It was suggested that chronic ethanol exposure could result in testicular germ cell apoptosis, but the mechanism is still unclear. In the present study, we use a model of transgenic mice ubiquitously overexpressing human FasL to investigate whether Fas ligand plays a role in ethanol-induced testicular germ cell apoptosis. Both wild-type (WT) mice and transgenic (TG) mice were treated with acute ethanol (20% v/v) by intraperitoneal injection for five times. After ethanol injection, WT mice displayed up-regulation of Fas ligand in the testes, which was shown by FITC-conjugated flow cytometry and western blotting. Moreover, TG mice exhibited significantly more apoptotic germ cells than WT mice did after ethanol injection, which was demonstrated by DNA fragmentation, PI staining flow cytometry and TUNEL staining. In addition, histopathological examination revealed that degenerative changes of epithelial component of the tubules occurred in FasL overexpressing transgenic mice while testicular morphology was normal in wild-type mice after acute ethanol exposure, suggesting FasL expression determines the sensitivity of testes to ethanol in mice. In summary, we provide the direct evidences that Fas ligand mediates the apoptosis of testicular germ cells induced by acute ethanol using FasL transgenic mice.

Keywords: *Fas ligand, ethanol, apoptosis, testes, transgenic mouse.*

INTRODUCTION

Apoptosis of testicular germ cells is critical for spermatogensis in mammals[1,2]. It is believed that apoptosis plays an important role in the removal of overproduced, genetically abnormal, or accidentally damaged germ cells. Furthermore, increases in germ cell apoptosis are observed in laboratory animals after exposure to various testicular toxicants[3,4] as well as in human after testicular injury or under certain disease conditions[5,6].

Apoptosis is a rapid, self-destructive process without damaging surrounding tissues[7]. Morphologically, this process of cell death is characterized by condensation and fragmentation of cell chromatin followed by cellular budding into apoptotic bodies[8,9]. The mechanisms controlling spontaneous or toxicant-induced germ cell apoptosis are the current focus of many investigations. Recent researches indicated that Fas-Fas ligand system involved in the regulation of germ cell apoptosis both in rodent model[10] and in human testes[11]. Fas ligand (FasL/CD95L) is a 31 kD type II transmembrane protein of tumour necrosis factor (TNF) family that induces apoptosis through interaction with Fas, a process best

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studied in lymphoid cells[12,13]. Fas (CD95/APO-1) is a type I transmembrane protein belonging to the family of TNF/nerve growth factor (NGF) receptor and has a broader distribution in vivo. In the rodent testes, FasL is constitutively expressed in Sertoli cells and Fas is located in selected germ cells[10].

Ethanol is among the most widely abused drug in the world, and chronic alcohol abuse leads to testicular atrophy, feminization and infertility in alcoholic men[14]. Histological examination of these patients demonstrated a marked reduction in seminiferous tubular diameter accompanied with a loss of germ cells[14]. The mechanisms of this injury were previously studied. One is that ethanol metabolism produces an oxidative stress within the testes[15]; another is that ethanol exposure changes either testosterone[16] or esterone levels[17]. In addition, Zhu et al showed that ethanol exposure adversely affected the secretory function of Sertoli cells[18] and their recent research revealed enhanced apoptosis within the testes after chronic ethanol exposure[19]. However, it is still not clear how ethanol can lead to testicular injury. Recently, Zhu et al mentioned that the expression of Fas ligand and p53 within the testes were enhanced after chronic ethanol exposure[19], but no further evidences were provided.

We have previously generated a model of transgenic mice ubiquitously expressing human FasL[20]. It is well known that human FasL possesses 76.7% similarity in amino sequence with murine FasL. Furthermore, cross-interaction between human Fas/FasL and murine Fas/FasL induces apoptosis at the same efficiency[21]. In this study, we use this FasL overexpressing transgenic model to evaluate the functional importance of FasL in the regulation of germ cell apoptosis in the testes after acute ethanol exposure.

