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수컷 랫드에서 정관절제술에 의한 생식세포의 Apoptosis

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Apoptosis of Germ Cells after Vasectomy in Rats

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Abstract: The pathological mechanism of impaired spermatogenesis after vasectomy has not been completely investigated. In this study, we examined pathological changes of the testis and the Fas-Fas ligand (FasL) mediated signaling pathway in apoptotic germ cell death after vasectomy in rats. Ten-weeks old Sprague-Dawley rats were underwent bilateral vasectomy and sacrificed after 1 day, 2 days, 3 days, 5 days, 1 week, 2 weeks, and 4 weeks of surgery and the testes were removed. Histopathological evaluation of spermatogenesis was performed by hematoxylin-eosin and periodic acid-Schiff-hematoxylin staining. To elucidate the pathophysiology of seminiferous tubule damage, terminal dUTP nick end labeling staining, electrophoresis assay of DNA fragmentation, and Western blotting analysis for Fas-FasL were performed. Relative weights of testes were decreased from 5 days after vasectomy. Germ cell degeneration were first found in the spermatogonia and spermatocytes at stages I-VI, and XII-XIV seminiferous tubules. Mean incidence of apoptotic germ cells after vasectomy progressively increased to peak in 5 days, and then gradually decreased to the control levels in 2 weeks after vasectomy. The expression of Fas-FasL reached maximum level at 5 days after vasectomy and then declined. In conclusion, impaired spermatogenesis after vasectomy associated with an increase in germ cell apoptosis, which is partly mediated by the activation of Fas-FasL.

Key words: vasectomy, spermatogenesis, apoptosis, Fas-Fas ligand

Introduction

During normal spermatogenesis, a number of spermatogenic germ cells die by apoptosis before reaching maturity [5, 6] in a variety of mammalian species [2, 17]. In addition, massive testicular germ cell loss is known to result from cryptorchidism [44], ischemia-repurfusion [38], radiation [10], hypophysectomy [36], treatment with gonadotropin-releasing hormone antagonist [14], heating [1] and drug treatment [4, 27, 29]. In many of these situations, germ cell death occurs via apoptosis [2]. Vasectomy is widely performed for male contraception and result in damage to spermatogenesis [20]. Recently, animal experiments indicate that apoptosis has a key role in the deterioration of spermatogenesis after vasectomy [38].

Fas is a transmembranous glycoprotein involved in the transduction of death signals through its binding to Fas lignad (FasL) in the immune system and other organs [9,

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26]. Recently, many reports has examined the possible involvement of the Fas-FasL system in the induction of apoptosis in various experimental systems, including rat ovary [11], liver [13], and thyroid [43], ischemia-reperfusion mouse kidney [28], and lipopolysaccharide-treated placenta of pregnant mice [8], as well as in human materials, such as osteoblast [16], ulcerative colitis [15], and colorectal cancer [30]. In the testis, several reports have shown up-regulation of testicular Fas gene expression in correlation to germ cell apoptosis caused by testosterone withdrawal [27], radiation [22] and Sertoli cell toxicants [23]. In addition, *in vitro* studies have shown that testicular germ cells are susceptible to Fas antibody-induced apoptosis, and that survival of germ cells is improved by inhibiting FasL activities with antagonistic FasL antibody [31] and antisense oligonucleotides [23]. However Fas-FasL system associated with apoptosis after vasectomy is unclear, although iNOS, NF kappa B [21], p53-Bax pathway [38] and mitogen activated protein kinases (MAPKs) [39] are associated with the apoptosis of germ cells after vasectomy in the rat.

Therefore, this study was performed to investigate the stage specific germ cell degeneration and the involvement of apoptosis in testicular atrophy induced by vasectomy. In addition, the possible involvement of the Fas-FasL system in the induction of apoptosis was examined.

Materials and Methods

Animals and Vasectomy

Nine-week old male Sprague-Dawley rats were used after 1-week of acclimation. Rats were randomized by body weight and housed 5 per cage with filter tops at 23±1℃, 55±5% of humidity and a 12 hr light/ 12 hr dark cycle, and given rodent chow and water *ad libitum*. All the testes looked normal in size and were located in the scrotal sac at the time of surgery.

Vasectomy was performed on a lower abdominal midline incision under the ether anesthesia. The bilateral testes and spermatic cord were exposed and the vas deferens were dissected, and cauterized at the middle of the vas deferens. Groups of five bilaterally vasectomized animals were sacrificed at intervals of 1 day, 2 days, 3 days, 5 days, 1 week, 2 weeks, and 4 weeks after surgery and the testes and epididymis were removed immediately and weighed.

