Effect of ethylene glycol monoethyl ether on the spermatogenesis in pubertal and adult rats

Chang Yong Yoon*, **Choong Man Hong[1]**, **Ji Young Song[1]**, **Yong-Yeon Cho**, **Kwang-Sik Choi**, **Beom Jun Lee[2]** and **Cheol Kyu Kim[1]**

Kwangju Regional Food & Drug Administration, Kwangju 500-480, Korea.
[1]*Department of Pathology, National Institute of Toxicological Research, Korea Food & Drug Administration, Seoul 122-704, Korea.*
[2]*Safety Research Center, Korea Testing and Research Institute for Chemical Industry, Seoul 150-038, Korea*

The effects of ethylene glycol monoethyl ether (EGEE) on testicular cell populations in pubertal (5 weeks old) and adult (9 weeks old) male rats were investigated by a flow cytometric method. A total of 50 rats (in number, 25 pubertal and 25 adult rats) was divided into 5 experimental groups including 0 (control), 50, 100, 200, and 400 mg EGEE/kg of body weight. The animals were administered by gavage for 4 weeks. In adult rats, the treatment of EGEE at the dose of 400 mg/kg of body weight decreased significantly the populations of haploid, while it increased those of diploid and tetraploid cells. In pubertal rats, the treatment of EGEE at the dose of 400 mg/kg of body weight caused only minimal changes in the relative percent of testicular cell types. These results suggest that the effects of EGEE on testicular function in pubertal rats appear to be less pronounced than in adult rats.

Key words: flow cytometry, ethylene glycol monoethyl ether, testis, epididymis, DNA contents

Introduction

Testicular damage by a toxicant is evaluated by analyzing parameters such as fertility, pregnancy outcome, testicular cell morphology, and sperm motility [7,9,12]. Traditional approaches involve histopathologic examination of testicular tissue, which includes the description of several cell types, the determination of spermatogenic stages, and the detection of morphologic and cell-kinetic abnormalies in the spermatogenic process [13]. However, these methods are subjective and time-consuming [10-12]. Moreover, the morphologic observation limits a local evaluation of the testicular tissue. Recently, flow cytometry (FCM) has become a useful tool for objective quantification of the types of testicular cell involved in spermatogenesis and it supplies valuable information for the detection of testicular toxicity [11,12]. As compared with current methods for the evaluation of spermatogenic impairment, FCM offers advantages in terms of objectivity, rapidity, analysis of large number of cells providing high statistical significance, and unbiased sampling of cells [10-12]. It also provides quantitative values for evaluating different cell types on the basis of their DNA ploidy/stainability level [10-13].

Ethylene glycol monoethyl ether (EGEE), a family of ethylene glycol ethers, has been used as a solvent in the industry and commercially as a deicing additive to fuel [1,3,16]. Several animal experiments demonstrated that EGEE were toxic to the reproductive system [1,3,5]. Exposure to EGEE in male animals caused testicular atrophy, degeneration of the germinal epithelium, infertiliy, and abnormal sperm morphology [1,3,5]. Embryotoxicity and teratogenicity were also observed in female animals [1,3,5]. EGEE is known to be converted initially to ethoxyacetaldehyde by the alcohol dehydrogenase present in cytoplasm of hepatocytes and then to ethoxyacetic acid (EAA) by the aldehyde dehydrogenase present in hepatocellular mitochondria [1,6,8]. The EAA, the final and major metabolite generated from EGEE, is considered to be a culprit in the testicular toxicity [6]. In the previous study, round and elongated spermatids appeared at the age of 4 weeks and 6 weeks, respectively, and an adult pattern occurred at the age of 8 to 10 weeks [15]. Rats at the age of 5 weeks showed a dramatic shift in the ratios of germ cells, which results from the increased wave of meiotic daughter cells.

In the present study, the effects of EGEE on testicular cells in pubertal (five weeks old) and adult (nine weeks old) rats were evaluated by flow cytometric description for the relative cell populations.