MATERIALS AND METHODS

Animals

The FasL overexpressed transgenic mice and wild-type littermates were developed as described[20]. Na ve adult mice aged 3-4 months and matched sex were used for all experiments. TG, as used throughout this manuscript, referred to homozygotes with FasL transgene (Tg2 in the reference 20), while WT (wild-type) referred to littermate mice without FasL transgene. Mice were group housed in plastic mouse cages (<4 mice per cage) with free access to standard rodent chow and water. The colony room was maintained at 22±2 ºC with a 12 h:12 h light:dark cycle. Each WT and TG mouse received a single dose of 20% (v/v) ethanol (5g/kg body weight) by intraperitoneal injection for five times while control mice received a same volume of saline. The animals were allowed to recover for 10~12 h between each injection. Then they were sacrificed and the testes were removed three hours after the last injection. At least three mice per experimental group were used. All the experimental protocols were performed in compliance with the National Institutes of Health guidelines for the care and use of animals and were approved by the local Animal Care and Use Committee.

Western blot analysis

Animals were sacrificed by cervical dislocation and testes were removed. Each tissue sample was homogenized in approximately 20 volumes of buffer using tissue protein extraction kit (Pierce) and cleared by centrifugation at 10000rpm for 5 min to produce a protein extract. Then the protein content of the preparation was determined with BCA kit (Pierce), using BSA as a standard. Equal concentrations of each sample (50 mg protein) were mixed with the loading buffer, boiled at 100 ºC for 5 min, separated by SDS-PAGE with a 10% gel gradient and then electrophoretically transferred onto nitrocellulose membranes. In staining procedure, the membrane was blocked for 1 h in TBST with 5% milk, and incubated with 1:100 rabbit anti-FasL (Santa Cruz, CA) at 4 ºC overnight and subsequently exposed to horseradish peroxidase- conjugated anti-rabbit IgG goat polyclonal antibody (Santa Cruz, CA). Between each step, the membrane was washed with TBST 10 minutes for three times. Bands were visualized with DAB according to the manufacturer's script.

Flow cytometry analysis

Single-cell suspensions of testicular cells were prepared by mechanical isolation and subsequently filtrated through a hole density-optimized nylon fiber. Samples were treated with 0.168 M NH4Cl to lyse erythrocytes and washed with 200 ml PBS for two times. Then cells were blocked with 1% BSA in PBS for 20 min on ice, incubated with 1:100 rabbit anti-FasL (Santa Cruz, CA) or with rabbit serum as background control for 1 h. Cells were washed with PBS for three times and subsequently exposed to FITC-conjugated anti-rabbit IgG goat polyclonal antibody (Santa Cruz, CA) in dark for 1 h. After three washes in PBS, samples were analyzed with a FACScan (Becton Dickinson, CA) to determine the expression of FasL. And data were processed in a Hewlettpackard (Palo Alto, CA) computer.

In PI-staining flow cytometry procedure, cells were stained with 100 ml 50 mg/ml propidium iodide (PI) in 300 ml PBS (1 h on ice in the dark), and then were measured by FACS as described above for relative PI fluorescence (FL-2).

Low-molecular-weight DNA isolation

Testes were decapsulated and mashed with a tip. Tissue was lysed by incubating at 53 ºC for one day with 0.2 mg/ml proteinase K in 500 μl of 100 m*M* Tris-HCl pH 8.0, 0.1 M EDTA and 0.5% SDS. And the supernatant was extracted with an equal volume of phenol for two times. The aqueous phase was ethanol precipitated for 2 h at -70 ºC , pelleted, and resulspended in TE buffer. Finally the resulting DNA was digested with DNase-free RNase. A total of 30 mg DNA was loaded on a 2% agarose gel and separated by electrophoresis. DNA was stained with ethidium bromide and visualized with anultraviolet transilluminator.

In situ TUNEL staining and quantitation

Testes were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer. Tissues were removed and dehydrated through up graded thanol, and then cleared with xylene, finally embedded in paraffin. 7 mm thick-sections were cut and TUNEL was performed using an ApopTag kit (Intergen). Tissues were counterstained with haematoxylin, then examined and photographed under Leica microscope. To quantitate the incidence of apoptosis, the percentage of seminiferous tubules containing three or more apoptotic cells of the total number of seminiferous tubules was calculated.

Histopathology

7 μ m thick paraffin-sections were prepared as described above and stained with haematoxylin and eosin according to the standard rotocol, then examined and photographed under Leica microscope.