Sample preparation

Right testes were decapsulated, and stored frozen at -70℃ until used for DNA agarose gel electrophoresis and Western blot assay. Left testes were fixed in Bouin's solution, after dehydration with ethanol, they were embedded in paraffin.

Histopathological examination

For light microscopy, serial paraffin sections of testis were processed for hematoxylin-eosin (H-E) and periodic acid-Schiff-hematoxylin (PAS) stain. Seminiferous tubules were devided into four groups can be identified by H-E stain, stages I-VI, VII-VIII, IX-XI, and XII-XIV, and germ cell types were identified by H-E and PAS stain according to the description by Russell *et al* [35].

TUNEL assesment

A serial section of testes was processed *in situ* terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphate biotin nick end labeling (TUNEL) stain using an ApopTag-peroxidase Kit (Intergen Co., NY, USA). Sections were counterstained with hematoxylin. TUNEL-labeled cells were identified by their brownish stain.

A total 40 seminiferous tubules (10 seminiferous tubules per stage) on circular cross sections in each testis were randomly chosen and evaluated in 1, 2, 3 days after vasectomy. But seminiferous tubules staging is difficult to identify from 5 days after vasectomy due to severe depletion of germ cells, since a total 40 seminiferous tubules on circular cross sections in each testis were randomly chosen and evaluated. The number of TUNEL positive nuclei per tubule was counted and expressed as the mean±standard division (SD) per group.

DNA laddering

Pooled testis tissue (250 mg) from each groups was homogenized in 2 ml lysis buffer (10 mM Tris-HCl, 100 nM EDTA, and 0.5% SDS). 200 μl Proteinase K (200 μg/ml) was added and digested at 56℃ for 2 hours. After addition of an equal volume of phenol, the preparations were mixed well by gently inverting the tube for 5 min, and centrifuged at 14,000 rpm at room temperature for 20 min. Slowly transfer the top, viscous, aqueous phase to a fresh tube with a wide-bore pipette. Repeat phenol extraction two to three times in order to maximally remove proteins

and cellular debris. The resultant solution was added 0.5 volume of 3 M sodium acetate and 2 to 2.5 volumes of 100% chilled ethanol and mixed gently. Carefully fish out the DNA pellet into fresh tube with a glass hook and briefly and gently rinse the DNA with 70% ethanol and dry the sample for 15 to 30 min. The pellet was resuspended the DNA in an appropriate amount of TE buffer (10 mM Tris-HCl, 1 mM EDTA). DNA (3 μg/10 μl) was loaded onto a 1.5% agarose gel and separated by electrophoresis. DNA was stained with ethidium bromide and visualized with an ultraviolet transilluminator. A 100 bp DNA ladder (Promega, Madison WI, USA) was used in electrophoresis as a size marker.

Immunoblotting analysis

The frozen testes were homogenized, as previously described method [18]. Immunoblotting was performed using antibody to Fas and FasL (Santa Cruz Biotechnology, CA, USA). Equal amounts of protein (10 μg/well) were applied to the gels, electrophoresed and blotted polyvinylidine difluoride (PVDF)-plus membranes (Osmonic, Westborough, MA). PVDF-plus membranes were incubated with 5% non-fat milk in TTBS (20 mM Tris, 0.5 M NaCl, 0.2 % Tween 20, pH 7.4) for 1 hr at room temperature. The membranes were then incubated at 4℃, overnight with a 1:1,000 dilution of rat antisera raised against Fas and FasL, and then washed in TTBS, incubated for 1.5 hr with a 1:1,000 dilution of rabbit anti-rat IgG conjugated with alkaline phosphatase (Boehringer Mannheim, GmbH, Germany). After washing with TTBS, immunoreactive bands on the membranes were developed with NBT/BCIP substrate (Boehringer Mannheim, Mannheim, Germany). The relative density of specific immunoreactive band was determined by scanning with laser computing densitometer using ImageQuant program (version 3.3, Molecular Dynamics, Sunnyvale, CA).

Statistical analysis

The results of testiculr weight and quantitative analysis of TUNEL-labeled germ cells in seminiferous tubules are expressed as mean±SD. Differences between groups were examined for statistical significance using Student's *t*-test at the level of $p<0.05$ and $p<0.01$.

Results

Relative weight of testes was significantly decreased in 5 days, 1 week, 2 weeks and 4 weeks after vasectomy compared with those of control group (Fig. 1).

