A FLOW CYTOMETRIC ANALYSIS OF CYTOTOXIC EFFECTS OF NITROBENZENE ON RAT SPERMATOGENESIS

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ABSTRACT — Cytotoxic effects of nitrobenzene (NB) on spermatogenesis of mature Sprague-Dawley (Crl:CD) rats were analyzed by measuring the DNA content distribution and testicular weight at 1, 2, and 3 weeks of daily oral dose of NB (60 mg/kg/day). Rats at the age of 9 weeks were used because the ratios of 1C, 2C, S, 4C to total testicular cells were stabilized after the age of 52 days. Within a week of administration, a large number of 1C cells were lost but 2C cells proliferated, resulting in little change of testicular weight. In another week that followed, the number of 1C cells and testicular weight were decreased, but the ratio of S-4C cells was increased, indicating that an appreciable number of 2C cells could progress to the 4C compartment. The data indicated that (1) 1C cells were destroyed, and (2) meiosis of secondary spermatocytes was suppressed, but (3) NB had little effect on the spermatocytes prior to the early pachytene stage. This interpretation was reinforced by the observation that (4) the ratio of 1C cells returned to a nearly normal level during a recovery period of 2 weeks. In conclusion, flow cytometry could offer an efficient method for the quantitative analysis of male reproductive toxicity.

KEY WORDS : Flow cytometry, Nitrobenzene, Rat, Spermatogenesis, Testis, Toxicity

INTRODUCTION

Nitrobenzene (NB) has been primarily employed as an oxidizing agent and a chemical intermediate in the manufacture of a variety of organic compounds. Major targets of NB toxicity include hematopoietic tissues, central nervous system, liver and male reproductive organs (e.g. Beauchamp et al., 1982). Toxic effects on the male reproductive organs were first studied in rats by Bond et al. (1981) and it was demonstrated that NB causes germ cell necrosis and a decrease in the number of sperm in the epididymis.

Testicular toxicity of NB has been investigated using various endpoints in vivo (e.g. Levin et al., 1988; Kawashima et al., 1995) and in vitro (e.g. Allenby et al., 1990).

Linder et al. (1992) reported that histopathology of the testis and epididymis was the most consistent indicator of reproductive damage. It reminds us of the importance of investigating the direct effects on spermatogenesis. It is also desirable to clarify the point in the process of spermatogenesis at which the chemical exerts its influence and to examine the effects quantitatively.

Spermatogenesis in rats is a linear process in which there are 14 stages as described by

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Leblond and Clermont (1952). During this process, cells go through different DNA ploidy compartments in the order of (i) diploid (2C) spermatogonia, (ii) tetraploid (4C) primary spermatocytes, (iii) diploid (2C) secondary spermatocytes and (iv) haploid (1C) spermatids and spermatozoa in a period of approximately 51 days (Clermont and Harvey, 1965). Percentages of cells in these compartments have been reported to be consistent in various species of laboratory animals (Suresh et al., 1992). For qualitative assessment of spermatogenesis, histological examination has been a standardized method for more than half a century. However, the method was laborious and time-consuming. By contrast, flow cytometry (FCM) is a rapid, objective, and precise technique and has been used in increased frequency in recent years by many investigators for quantitating spermatogenesis in reproductive biology (e.g. Hirsch et al., 1993; Hittmair et al., 1992; Spano and Evenson, 1993) and in reproductive toxicology (e.g. Evenson et al., 1989; Jagetia et al., 1996; Spano et al., 1996; Takizawa et al., 1995). However, as far as the authors are aware, testicular toxicity of NB has never been investigated using flow cytometry.

The present investigation was undertaken to evaluate quantitatively the spermatotoxic effects of NB in rats using flow cytometry for the measurement of cell distribution in three different DNA ploidy compartments. In combination with the data of testicular weight, the observed changes in the DNA distribution pattern will be discussed in terms of disturbance of spermatogenesis to specify the point of action of the toxicant.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley (Crlj:CD) rats were used. All rats were housed in hanging cages with stainless mesh and were given a rodent chow (MBR-1; Funabashi Farms, Funabashi, Chiba) and tap water ad libitum. The animal room was kept in a 12-hr light/dark cycle under conditions of constant temperature (24 ± 2°C) and constant humidity (55 ± 10%).