RESULTS

Up-regulation of FasL in testes after ethanol exposure

Firstly, we examined the expression of FasL in the testes at protein level after ethanol exposure. FITC-conjugated flow cytometry analysis showed that after the subtraction of the background control, the percentage of FasL-positive cells was 6.47% in testes of saline-injected WT control mice, while that was 10.72% in testes of ethanol-injected WT mice (Fig 1A). Therefore, FasL-positive cells were increased by 4.25% in mouse testes induced by ethanol. In addition, western blotting analysis revealed that the band of 31 kD FasL was very weak in the saline treated mouse testis, while a very clear single band of FasL was detected after ethanol exposure (Fig 1B). These results suggested the expression of Fas ligand protein in testes was enhanced after acute ethanol exposure. We also measured the exoression in TG mice, and a higher intensity of FasL was observed (Fig 1B), which indicated that FasL is overexpressed in testes of transgenic mice as we previously reported[20].

Increasement of germ cell apoptosis in transgenic mice after ethanol exposure

As an indirect measure of endonuclease activity and an initial conformation of ethanol-induced apoptosis, DNA fragmentation was assessed by gel electrophoresis. No visible ladders were observed in testes of saline-treated control mice when DNA samples were separated by 2% agarose gel electrophoresis, but they were detectedin

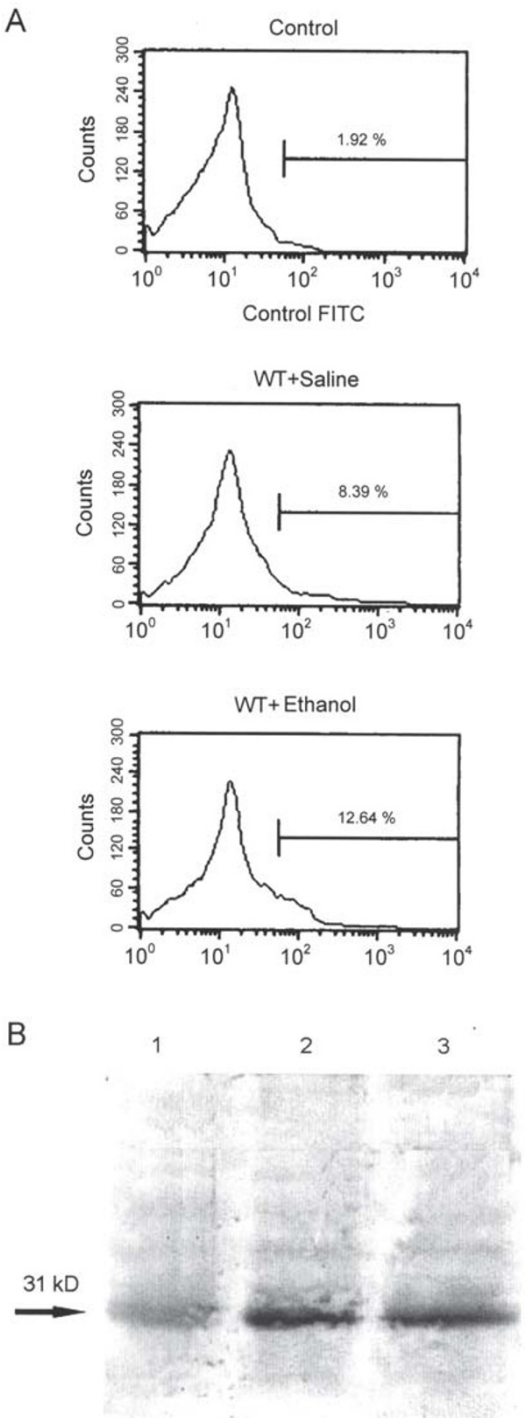


Fig 1. The expression of FasL protein in the testes after ethanol exposure. (**A**) Flow cytometry analysis showed that the percentage of FasL-positive cells in the testes was increased after ethanol exposure. n=3 for each group, $p<0.05$. (**B**) Western blot analysis showed that FasL protein was increased in the testes after ethanol exposure. Lane 1: Wild-type mice with saline injection as control, Lane 2: Wild-type mice after ethanol exposure, Lane 3: Transgenic mice, n=3 for each group.

testes of both WT and TG mice treated with acute ethanol (Fig 2). However, the intensity of the laddering bands in transgenic mice was significantly greater than that in wild-type mice (Fig 2).