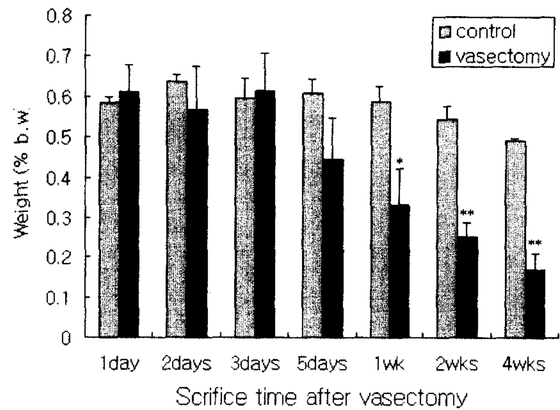

Fig. 1. Relative testicular weight (testicular weights (g)/ body weights (g) × 100) after vasectomy. Data are expressed as mean±SD. (*$p<0.05$, **$p<0.01$).

Histopathologically, testes after vasectomy showed various degrees of seminiferous tubular degeneration, including intraepithelial vesicle formation and loss or degeneration of germ cells. Germ cell degeneration was first found in spermatogonia and spermatocytes (Fig. 2A) at the stages I-VI, and XII-XIV seminiferous tubules in 1 day after vasectomy. Depletion of the spermatocytes was notable with the intraepithelial vacuolation of I-VI stage tubules in 3 days after vasectomy (Fig. 2B). The denaturation of germ cells was particularly evident at the stages I-VI rather than the other stages. In addition, marginal condensation of chromatin in round spermatids and multinucleated giant cells was noted (Fig. 2C). The testicular damage had markedly progressed by day 5 with severe depletion and exfoliation of germ cells and atrophy of overall seminiferous tubules (Fig. 2D). At 1 weeks after vasectomy, seminiferous epithelial cycle was completely disrupted.

Quantitative data for TUNEL-labeled germ cells are shown in Figs. 3 and 4. Preferentially TUNEL-labeled germ cells were spermatogonia and spermatocytes in control and vasectomized rats. In the testes from the control rats, a few TUNEL-labeled germ cells were found in the stages I-VI and XII-XIV seminiferous tubules (Fig. 5A). Mean incidence of apoptotic germ cells after vasectomy increased in a stage-specific and time-dependent manner. As shown in Fig. 4, the total number of TUNEL-labeled germ cells

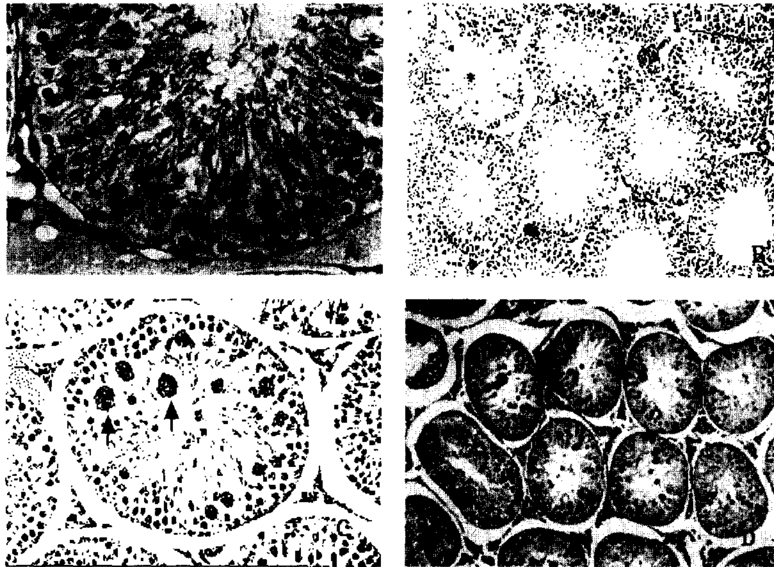

Fig. 2. Histopathological lesions of testis Stained with H-E. A, seminiferous tubule at stage I-VI in 1 day after vasectomy, showing degenerating germ cells (arrow heads), ×400. B, seminiferous tubules in 3 days after vasectomy, showing depletion of the spermatocytes with the intraepithelial vacuolation (*), ×100. C, seminiferous tubule in 3 days after vasectomy, showing multinucleated giant cells (arrows), ×200. D, seminiferous tubules in 5 days after vasectomy, showing severe depletion and exfoliation of germ cells and atrophy of overall seminiferous tubules, ×100.