Materials and Methods

Chemicals

Ethylene glycol monoethyl ether (EGEE) was pur-

*Corresponding author
Phone: +82-62-602-1507; Fax: +82-62-602-1500
E-mail: yoonchangyong@hanmail.net

chased from Wako Chemicals Co. (Japan). Trisodium citrate, spermine tetrahydrochloride, and propidium iodide were purchased from Sigma Chemical Company (St. Louis, MO, USA).

Experimental Animals

Four weeks and eight weeks old male Sprague-Dawley (SD) rats were obtained from laboratory animal resources of Korea Food and Drug Administration (KFDA) and acclimated for 1 week before the start of experiments. Five weeks old rats as the pubertal stage and 9 weeks old rats as the adult stage were used in this experiment. The animals were kept in plastic cages and fed pellet food and tap water *ad libitum*. Animal quarters were maintained at the temperature of $21 \pm 2^{\circ}C$, the relative humidity of 60%, and a 12 h-light/dark cycle.

Experimental Design

Twenty-five pubertal rats and 25 adult rats were assigned respectively to five experimental groups (5 rats in each group). At the five doses of 0 (control), 50, 100, 200, and 400mg/kg of body weight, EGEE were administered daily by gavage for 4 weeks (6 times per week). Rats were examined daily for treatment-related behavioral effects and were weighed once a week.

Organ weight

Rats were anesthetized with carbon dioxide. After collection of blood by heart puncture, rats were sacrificed by cervical dislocation. The testis and epididymis were removed and weighed. The testes were stored in citrate buffer at $-80^{\circ}C$ in polypropylene tubes (52×17 mm, with screw cap, Wheaton, Millville, N.J. USA) until use.

Preparation of testicular cells

Testes were thawed, minced, and then incubated for 30 min at room temperature (RT) by gentle magnetic stirring in citrate buffer. Cell suspension was filtered with a 149-μm pore size polypropylene filter (Spectrum Laboratories, Inc.) in order to discard tissue debris and it was resuspended to 1×10^7 cells/ml with citrate buffer. For staining of the cells, an integrated set of methods was applied [2]. Briefly, 1800 μl of solution A [Stock solution (3.4 mM Trisodium citrate · $2H_2O$, 0.1% v/v NP-40, 1.5 mM Spermine tetrahydrochloride, 0.5 mM Tris) containing 30 mg of Trypsin/L, pH 7.6] was added to 200 μl of cell suspension (1×10^7 cells/ml). After standing for 10 min at RT, 1500 μl of solution B (Stock solution containing 500 mg of Trypsin inhibitor and 100 mg of RNase A/L, pH 7.6) was added. After incubation for 10 min at RT, 1000 μl of ice-cold solution C [Stock solution containing 416 mg of Propidium Iodide (PI) and 1160 mg of Spermine tetrahydrochloride] was added. The solutions were mixed and filtered with a 60-μm nylon filter (Spectrum Laboratories, Inc.) into a test

tube wrapped with aluminum foil for protection of the propidium iodide (PI) against light. After addition of solution C, the samples were kept in an ice bath for 30 min to 3 h until analysis.