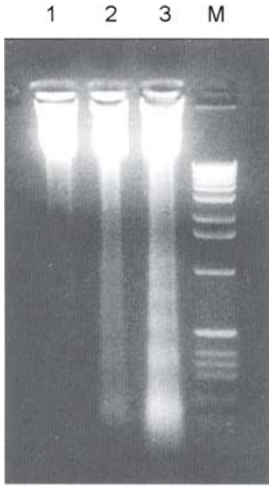


Fig 2. Electrophoretic analysis of DNA extracted from testes after ethanol injection; 30 mg of DNA was applied to each lane. Lane 1: Wild-type mice with saline injection as control, Lane 2: Wild-type mice after ethanol exposure, Lane 3: Transgenic mice after ethanol exposure, n=3 for each group.

Determination of the relative DNA content of apoptotic nuclei (which is hypodiptoid due to loss of fragments) by PI staining and cytofluorimentric analysis is a more sensitive way to demonstrate apoptotic cell death[22]. As shown in Fig 3, ethanol-treated WT mice displayed increased apoptotic cells compared with saline-treated WT mice. Transgenic mice also showed slightly more apoptotic cells than their wild-type littermates, which may underlie the slight alteration of testis morphology in transgenic mice as we previously observed[20]. However, the percentage of apoptotic cells in TG mice (15.72%) was significantly greater than that in WT mice (7.43%) after ethanol exposure.

Apoptotic germ cells could be labeled using TUNEL method. Within the testes of saline-treated WT mice, only a few TUNEL-positive cells were observed (Fig 4A). However, TG mice treated with saline showed a slight but significant augmentation of TUNEL-positive cells compared with saline-treated WT mice (Fig 4B), which is coherent with PI staining study.

After ethanol injection, more cells undergoing apoptosis were detected in both WT and TG mice, but TG mice showed a strongly more significant apoptosis (Fig 4C,4D). For statistical analysis, a scoring system based on the percentage of seminiferous with high (>=3 events per seminiferous tubules across section) number of cells undergoing apoptosis was used. Because in saline-treated WT mice testes, the percentage of tubules with more than 3 TUNEL-positive cells is less than 5%, an increase in apoptosis is easily determined using this counting approach. TUNEL-stained multinucleated cells were counted as a single event. The result of statistical analysis was shown in Fig 4E. TG mice testes displayed a significant augmentation of apoptosis than those of WT mice after ethanol exposure, which suggested that FasL played an important role in apoptosis of germ cells induced by ethanol.

Histopathology

To examine the morphological appearance of seminiferous tubules following ethanol exposure, histopathology was evaluated in 7 mm cross section of paraffin-embedded testes stained with haematoxylin and eosin. The seminiferous epithelium appeared similar in both saline-treated WT and TG mice. However, in local region of transgenic mice, vacuolated spermatogenic epithelium of the seminiferous tubules with expanded lumen in the testis was observed, as we previously studied

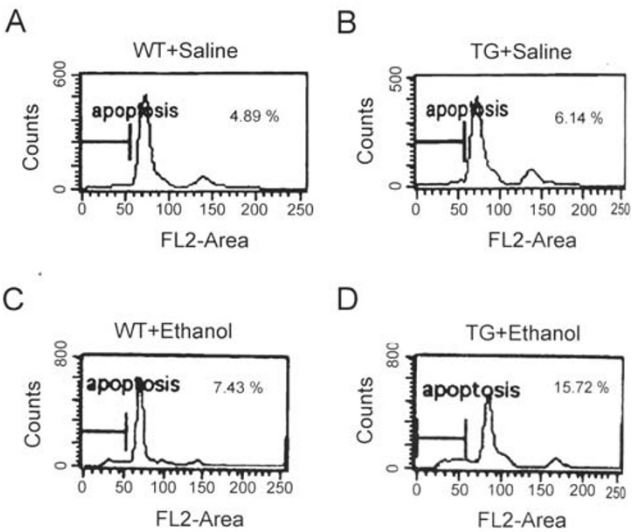
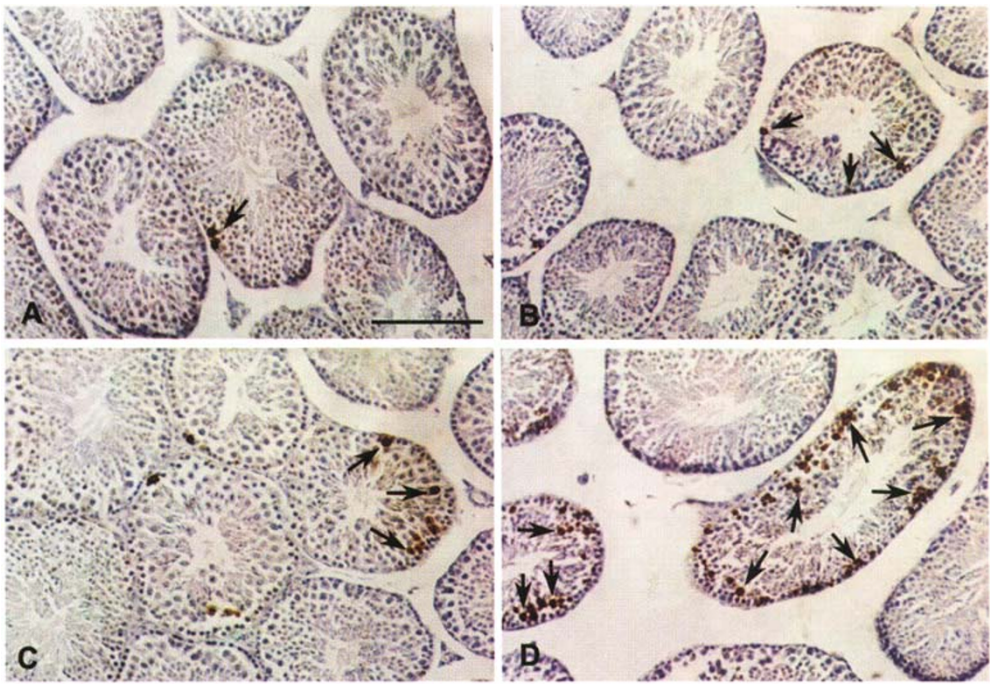