Determination of Maturation Time of Testis

Seventy young rats at the age of 3 weeks were divided into 14 groups, each comprising 5 rats. They were used to determine how a DNA content distribution in the testis changes with age. Animals of each group were killed under deep ether anesthesia at 3- or 4-day intervals up to 8 weeks postpartum (at 11 different ages) and at the ages of 9, 10 and 15 weeks. Each time, the right testis was removed, weighed, and a DNA content distribution was measured using a flow cytometer (see below).

Experimental Design

Forty rats at the age of nine weeks were used for toxicity testing because DNA content distributions of testicular cells are stabilized by this time. These animals were randomized with respect to body weight and then divided into 8 groups, each comprising 5 rats. Nitrobenzene (Wako Chemical Industries, Ltd., Osaka) was dissolved in corn oil (Sigma Chemical Co., St. Louis, MO) at the concentration of 24 mg/ml and given to five groups of animals by gavage at a dose of 60 mg/kg/day, a dose which was found to cause a marked decrease of testicular weight in our preliminary experiment. Corn oil was given to the control animals. To determine the dose level for the individual animals, body weights were measured twice a week. The daily oral administration was continued for 1, 2, and 3 weeks to group 1, group 2 and group 3, respectively. For analysis of the recovery process, the animals in groups 4 and 5 were given NB for 2 weeks and sacrificed after 2 and 4 weeks of a recovery period, respectively. At the time of sacrifice, the weight of the right testis and right epididymis as well as body weight were measured and relative organ weights were calculated.

Cell Dispersion for Flow Cytometry

Cell dispersion protocols used by Clausen et al. (1977) and Iwamoto et al. (1993) were modified slightly as described below. A right testis taken from each animal was stripped of tunica albuginea, placed in cold Dulbecco's phosphate-buffered saline (PBS) (Dainippon Pharmaceutical Co. Ltd., Osaka), and minced with razors. Cells and tissue fragments of the testis were collected by centrifugation at 400G for 5 min, and treated with 0.05 % collagenase (Wako Pure Chemical Industries, Ltd., Osaka) solution in PBS containing 1 mM CaCl₂ (Wako Pure Chemical
Industries, Ltd., Osaka) at 37°C for 60 min under shaking. The cell suspension was filtered through a 36 μm nylon mesh (NBC Industries, Ltd., Tokyo) to remove cell aggregates and centrifuged at 400G for 5 min. The resulting pellet was washed twice by centrifugation at 400G for 5 min and resuspension in 10 ml PBS. The dispersed cells were fixed in cold 70 % ethanol and kept overnight at -20°C.

DNA Staining for Flow Cytometry

DNA staining protocols used by Hashimoto (1984) and Kawai (1984) were modified slightly as described below. Twenty to 30 million cells fixed in ethanol were washed twice in 10 ml PBS and incubated with 10 ml of 0.1 % ribonuclease (Sigma Chemical Co., St. Louis, MO) solution in PBS at 37°C for 30 min. The cells were washed once in PBS and incubated with 10 ml of 0.1 % pepsin (Sigma Chemical Co., St. Louis, MO) solution in 0.2 % HCl (pH 2.0) (Wako Pure Chemical Industries, Ltd., Osaka) at 37°C for 15 min. After washing twice in PBS, 2 x 10⁶ cells were stained in 1 ml of 50 μg/ml propidium iodide (PI) (Sigma Chemical Co., St. Louis, MO) solution in PBS, placed in the dark for 30 min on ice and filtered through a 36 μm nylon mesh to remove cell aggregates. Rat thymocytes were also stained using the same PI solution to set a diploid DNA peak at the 200th channel at the beginning of each measurement.

Flow Cytometric Measurement of DNA Histogram

DNA content distribution of testicular cells was measured using a flow cytometer, Coulter EPICS Elite (Coulter Electronics, Hialeah, FL). The machine was set for alignment by running 2 μm fluorescent microspheres (Polysciences, Inc., Warrington, PA) at the beginning of each measurement or whenever instrumental drift became a problem. The PI was excited at 488 nm

Fig. 1. Representative DNA histograms of testicular cells sampled at the ages of 21, 28, 35, and 56 days.
with an 10 mW argon laser, and red fluorescence (> 625 nm; PMT4) emitted from individual cells was detected. Ten thousand fluorescent events were recorded per sample at a rate of approximately 100 fluorescent events per second, and the list mode data were stored on the hard disk. Cell fraction in each cell-cycle phase was calculated by analyzing the DNA histograms using software MacCycle (Phoenix Flow Systems Inc., San Diego, CA).