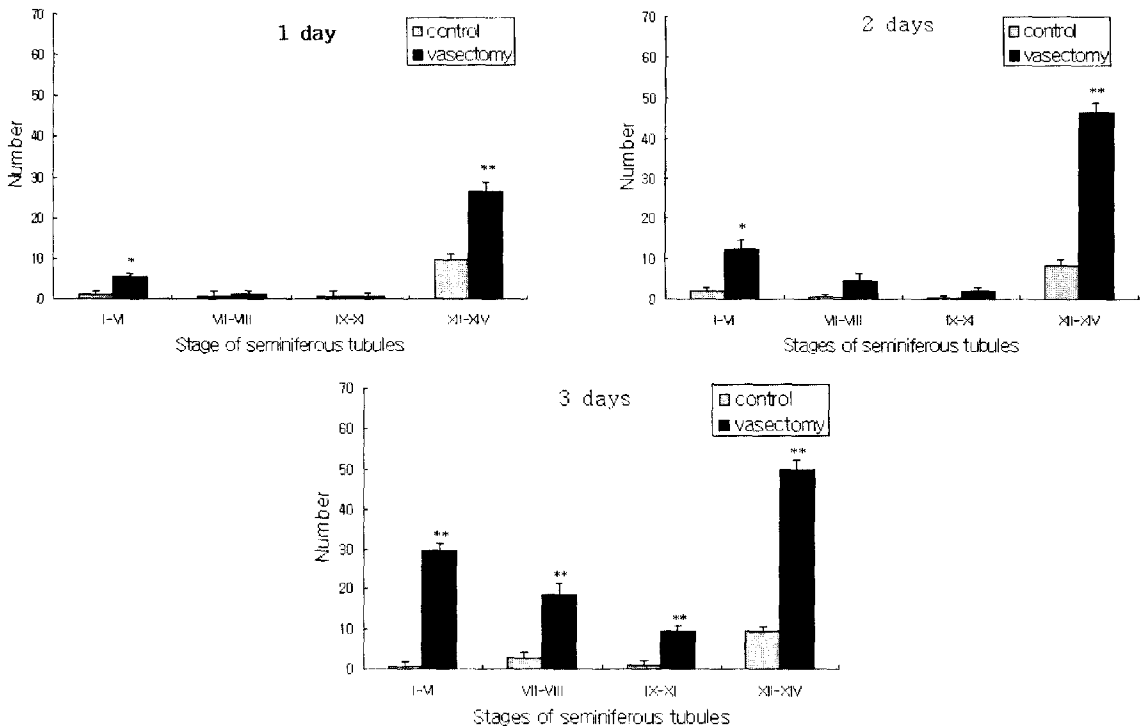

Fig. 3. Stage-specific quantification of TUNEL-labelled germ cells per 40 seminiferous tubules in each time point. Values are mean±SD. Asterisks on the bars mean significant difference from control group value (*$p<0.05$, **$p<0.01$).

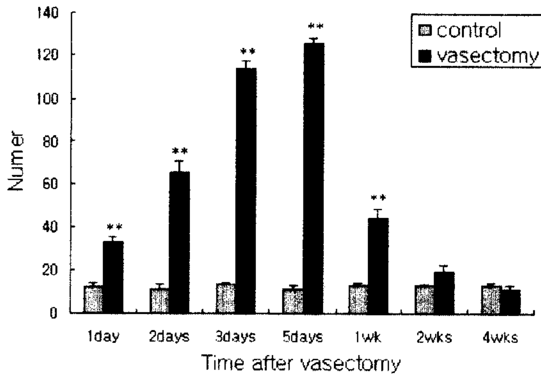

Fig. 4. Total quantification of TUNEL-labeled germ cells per 40 seminiferous tubules in each time point. Values are mean ± SD. **Significant difference from control group value at p<0.01.

increased progressively to peak by 5 days, and then gradually decreased to the same level of control by 2 weeks after vasectomy. Significantly increased incidence of apoptosis at the stages I-VI and XII-XIV seminiferous tubules over control values was noted by day 1. In 3 days after vasectomy, apoptotic cells were also noted at the stages VII-VIII and IX-XI seminiferous tubules. During the study, the highest number of apoptotic cells occurred by 5 days after vasectomy (Fig. 5B). In 1 week after vasectomy, seminiferous tubules still exhibited the higher number of cells undergoing apoptosis, and then gradually decreased within normal level at the end of study.

