Flow Cytometric Assessment of Ethylene Glycol Monoethyl Ether on Spermatogenesis in Rats

Chang Yong YOON1), Choong Man HONG1), Yong -Yeon CHO1), Yong Hyun CHUNG2), Hong Ki MIN1), Young Won YUN1), Beom Jun LEE3), Ki Hwa YANG1), Yong Soon LEE4) and Cheol Kyu KIM1)

1)Department of Pathology, National Institute of Toxicological Research, Korea Food & Drug Administration, Seoul 122–704,
2)Occupational Safety and Health Research Institute, Taejon 305–380, 3)College of Veterinary Medicine, Chungbuk National University, Cheongju 361–763 and 4)College of Veterinary Medicine, Seoul National University, Savon 441–744, Korea

(Received 2 April 2002/Accepted 7 November 2002)

ABSTRACT. The effects of ethylene glycol monoethyl ether (EGEE) on testicular cell populations in rats were investigated by a flow cytometric method. Rats were administered by gavage with EGEE at the various doses of 0 (saline alone), 100, 200, 400, and 800 mg/kg body weight/day for 4 weeks. The treatment of EGEE caused decreases in the weight of testis and epididymis and in the number of testicular cells. Histopathologically, exfoliation of germ cells into the tubular lumen was observed at the doses of above 200 mg/kg. The treatment of EGEE at the dose of 400 mg/kg caused moderate testicular degeneration. A significant depletion of haploid cells and a disproportionate ratio of diploid and tetraploid cells were observed as determined by flow cytometric analysis. These results indicate that the toxic effect of EGEE on the male reproductive system may be strongly associated with the disproportion of testicular germ cells.

KEY WORDS: DNA content, epididymis, ethylene glycol monoethyl ether, flow cytometry, testis.

Testicular damage by a toxicant is generally evaluated by analyzing parameters such as fertility, pregnancy outcome, testicular cell morphology, and sperm production and motility [7, 9, 12]. Traditional evaluation approaches also 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 abnormalities 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]. 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, infertility, and abnormal sperm morphology [1, 3, 5]. Embryotoxicity and teratogenicity were also observed in female animals [1, 3, 5]. EGEE is initially converted to ethoxycetaldehyde by alcohol dehydrogenase present in the cytoplasm of hepatocytes and then to ethoxyacetic acid (EAA) by aldehyde dehydrogenase present in the 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 toxic effects of EGEE on spermatogenesis in rats were evaluated histopathologically and by flow cytometric description for the alterations of testicular cell population.

MATERIALS AND METHODS

Chemicals: EGEE was purchased from Wako Chemicals Co. (Japan). Trisodium citrate, spermine tetrahydrochloride, and propidium iodide were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Animals and treatments: 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. The animals were kept in plastic cages and fed pellet food and tap water ad libitum. Animal quarantines were maintained at the temperature of 21 ± 2°C, the relative humidity of 60%, and a 12 hr-light/dark cycle. Twenty-five rats were assigned to five experimental groups (5 rats in each group). At the five doses of 0 (control), 100, 200, 400 and 800 mg EGEE/kg of body weight, rats 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 measurement and histopathological observation: Rats were anesthetized with carbon dioxide. After collection of blood by heart puncture, rats were sacrificed by cervical dislocation. The testes and epididymis were removed and weighed. The testes were stored in citrate buffer (250 mM sucrose, 40 mM trisodium citrate, 5% DMSO, pH 7.6) at –80°C in polypropylene tubes (52 × 17 mm, with screw cap, Wheaton, Millville, N.J., U.S.A.) until use. The testes and epididymis were also fixed in 10% phosphate buffered formalin solution, embedded in paraffin, stained with hematoxylin & eosin, then examined under a light microscope.

Testicular cell counts: Testes stored in citrate buffer at –80°C were thawed and minced with surgical scissors in 6 ml of citrate buffer. The minced tissue was then shaken with continuous gentle magnetic stirring for 30 min at room temperature (RT). An aliquot of the cell suspension was loaded on a hemocytometer and individual cells were counted under a light microscope.

Preparation of testicular cells: Testes were thawed, minced, and then incubated for 30 min at 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, 1,800 µl of solution A [Stock solution (3.4 mM Trisodium citrate·2H2O, 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, 1,500 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, 1,000 µl of ice-cold solution C [Stock solution containing 416 mg of Propidium Iodide and 1,160 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 against light. After addition of solution C, the samples were kept in an ice bath for 30 min to 3 hr until analysis.

Flow cytometry: The DNA contents of the dispersed testicular cells were measured by FCM (Coulter Epics XL, Coulter Corp., U.S.A.) 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 Acquisition and Display Software. The relative proportions of haploid, diploid, and tetraploid cells were calculated from the area under peak in the DNA histogram.

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’s test was carried out.