Flow cytometry

The DNA contents of the dispersed testicular cells were measured by FCM (Coulter Epics XL, Coulter Corp., USA) equipped with a 2-W argon laser and operated on 488 nm. Propidium iodide fluorescent emissions were monitored using a 620 nm band-pass filter, along with a dichroic long-pass filter, 645 DL. The degree of fluorescence was directly proportional to the amount of stain absorbed, thereby directly corresponding to the DNA content of each cell. A total of 2×10^4 events was accumulated for each histogram. The histograms were analyzed with the curve-integration routines provided by the Coulter Multiparameter Data Aquisition and Display Software. The relative proportions of haploid, diploid, and tetraploid cells were calculated from the area under peak in the DNA histogram.
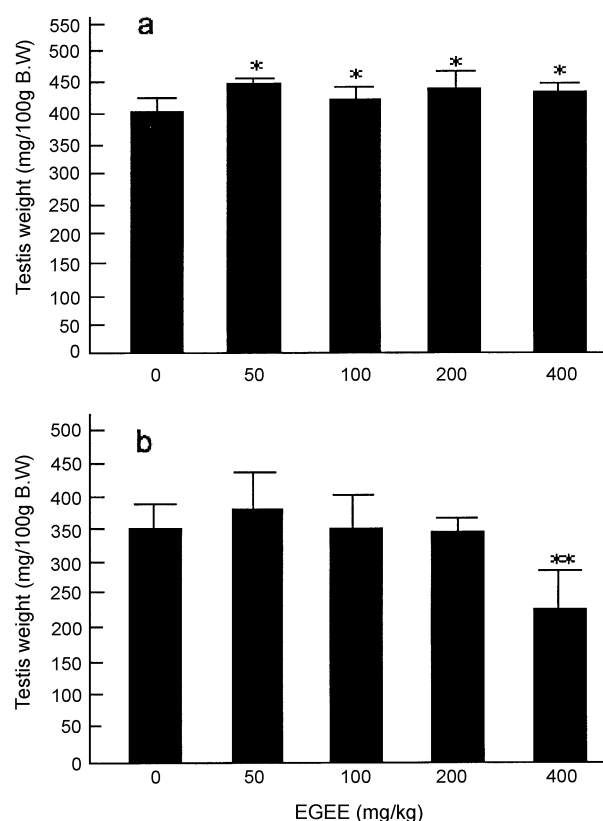
Fig. 1. Testis weight of pubertal (a) and adult (b) rats administered with various doses of EGEE for 4 weeks. Weights were normalized to mg/100g of body weight. Each bar represents the mean±SD of 5 rats per group. *indicates a significant difference at p<0.05 and **indicates a significant difference at p<0.01, compared to the control.
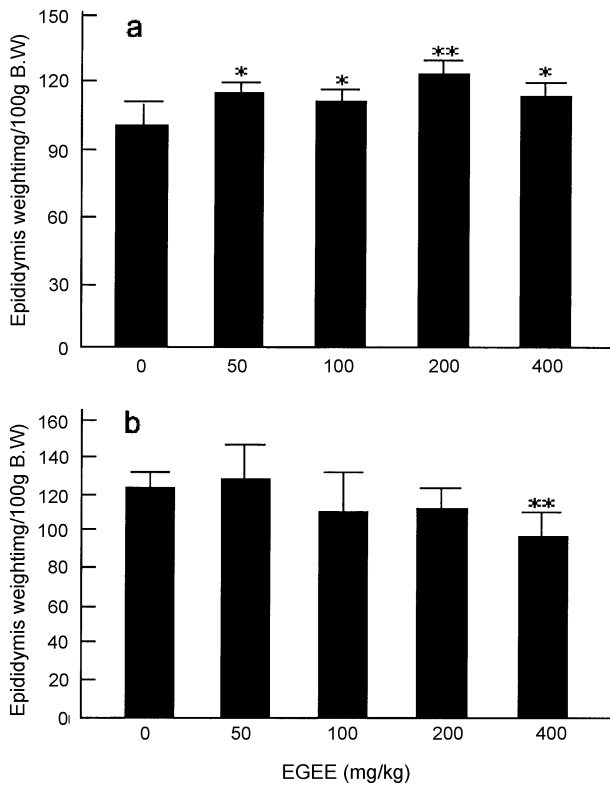
Fig. 2. Epididymis weight of pubertal (a) and adult (b) rats administered with various doses of EGEE for 4 weeks. Weights were normalized to mg/100g of body weight. *indicates a significant difference at p<0.05 and **indicates a significant difference at p<0.01, as compared to the control.