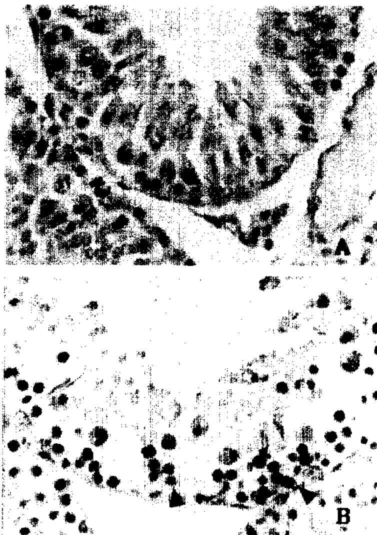

Fig. 5. Representative photomicrograph of seminiferous tubules stained with TUNEL. A, control seminiferous tubule at stages XII-XIV, a few TUNEL-labeled germ cells are noted, ×400. B, seminiferous tubule at stages I-VI in 5 days after vasectomy, arrow heads showing significantly increased apoptotic germ cells. ×400.

In analysis of testicular apoptotic DNA fragmentation, DNA ladders were apparent after 3 days of vasectomy (Fig. 6). Apoptotic DNA fragmentation of germ cells fully corroborated the observed increase in the degree of time-elated apoptosis of germ cells.

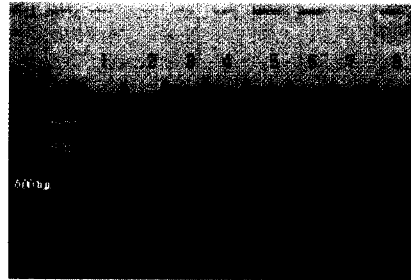

Fig. 6. Agarose gel electrophoresis of testicular 180-200 bp nucleosomal fragments DNA isolated from control and vasectomized rats. Lane 1, 2, 3, 4, 5, 6, 7 and 8 represents the control, 1 day, 2 days, 3 days, 5 days, 1 week, 2 weeks, 4 weeks respectively after vasectomy.

Analysis of Fas-FasL expression revealed a detectable increase at day 3 and a maximal increase at 5 days after vasectomy (Fig. 7) and then gradually decreased, but the level was higher than that of control even at the end of the study. Fas-FasL expression are correlated with the observed increase in the degree of germ cell apoptosis with time.

Discussion

This study provides the evidence that impairment of spermatogenesis after vasectomy occur via apoptosis, in which the Fas-FasL system is involved. The TUNEL study demonstrated the presence of a few apoptotic cells in the control testes. This is in agreement with previous reports [32, 33]. And a significant increase of apoptotic germ cells was observed in the testes after vasectomy, which is consistent with the results of Kubota *et al* [21], and Shiraishi *et al* [38, 39]. These TUNEL findings were confirmed by electrophoresis assay of DNA fragmentation and observation of giant cells, a marker of enhanced germ cell degeneration [44].

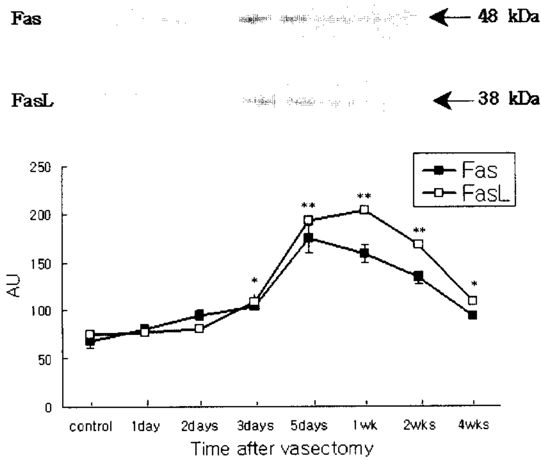

Fig. 7. Effect of vasectomy on Fas and FasL expression. The amounts of Fas and FasL expression were determined by immunoblot analysis and densitometry (lower graph). AU is arbitrary densitometric units (* $p<0.05$, ** $p<0.01$).

In stage specificity analysis of apoptosis, the most sensitive spermatogenic cells were spermatogonia and spermatocytes of seminiferous epithelium stages I-VI and XII-XIV. This result is in line with previous study that vasectomy induced germ cell apoptosis at stages XIII-I seminiferous tubules in the hamster [24]. The underlying mechanism remains as to why germ cell at these stages are specifically targeted for apoptosis. Germ cells are most vulnerable to apoptotic stimuli at the phases of meiosis and spermatogenesis based on the observation that germ cells undergo apoptosis most frequently at stages VII to VIII [40]. Indeed, the earliest morphological signs of germ cell degeneration involving preleptotene and pachytene spermatocytes, step 7 spermatids (at stage VII), and step 19 spermatids (at both stages VII and VIII) were detected 5 days after the commencement of GnRH-A treatment [14]. However, treatment with cyclophosphamide increased apoptosis, predominantly in spermatogonia and spermatocytes at stages I-VI and XI-XIV [4]. After short-term (24 hr and 48 hr) experimental cryptorchidism in rats, a significant increase in number of apoptotic germ cells was evident in all stages, except for VI and VII [12]. In the testes reperfused after 1 hr of ischemia, a high number of TUNEL-positive cells were identified in all stage, except at stages XI and XII [19]. After hypophysectomy the incidence of degenerating spermatocytes, and spermatids which are present in stages VII or VIII seminiferous tubules [36]. Thus, the cell- and stage-specific induction of apoptosis is stimulus-specific.