RESULTS

Body and organ weights: The treatment of EGEE at the doses of 200–800 mg/kg B.W. decreased body weight gain in a dose-dependent manner. However, the treatment of EGEE at the dose of 100 mg/kg B.W. slightly increased body weight compared to the control (Fig. 1). The administration of EGEE at the doses of 100 and 200 mg/kg did not affect the weight of testes of rats. Meanwhile, the administration of EGEE at the high doses of 400 and 800 mg/kg decreased significantly (p<0.01) the weight of testes by 35.7% and 50.4%, respectively, as compared with the control (Fig. 2). The administration of EGEE significantly decreased the weight of epididymis in a dose-dependent manner in all the EGEE-treated groups as compared with the control (Fig. 3).
EFFECTS OF EGEE ON SPERMATOGENESIS

Effects of EGEE on the number of testicular cells: The treatment of EGEE at the doses of 100 and 200 mg/kg B.W. did not affect the testicular cell numbers. However, at the doses of 400 and 800 mg/kg B.W., the treatment of EGEE decreased the testicular cell number by approximately 43.4% and 20.0%, respectively, compared to the control (Fig. 4). Among the individually identified germ cell subpopulations, the most affected cell type was the haploid cells. The cell types ranging from round spermatids to elongated spermatids were significantly depleted. In the group treated with 400 mg EGEE/kg B.W., mature and immature haploid cells were reduced by 24% and 5%, respectively, compared to the control. Especially, in the group treated with 800 mg/kg B.W., round and elongated spermatids were almost completely depleted. Concomitantly with the reduction of the proportion of haploid cells, the relative proportion of diploid cells was significantly increased at the doses of above 400 mg EGEE/kg B.W., compared with the control. The proportion of tetraploid cells was also significantly increased at the dose of 400 mg EGEE/kg B.W.

DISCUSSION

The administration of EGEE by gavage for 4 weeks caused a dose-dependent and significant decrease in body weight gain at the doses of 200, 400, and 800 mg/kg B.W.,
suggesting a systemic effect of EGEE. In the EGEE-treated groups, a noticeable decrease in the weight of testis and epididymis associated with cellular damage indicates that these organs are the targets for EGEE-induced toxicity [1, 3].
degree of damage was actually related to the administered dosage. In addition, the testes of rats treated with EGEE had a variety of pathological lesions, including exfoliation of germ cells into the tubular lumen, appearance of giant cells, and...
disappearance of spermiogenic cells with moderate testicular degeneration, and considerable depletion of all types of spermatids. As far as the cytotoxic effects from histological findings are concerned, EGEE affects mainly germ cells such as spermatogonia and spermatocytes [15], and primary spermatocytes undergoing postzygote meiotic maturation and division [4, 5]. In contrast, Foster et al. [5] reported that the Sertoli and Leydig cells, spermatogonia, prepauchytoe spermatocytes, and spermatids were unaffected by EGEE treatment at the doses of 250 to 1,000 mg/kg B.W. for 11 days apart from partial maturation depletion of early stage spermatids. Although the results obtained by the authors showed similar histopathological findings, there were some discrepancies concerning the cell types affected by EGEE treatment.

In this study, EGEE exposure altered the normal ratio of testis cell types as determined by FCM, reflecting an interference with the spermatogenic process, thereby resulting in killing and blocking of the cells at specific stages of development. It has been clearly demonstrated that EGEE treatment for 4 weeks caused a marked depletion of haploid cells and a relative increase in diploid and tetraploid cells. An intriguing finding in this FCM study is that germ cells such as spermatogonia and spermatocytes are not the only target cells of EGEE toxicity during spermatogenesis. Immature and mature haploid cells appeared to be alternative target cell types to EGEE toxicity as evidenced by considerable depletion of haploid population. It is known that EGEE is highly toxic to spermatogonia and primary spermatocytes [5, 6, 15]. The reduction in the relative percentage of immature and mature haploid cells observed at the high doses of EGEE (400 mg/kg B.W. and 800 mg/kg B.W.) could be due to the lethal effects of EGEE on earlier cell types like spermatogonia which eventually differentiates into spermatids. In addition, the rapid regeneration of the surviving spermatogonia and a mitotically active cell type and the accumulation of the maturation-arrested zygote spermatocytes may contribute to the relative increase in the tetraploid cell compartment [4, 7]. The relative increases in diploid and tetraploid cells at the doses of 400 and 800 mg/kg B.W. of EGEE may also be due to the flow cytometric acquisition of cells, associated with killing other cell types, especially immature and mature haploid cells. The flow cytometer may acquire the diploid and tetraploid cells that are resistant to the EGEE-induced cytotoxicity, resulting in an increase in cell proportions. However, it could be considered that the depletion in spermatid populations may be due to direct cytotoxic effect of EGEE on these cell types.

In conclusion, our study demonstrated that the exposure to EGEE induced reproductive cytotoxicity including the spermatid compartments.

ACKNOWLEDGEMENT. This study was supported by a grant from the Safety Research Budget of National Institute of Toxicological Research (NITR).

REFERENCES