Statistical Analysis

Data were statistically evaluated by analysis of variance analysis (ANOVA, one way) with p≤0.05. For a significant difference between experimental groups, the Scheffe test was carried out.

Results

Weight of Testis and Epididymis

The weights of testes and epididymis were normalized by 100 g of body weight. The administration of EGEE at the doses of 50, 100, 200, and 400 mg/kg increased significantly (p<0.05) the weight of testes in pubertal rats as compared with the control (Fig.1a). In adult rats, the administration of EGEE at the highest dose of 400 mg/kg decreased significantly (p<0.01) the weight of testes as compared with the control (Fig. 1b).

In pubertal rats, the administration of EGEE significantly increased the weight of epididymis in all EGEE-treated groups as compared with the control (Fig. 2a). However, the administration of EGEE at the highest dose of 400 mg/kg significantly (p<0.01) decreased the weight of epididymis in adult rats as compared with the control (Fig. 2b).
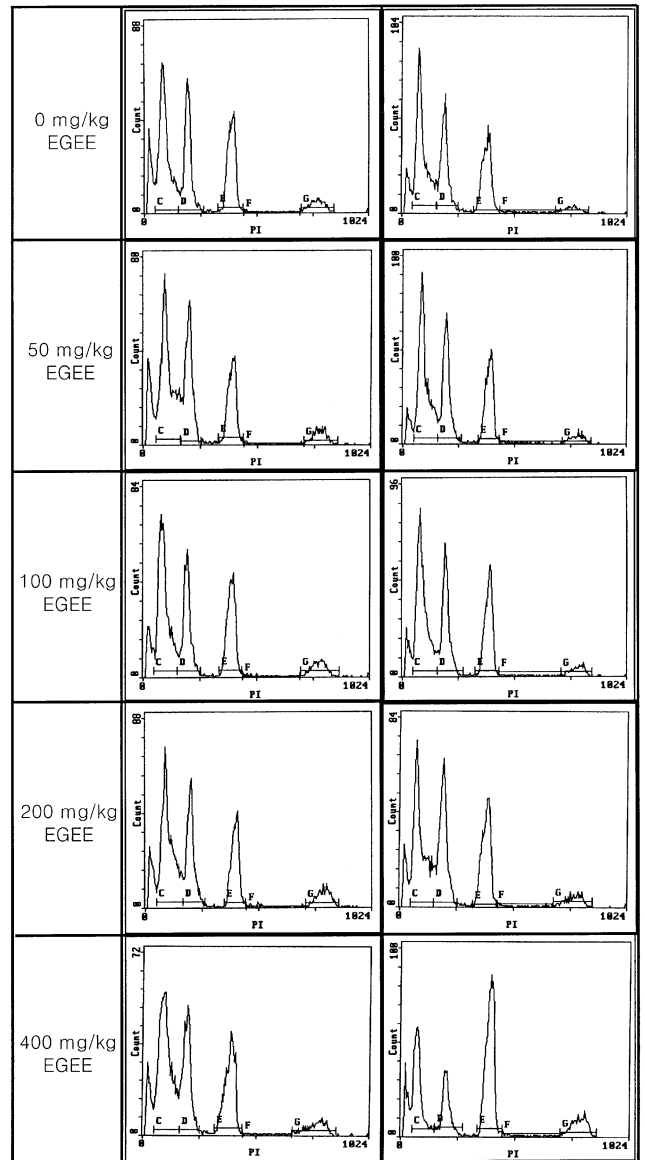
Fig. 3. Representative DNA content histograms of testicular cells in pubertal (left column) and adult (right column) rats administered with various doses of EGEE for 4 weeks. The letters, C, D, E and G represent mature haploid, immature haploid, diploid and tetraploid cell peaks, respectively. F represents S-phase (DNA synthesis).