**Statistical Analysis**

The ratio of cells in each ploidy compartment, relative weight of testis and relative weight of epididymis were calculated for the treated animals and compared with those of control animals using Student's t-test after homogeneity of variance was examined using F test. For the comparison of data from recovery groups with those of control, Tukey's parametric method was used after examining homogeneity of variance using Bartlett's test.

**RESULTS**

**Normal Course of Testicular Maturation**

Representative DNA histograms of testicular cells taken from animals at the ages of 21, 28, 35 and 56 days were quite different (Fig. 1). There was no 1C peak at the age of 21 days. First, at the age of 28 days, a small number of 1C cells appeared and the ratio to total cell population was 6.7 ± 1.8 % (Fig. 2). Henceforth, the ratio of 1C cells increased progressively (at the cost of 2C and 4C cells) and reached a plateau level of 64.4 ± 2.5 % at the age of 52 days. The ratio of 2C cells decreased almost monotonously from 63.7 ± 2.5 % to 20.9 ± 2.0 %. The ratio of 4C cells followed a rather irregular course from 25.5 ± 1.6 % to the level of 4.6 ± 0.9 %. The irregularity was marked before the age of 7 weeks. However, the ratio of 4C/2C varied within the range from 0.2 to 0.9. Cell fraction in S-phase changed only slightly around the level of 10 % throughout the period of observation. The relative numbers of cells in the four compartments were

![Graph](image_url)  
**Fig. 2.** Fraction of cells in 1C, 2C, 4C peaks and S-phase plotted against age of animals (○—○ : 1C, □□□□ : 2C, □□□□ : 4C, S-phase cells). Each point represents a mean value of 5 rats and a vertical line represents a standard deviation.
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**Fig. 3a.** Relative weights of testis measured at 1, 2, and 3 weeks of daily oral administration of NB (60 mg/kg/day) (■) and corn oil (□). Relative weights of testis were calculated based upon the body weight. The columns with ** were significantly lower than control (P < 0.01).

**Fig. 3b.** Relative weights of epididymis measured at 1, 2, and 3 weeks of daily administration of NB (60 mg/kg/day) (■) and corn oil (□). Relative weights of epididymis were calculated based upon the body weight. A column with * was significantly lower than control (P < 0.05).
stabilized after the age of 52 days.

**Changes in the Weight of Testis and Epididymis**

Even without a dose of NB, the relative weights of testis and epididymis were decreased slightly (white columns in Fig. 3a and 3b) due to increase of body weight. However, after 2 weeks of daily oral dose, the testis weight was remarkably decreased to approximately 50% of control. Decrease in relative weight of testis was statistically significant (black columns in Fig. 3a). Atrophy of the epididymis was less pronounced and became statistically significant only after dosing over 3 weeks (black columns in Fig. 3b).

After discontinuation of NB administration, the relative weight of the testis continued to decrease very slowly for the first 2 weeks (compare light and black columns in Fig. 4a) and then increased slowly during the succeeding 2 weeks. Finally it became significantly higher (P < 0.05) than the value before the recovery period (compare gray and black columns in Fig. 4a). Atrophy of the epididymis was marked and the weight was kept significantly lower than the value before the recovery period (compare light, gray and black columns in Fig. 4b).

**Cell Distribution in Different Ploidy Levels**

The ratio of 1C cells dropped rapidly to 34% (to 60% of the control) within a week of NB administration. The increase of 2C cells was significant, but the increase of S-4C was not (Fig. 5b). During the succeeding 2 weeks, the ratio of 1C cells was decreased more remarkably (Fig. 5b) accounting for a marked decrease in testis weight (Fig. 3a). The ratio of 2C was slightly decreased but not significant. By contrast, S-4C increased to 78% (Fig. 5b). After dosing of 3-week duration, 1C cells became practically absent. The S-4C cells were still the largest subpopulation, but the ratio was less than a week previously. Instead, 2C cells recovered to 37% (Fig. 5c).

During 2 weeks of the recovery period, the ratio of testicular cells in three different ploidy compartments changed rapidly toward the normal value (Fig. 6). During the next 2 weeks, the ratios were little changed (Fig. 6).