In the testes, p53 [41], bcl-2/bcl-xL/bax [34] and Fas-FasL system [42] have been shown to be present. Such an expression pattern would provide and efficient way for regulating germ cell apoptosis [7]. Previously vasectomy increased p53 [18] and phospho-p38 [19] immunoreactivity in primary spermatocytes. In the present study, vasectomy increased in the amount of Fas-FasL protein. Fas is expressed in germ cells and FasL is detected in Sertoli cells [25]. Fas-FasL system has been identified as a key regulator of Sertoli cell-directed germ cell apoptosis in response to specific stimuli [23]. On exposure to specific Sertoli cell toxicants, injured Sertoli cells overexpress FasL which translocates and diffuses towards the apical side of seminiferous tubules, secondarily inducing apopotic deletion of Fas-expressing germ cells [3]. Vasectomy induces hitopathological and physiological changes in the Sertoli cells [37]. Therefore, vasectomy may not only primarily up-regulate Fas in damaged germ cells, but also enhance FasL expression in concomitantly injured Sertoli cells resulting in secondary germ cell apoptosis.

In conclusion, vasectomy induces testicular germ cell apoptosis in a time-dependent and stage-specific pattern, and spermatogonia and early spermatocytes at stages I-VI, XII-XIV were the main target cells. And enhanced apoptosis of germ cells in the testes after vasectomy is associated with up-regulation of the Fas-FasL signal pathway.

Acknowledgements

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References

1. **Allan, D., Harmon, B. and Kerr, J.** Cell death in spermatogenesis. *In* Perspectives on Mammalian Cell Death. Potten, C. (ed), pp. 229-258. Oxford University Press, London, 1987.
2. **Blanco-Rodriguez, J. and Martinez-Garcia, C.** Spontaneous germ cell death in the testis of the adult rat takes the form of apoptosis: re-evaluation of cell types that exhibit the ability to die during spermatogenesis. Cell Prolif. 1996, **29**, 13-31.