Flow cytometric analysis

Testicular cells obtained from pubertal and adult rats were placed in suspension, stained with PI, and measured by flow cytometry. Fig. 3 displays representative DNA content histograms of the testicular cells in pubertal (left colum) and adult (right column) rats. A typical pattern of four major testicular cells including mature and immature haploid (1n), diploid (2n), and tetraploid (4n) cells were shown in Fig 3. The treatment of EGEE up to the doses of 400 mg/kg in pubertal rats did not affect the relative population of these four cell types, indicating no effect on the
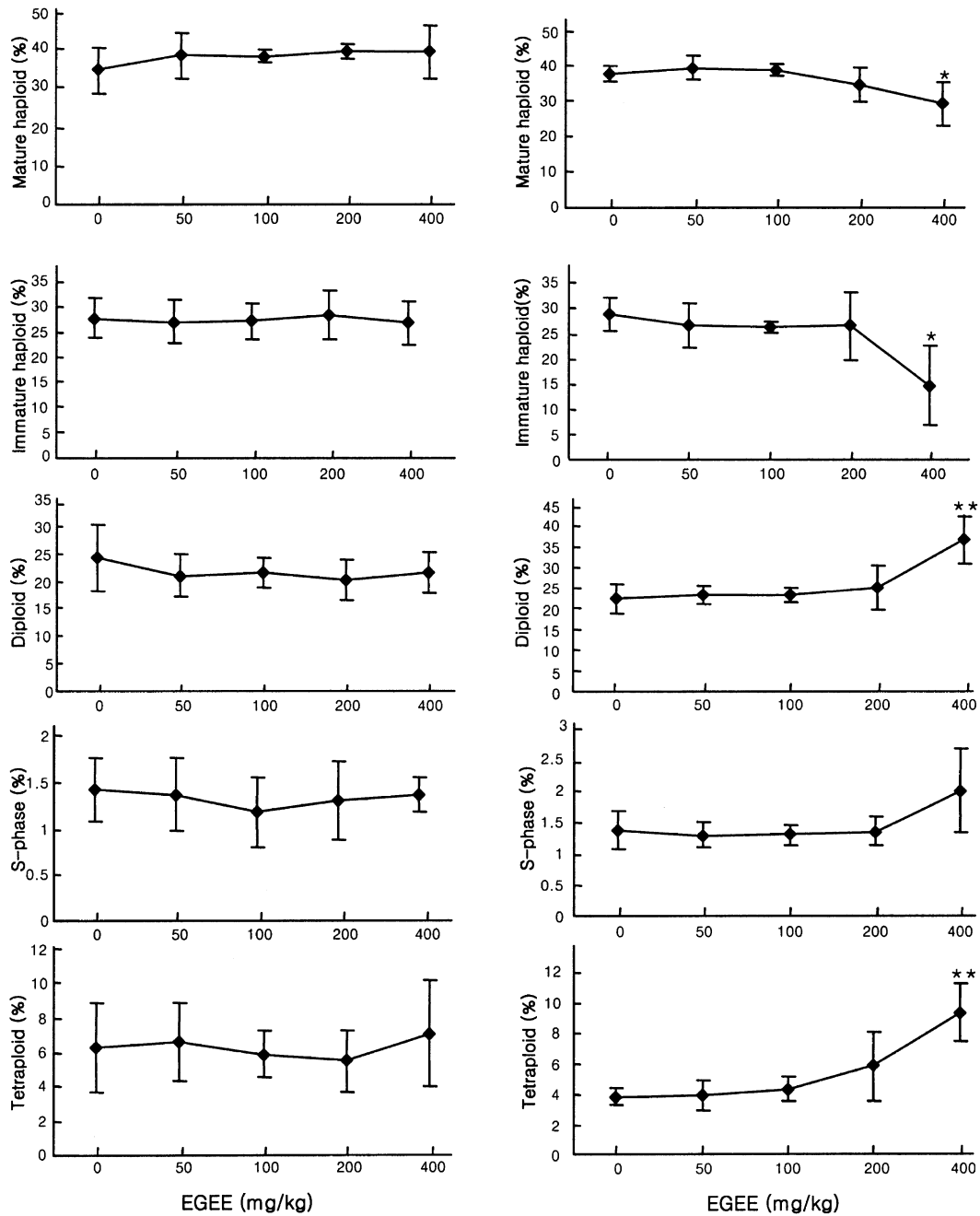
Fig. 4. Alteration of testicular cell populations from pubertal (left column) and adult (right column) rats administered with various doses of EGEE for 4 weeks. Each point represents the percentage of each cell population (mean±SD). *indicates a significant difference at $p < 0.05$ and **indicates a significant difference at $p < 0.01$, as compared to the control.