Fig 3. Flow cytometry analysis of apoptosis in the testes of wild-type and transgenic mice after ethanol exposure by PI staining. The percentage of apoptotic cells in the total testes cells was indicated by the cursor. n=6, mice were individually analyzed in two separate experiments for each group ($p<0.05$).



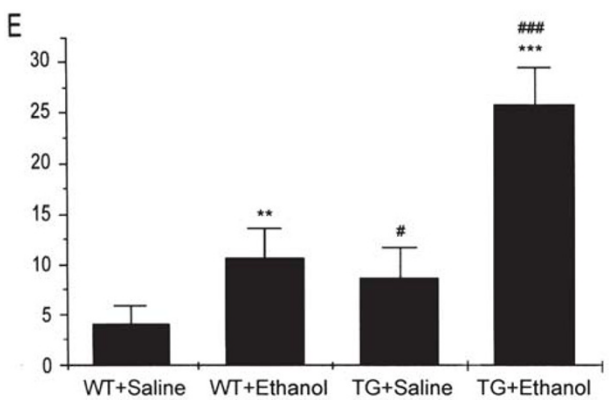


Fig 4. Representative photomicrographs of TUNEL-stained testis cross sections from control and ethanol-exposed mice. Occasional cells within the seminiferous epithelium of control WT mice (**A**) were TUNEL stained (Arrows). And TG mice (**B**) displayed a slight but significant augmentation of TUNEL-positive cells. After ethanol exposure, TG mice showed significantly more apoptotic cells (**D**) compared with WT mice (**C**). Quantitation of apoptosis in situ after ethanol exposure was shown in (**E**). Only a high level (>3 events per seminiferous tubule cross section) of apoptosis was counted. Values are presented as means±s.e.m. **p<0.01, versus WT+Saline; ***p <0.001, versus TG+Saline. #p<0.05, versus WT+Saline; ###p<0. 001, versus WT+Ethanol; n=5 for each group, bar=100 μm.

[20] (Fig 5A,5B). After acute ethanol exposure, WT mice showed normal testicular morphology (Fig 5C), whereas TG mice displayed a severe injury of spermatogonia and spermatocytes. In some region, degenerative changes of epithelial component of the tubules occurred (Fig 5D).