3. **Boekelheide, K., Fleming, S. L., Johnson, K. J., Patel, S. R. and Schoenfeld, H. A.** Role of Sertoli cells in injury-associated testicular germ cell apoptosis. Proc. Soc. Exp. Biol. Med. 2000, **225**, 105-115.
4. **Cai, L., Hales, B. F. and Robaire, B.** Induction of apoptosis in the germ cells of adult male rats exposure to cyclophosphamide. Biol. Reprod. 1997, **56**, 1490-1497.
5. **De Rooij, D. G.** Proliferation and differentiation of spermatogonial stem cells. Reproduction. 2001, **121**, 347-354.
6. **Dunkel, L., Taskinen, S., Hovatta, O., Tilly, J. and Wikstrom, S.** Germ cell apoptosis after treatment of cryptorchidism with human chorionic gonadotropin is associated with impaired reproductive function in the adult. J. Clin. Invest., 1997, **100**, 2341-2346.
7. **Eddy, E. M.** Male germ cell gene expression. Recent Prog. Horm. Res. 2002, **57**, 103-128.
8. **Ejima, K., Koji, T., Tsuruta, D., Nanri, H., Kashimura, M. and Ikeda, M.** Induction of apoptosis in placentas of pregnant mice exposed tolipopoly-saccharides: possible involvement of Fas/Fas ligand system. Biol. Reprod. 2000, **62**, 178-185.
9. **Friesen, C., Herr, I., Krammer, P. H. and Debatin, K. M.** Involvement of the CD95 (APO-1/Fas) receptor/ligand system in drug-induced apoptosis in leukemia cells. Nat. Med. 1996, **2**, 574-577.
10. **Grossfeld, G. D. and Small, E. J.** Long-term side effects of treatment for testis cancer. Urol. Clin. North. Am. 1998, **25**, 503-515.
11. **Hakuno, N., Koji, T., Yano, T., Kobayashi, N., Tsutsumi, O., Taketani, Y. and Nakane, P. K.** Fas/APO-1/CD95 system as a mediator of granulosa cell apoptosis in ovarian follicle atresia. Endocrin. 1996, **137**, 1398-1348.
12. **Henriksens, K., Hakovirta, H. and Parvinen, M.** *In situ* quantification of stage specific apoptosis in the rat seminiferous epithelium: effects of short-term experimental cryptorchidism. Int. J. Androl. 1995, **18**, 256-262.
13. **Higami, Y., Shimokawa, I., Tomita, M., Okimoto, T., Koji, T., Kobayashi, N. and Ikeda, T.** Aging accelerates but life-long dietary restriction suppresses apoptosis related Fas expression on hepatocytes. Am. J. Pathol. 1997, **151**, 659-663.
14. **Hikim, A. P., Wang, C., Leung, A. and Swerdloff, R. S.** Involvement of apoptosis in the induction of germ cell degeneration in adult rats after gonadotropin-releasing hormone antagonist treatment. Endocrinology. 1995, **136**, 2770-2775.
15. **Iwamoto, M., Koji, T., Makiyama, K., Kobayashi, N. and Nakane, P. K.** Apoptosis of crypt epithelial cells in ulcerative colitis. J. Pathol. 1996, **180**, 152-259.
16. **Kawakami, A., Eguchi, K., Matsuoka, N., Tsuboi, M., Koji, T., Urayama, S., Fujiyama, K., Kiriyama, T., Nakashima, T., Nakane, P. K. and Negataki, S.** Fas and Fas ligand interaction is necessary for human osteoblast apoptosis. J. Bone Miner. Res. 1997, **12**, 1637-1646.
17. **Kerr, J. B.** Spontaneous degeneration of germ cells in rat testis: Assessment of cell types and frequency during the spermatogenic cycle. J. Reprod. Fertil. 1992, **95**, 825-830.
18. **Koji, T., Hishikawa, Y., Ando, H., Nakanishi, Y. and Kobayashi, N.** Expression testes: involvement of the Fas system in the induction of germ cell apoptosis in the damaged mouse testis. Biol. Reprod. 2001, **64**, 946-952.
19. **Koji, T., Ohta, H., Kobayashi, N., Nakanishi, Y. and Nakne, P. K.** Involvement of Fas/Fas ligand system in the induction of germcell apoptosis in normal and ischemia-reperfusion testis of adult mouse. Cell Struct. Funct. 1997, **22**, 757-761.
20. **Kothari, L. K., Mishra, P. and Mishra, R. K.** Effect of bilateral vasectomy on the structure and function of the testes. Am. J. Surg. 1973, **126**, 84-88.
21. **Kubota, Y., Sasaki, S., Kubota, H., Tatsura, H. and Kohri, K.** A study on the mechanism of the spermatogenic damage after vasectomy in rats. Nippon Hinyokika Gakkai Zasshi. 2001, **92**, 13-22.
22. **Lee, J., Richburg, J. H., Shipp, E. B., Meistrich, M. L. and Boekelheide, K.** The Fas system, a regulator of testicular germ cell apoptosis, is differentially up-regulated in Sertoli cell versus germ cell injury of the testis. Endocrin. 1999, **140**, 852-858.
23. **Lee, J., Richburg, J. H., Younkin, S. C. and Boekelheide, K.** Fas system is a key regulator of germ cell apoptosis in the testis. Endocrin. 1997, **138**, 2081-2088.
24. **Lue, Y., Sinha Hikim, A. P., Wang, C., Bonavera, J. J., Baravarian, S., Leung, A. and Swerdloff, R. S.** Early effect of vasectomy on testicular structure and

on germ cell and macrophage apoptosis in the hamster. J. Androl. 1997, **18**, 166-173.