spermatogenesis of rats (Fig. 4). In adult rats, the treatment of EGEE at the dose of 400 mg/kg caused a significant decrease of relative proportion in mature and immature haploid cells ($p < 0.05$) and a significant increase of relative proportion in diploid and tetraploid cells ($p < 0.01$), as compared to that of the control.

Discussion

On the basis of DNA contents, four main germ cell peaks including mature haploid (elongated spermatids), immature haploid (round and elongating spermatids), diploid (spermatogonia, secondary spermatocytes, tissue somatic cells), and tetraploid (mostly primary spermatocytes) could be identified by flow cytometry in the control animals. The region between the diploid and tetraploid peaks is S-phase, comprised of cells actively synthesizing

DNA. The haploid region can be split into two peaks based on the differential stainability of elongated and round/elongated spermatids. The chromatin of the elongated spermatids is highly condensed and binds less to fluorescent dye when compared to that of the round spermatids. The elongated spermatids appear as the first peak in the flow cytogram [12,15].

The treatment of EGEE has been to cause severe testicular toxicity on the male reproductive system with atrophy of testis in a number of animal species including man [1,3-6,14,16]. As far as the cytotoxic effects from histological findings are concerned, EGEE was reported to affect mainly germ cells such as spermatogonia and spermatocytes [16], and primary spermatocytes undergoing postzygotene meiotic maturation and division [4,5]. In contrast, Foster *et al*. reported that Sertoli and Leydig cells, spermatogonia, prepachytene spermatocytes and spermatids were unaffected by EGEE administration from 250 to 1000 mg/kg for 11 days apart from partial maturation depletion of early spermatid stage [5]. Reproductive toxicity of EGEE is still in controversy from these studies.

In the present study, we evaluated the testicular toxicity induced by EGEE in the pubertal and adult rats by flow cytometric and histological description of testicular cell populations. In adult rats, the exposure of 400 mg EGEE/kg caused abnormal spermatogenesis, resulting in the reduced testicular and epididymal weight (Fig. 1 & 2), and the altered ratios of testicular germ-cell types (Fig. 3 & 4). Meanwhile, in pubertal rats, the treatment of EGEE at the dose of 400 mg/kg of body weight caused a slight increase in the testicular and epididymal weight, which might be induced by a relative decrease of body weight in this group (data not shown). In addition, the treatment of EGEE up to the dose of 400 mg/kg did not produce any major change in the testicular growth and relative percentage of testicular cell types.

The reasons for lack of major effects of EGEE on spermatogenesis of pubertal rats are not clear at present. However, the toxicity of EGEE was evidenced by the systemic effect such as the decrease of body weight in both adult and pubertal rats. In addition, our results indicate that the effects of EGEE on the testicular toxicity in pubertal rats appear to be less pronounced than in adult rats.