DISCUSSION

It was demonstrated that Bcl-2 family[23] and p53 [24] were important in the regulation of germ cell apoptosis. Fas system was also supposed to be a key regulator of germ cell apoptosis in testis[10] and up-regulation of Fas ligand was reported in many toxicant-induced testicular germ cell apoptosis[25,26]. In addition, numerous studies showed that apoptosis was an important mechanism by which ethanol caused tissue injury[27, 28]. Recently, Zhu et al firstly showednumerous studies showed that apoptosis was an important mechanism by which ethanol caused tissue injury that chronic ethanol exposure enhanced apoptosis of testicular germ cells and measured expression of some apoptosis-related proteins such as Bcl-2, p53 and Fas ligand[19]. During the time of writing this paper, Eid et al suggested that Fas system might involve in germ cell apoptosis in rats treated with chronic ethanol for 12 weeks[29]. However, till now no direct evidences are provided that FasL leads to ethanol-induced testicular germ cell apoptosis. In this paper, we demonstrated that induction of testicular germ cell apoptosis after acute ethanol exposure was associated with increased Fas ligand using the model of

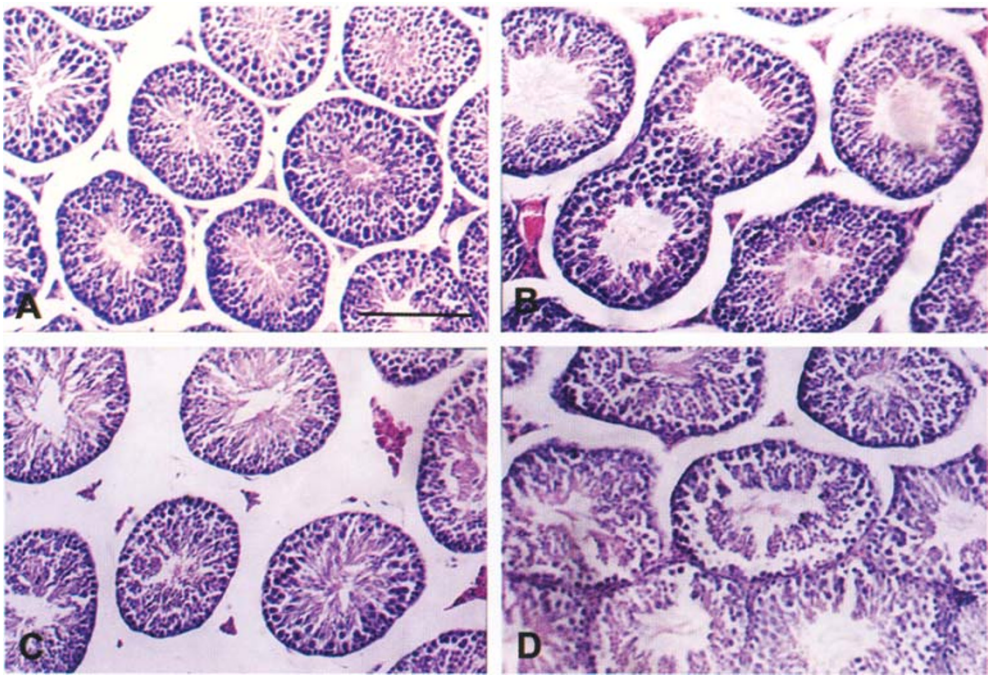


Fig 5. Testicular histopathology induced by ethanol. Note expanded lumen in TG mice, and degenerative changes of epithelial component of the tubules in TG mice compared with wild-type mice after ethanol exposure. (A) WT+Saline, (B) TG+Saline, (C) WT+Ethanol, (D) TG+Ethanol, n=5 for each group, bar=100 μ m.

trantransgenic mice. Furthermore, we observed a severe injury in spermatocyte in some region and degenerative changes of epithelial component of the tubules in transgenic mouse testes after acute ethanol exposure. It means that the background expression of FasL has direct correlation with the sensitivity of testes to ethanol and indicates that the expression level of FasL could be used as an important factor to evaluate the risk of tissue damage by ethanol.This result also could be used to explain (at least in part) the different sensitivity to ethanol-induced tissue damage existed in human subpopulations.

It is noticeable that in local region of FasL-overexpressing transgenic mice, slight vacuolated spermatogenic epithelium of seminiferous tubules with expanded lumen in the testes was observed using haematoxylin and eosin staining (Fig 5), which was also found by our group previously[20]. We assumed that this damage possibly resulted from enhanced apoptosis as a consequence of human FasL expression, and this assumption was demonstrated in the present study. PI staining and TUNEL staining showed that transgenic mice displayed slightly but significantly more apoptotic cells compared with WT mice (Fig 3A,3B,4A,4B), although no visible DNA fragmentations were seen in saline-treated TG mice (Data not shown).

Taken together, we directly demonstrated that Fas ligand participated in the apoptosis of testicular germ cells after acute ethanol exposure using FasL overexpressing transgenic mice.

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