25. **McClure, R. F., Heppelmann, C. J. and Paya, C. V.** Constitutive Fas ligand gene transcription in Sertoli cells is regulated by Sp1. J. Biol. Chem. 1999, **274**, 7756-7762.
26. **Nagata, S. and Golstein, P.** The Fas death factor. Science. 1995, **267**, 1449-1456.
27. **Nandi, S., Banerjee, P. P. and Zirkin, B. R.** Germ cell apoptosis in the testes of Spraque (-) Dawley rats following testosterone withdrawal by ethane 1,2-dimethane sulfonate administration: relationship to Fas? Biol. Reprod. 1999, **61**, 70-75.
28. **Nogae, S., Miyazaki, M., Kobayashi, N., Saito, T., Abe, K., Saito, H.,Nakane, P. K., Nakanishi, Y. and Koji, T.** Induction of apoptosis in ischemia-reperfusion model of mouse kidney: possible involvement of Fas. J. Am. Soc. Nephrol. 1998, **9**, 620-631.
29. **Nonclercq, D., Reverse, D., Toubeau, G., Beckers, J. F., Sulon, J. L., Zanen, J. and Heuson-Stiennon, J. A.** *In situ* demonstration of germinal cell apoptosis during diethylstilbestrol-induced testis regression in adult male Syrian hamsters. Biol. Reprod. 1996, **55**, 1368-1376.
30. **Okada, K., Komuta, K., Hashimoto, S., Matsuzaki, S., Kanematsu, T. and Koji, T.** Frequency of apoptosis of tumor-infiltrating lymphocytes induced by Fas counterattack in human colorectal carcinoma and its correlation with prognosis. Clin. Cancer Res. 2000, **6**, 3560-3564.
31. **Pentikainen, V., Erkkila, K. and Dunkel, L.** Fas regulates germ cell apoptosis in the human testis *in vitro*. Am. J. Physiol. 1999, **276**, E310-E316.
32. **Richburg, J. H., Nanez, A. and Gao, H.** Participation of the Fas-signaling system in the initiation of germ cell apoptosis in young rat testis after exposure to mono-(2-ethylhexyl) phthalate. Toxicol. Appl. Pharmacol. 1999, **160**, 271-278.
33. **Richburg, J. H., Nanez, A., Williams, L. R., Embree, M. E. and Boekelheide, K.** Sensitivity of testicular germ cells to toxicant-induced apoptosis in gld mice that express a non-functional form of Fas ligand. Endocrin. 2000, **141**, 787-793.
34. **Rodriguez, I., Ody, C., Araki, K., Garcia, I. and Vassalli, P.** An early and massive wave of germinal cell apoptosis is required for the development of functional spermatogenesis. EMBO J. 1997, **16**, 2262-2270.
35. **Russell, L. D., Ettlin, R. A., Sinha Hikim, A. P. and Clegg, E. D.** Staging for laboratory species. *In* Histological and Histopathological Evaluation of the Testis, pp. 62-118, Cache River Press, Clearwater, 1990.
36. **Russell, L. D. and Clermont, Y.** Degeneration of germ cells in normal, hypophysectomized and hormone treated hypophysectomized rats. Anat. Rec. 1997, **187**, 347-366.
37. **Sarrat, R., Whyte, J., Torres, A., Lostale, F. and Diaz, M. P.** Experimental vasectomy and testicular structure. Histol. Histopathol. 1996, **11**, 1-6.
38. **Shiraishi, K., Naito, K. and Yoshida, K.** Vasectomy impairs spermatogenesis through germ cell apoptosis mediated by p53-Bax pathway in rat. J. Urol. 2001, **166**, 1565-1571.
39. **Shiraishi, K., Yoshida, K., Fujimiya, T. and Naito, K.** Activation of mitogen activated protein kinases and apoptosis of germ cells after vasectomy in the rat. J. Urol. 2002, **168**, 1273-1278.
40. **Sinha Hikim, A. P., Rajavashisth, T. B., Sinha Hikim, I., Lue, Y., Bonavera, J. J., Leung, A., Wang, C. and Swerdloff, R. S.** Significance of apoptosis in the temporal and stage-specific loss of germ cells in the adult rat after gonadotropin deprivation. Biol. Reprod. 1997, **57**, 1193-1197.
41. **Stephan, H, Polzar, B., Rauch, F., Zanotti, S., Ulke, C. and Mannherz, H. G.** Distribution of deoxyribonuclease I (DNase I) and p53 in rat testis and their correlation with apoptosis. Histochem. Cell Biol. 1996, **106**, 383-393.
42. **Suda, T., Takahashi, T., Golstein, P. and Nagata, S.** Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. Cell. 1993, **75**, 1169-1178.
43. **Tamura, M., Kimura, H., Koji, T., Tominaga, T., Ashizawa, K., Kiriyama, T., Yokoyama, N., Yoshimura, T., Eguchi, K., Nakane, P. K. and Nagataki, S.** Role of apoptosis of thymocytes in a rat model of goiter. A possible involvement of Fas system. Endocrin. 1998, **139**, 3646-3653.
44. **Yin, Y., DeWolf, W. and Morgentaler, A.** Experimental crytorchidism induces testicular germ cell apoptosis by p53-dependent and -independent pathway in mice. Biol. Reprod. 1998, **58**, 492-496.