References

1. **Chung, W. G., Yu, I. J., Park, C. S., Lee, K. H., Roh, H. K. and Cha, Y. N.** Decreased formation of ethoxyacetic acid from ethylene glycol monoethyl ether and reduced atrophy of testes in male rats upon combined administration with toluene and xylene. Toxicol. Lett. 1999, **104**, 143-50.
2. **Darzynkiewicz, Z. and Crissman, H. A.** (ed.), Methods in cell biology. In: Vindelov L, Christensen IBJ, eds. An integrated set of methods for routine flow cytometric DNA analysis. San Diego,California: Academic Press, Inc, 1990, 127-37.
3. **Donald, Miller J.** DHHS (NIOSH) Publication No. 83-112: Glycol ethers, 2-methoxyethanol and 2-ethoxyethanol. May 2, 1983.
4. **Foster, P. M. D., Creasy, D. M., Foster, J. R. and Gray, T. B. J.** Testicular toxicity produced by ethylene glycol monomethyl and monoethyl ethers in the rat. Environ. Health Perspect. 1984, **57**, 207-217.
5. **Foster, P. M. D., Creasy, D. M., Foster, J. R., Thomas, L. V., Cook, M. W. and Gangolli, S. D.** Testicular toxicity of ethylene glycol monomethyl and monoethyl ether in the rat. Toxicol. Appl. Pharmacol. 1983, **69**, 385-399.
6. **Gray, T. B. J., Moss, E. J., Creasy, D. M. and Gangolli, S. D.** Studies on the toxicity of some glycol ethers and alkoxyacetic acids in primary testicular cell cultures. Toxicol. Appl. Pharmacol. 1985,**79**, 490-501**.**
7. **Jagetia, G. C., Jyothi, P. and Krishnamurthy, H.** Flow cytometric evaluation of the effect of various doses of vindesine sulphate on mouse spermatogenesis. Reprod. Toxicol. 1997, **11(6)**, 867-874.
8. **Jonsson, A. K., Pedersen, J. and Steen, G.** Ethoxyacetic acid and N-ethoxyacetylglycine: metabolite of ethoxyethanol(ethylcellosolove) in rats. Acta Pharmacol. Toxicol. 1982, **50**, 358-362.
9. **Spano, M., Bartoleschi, C., Cordelli, E., Leter, G. and Segre, L.** Flow cytometric and histological assessment of 1,2 : 3,4-diepoxybutane toxicity on mouse spermatogenesis. J. Toxicol. Environ. Health 1996, **47**, 423-441.
10. **Spano, M., Bartoleschi, C., Cordelli, E., Leter, G., Tiveron, C. and Pacchierotti, F.** Flow cytometric assessment of trophosphamide toxicity on mouse spermatogenesis. Cytometry 1996, **24,** 174-180.
11. **Spano, M. and Evenson, D. P.** Flow cytometric analysis for reproductive biology. Biol. Cell 1993,**78**, 53-62.
12. **Suter, L., Bobadilla, M., Koch, E. and Bechter, R.** Flow cytometric evaluation of the effects of doxorubicin on rat spermtogenesis. Reprod. Toxicol. 1997, **11**(4), 521-531.
13. **Suter, L., Clemann, N., Koch, E., Bobadilla, M. and Bechter, R.** New and traditional approaches for assessment of testicular toxicity. Reprod. Toxicol. 1998, **12(1)**, 39-47.
14. **Welch, L. S., Schrader, S. M., Turner, T. W. and Cullen, M. R.** Effects of exposure to ethylene glycol ethers on shipyard painters: Male reproduction. Am. J. Ind. Med. 1988, **14**, 509-526.
15. **Yoon, C. Y., Hong, C. M., Cho, Y. Y., Song, J. Y., Hong, I. J., Cho, D. H., Lee, B. J., Song, H. J. and Kim, C. K.** Flow cytometric evaluation on the age-dependent changes of testicular DNA contents in rats. J. Vet. Sci. 2001, **2(1)**, 43-46.
16. **Yu, I. J., Lee, J. Y., Chung, Y. H., Kim, K. J., Han, J. H., Cha, G. Y., Chung, W. G., Cha, Y. N., Park, J. D., Lee, Y. M. and Moon, Y. H.** Co-administration of toluene and xylene antagonized the testicular toxicity but not the hematopoietic toxicity caused by ethylene gylcol monoethyl ether in Sprague-Dawley rats. Toxicol. Lett. 1999, **109**, 11-20.