**DISCUSSION**

Since germ cells in the testis go through different ploidy levels before they are terminally differentiated to spermatozoa, disturbances of spermatogenesis are reflected in the change of DNA content distribution which can easily be detected by flow cytometry. To clarify the effect of NB, mature animals (9 weeks postpartum) with a constant distribution of three different ploidy compartments (i.e. 1C : 65%, 2C : 20%, S-4C : 15%) were used. The ratios are approximately equal to the values reported by other investigators (e.g. Clausen et al., 1977).

In the present investigation, severe spermatototoxicity of NB was evident from the change of testicular weight. Recovery from damage was slow and unsatisfactory. Effect on the epididymis was less pronounced but the weight continued to decrease for a longer period, due probably to damage of seminiferous tubules which supply cells to the epididymis.

DNA content distribution of testicular cells also disclosed NB cytotoxicity. Of various changes observed, most marked was the loss of haploid cells, and the percentage was decreased to nearly 60% of control within a week after daily dose of NB. This seems to be consistent with the result of Hess et al. (1988) that 1,3-dinitrobenzene, a similar compound, reduced the number of spermatids. By contrast, the ratio of diploid cells was increased, and it can be considered that the diploid cells were increased not only in ratio but also in number because testicular weight was not decreased appreciably. This implies that diploid cells underwent mitosis even in the presence of NB. A small fraction of them might have moved into the region of S-4C, but in reality the latter cell fraction was not significantly increased by this time. This may be explained by the degeneration of pachytene spermatocytes as observed by Levin et al. (1988).

A decrease of testicular weight (to 60% of the control) during 2 weeks of daily administration can be regarded to represent (although not exactly) a decrease in the number of testicular cells, especially in the 1C compartment. To the contrary, cells in the S-4C compartment were increased both in ratio and in number, because the ratio of S-4C cells was increased enormously to
Fig. 4a. Relative weights of testis after recovery periods (0 week : ■ ; 2 weeks : ○ ; 4 weeks : □) were compared with the value of control animals given corn oil for 2 weeks (□). Relative weights of testis were calculated based upon the body weight. The columns with ** ( ■, ○ ) were significantly lower than control ( □ ) (P < 0.01). A column with # ( □ ) was significantly higher than ■ (P < 0.05).

Fig. 4b. Relative weights of epididymis after recovery periods (0 week : ■ ; 2 weeks : ○ ; 4 weeks : □) were compared with the value of control animals given corn oil for 2 weeks (□). Relative weights of epididymis were calculated based upon the body weight. The columns with ** ( ■, ○ ) were significantly lower than control ( □ ) (P < 0.01). The columns with ## ( □ ) were significantly higher than ■ (P < 0.01).
470% of control while the testicular weight was decreased to 60%. This indicates that, even in the presence of NB, 2C cells could synthesize DNA and enter into S-4C compartments. The interpretation described above seems to be consistent with the result of Shinoda et al. (1989) that 1,3-dinitrobenzene had no effects on DNA synthesis of spermatogonia and preleptotene spermato-

![Bar charts](chart1.png)  ![Bar charts](chart2.png)  ![Bar charts](chart3.png)

**Fig. 5.** Ratios of testicular cells after daily oral dose of NB (60 mg/kg/day) (■) and corn oil (□) for 1 week (a), 2 weeks (b), and 3 weeks (c). The columns with ** were significantly different from control (P < 0.01).
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cytes.

During the third week of NB administration, the ratio of S-4C cells was decreased (from 78% to 61%) with a concomitant rise in the ratio of 2C cells (from 18% to 37%). This is merely a rise in ratio of 2C cells because of a marked testicular atrophy (Fig. 3a right). Whether or not some fraction of 4C cells moved into the 2C compartment (so as to be secondary spermatocytes) is uncertain, but it is doubtless that spermatids and spermatozoa were not generated because 1C cells were almost extinct (Fig. 5c).

During a recovery period of 4 weeks, testicular weight was increased slightly but remained significantly lower than at the pre-treatment level. Nevertheless, the percentage of 1C subpopulation returned to 74% of control within 2 weeks. In reference to the time table of spermatogenesis reported by Adler (1996), recovery within 2 weeks seems to suggest that a main target of NB toxicity is the germ cells after the early pachytene stage.

The data described above indicate that (i) haploid cells are quickly destroyed by the cytotoxic effect of NB, (ii) spermatocytes before early pachytene stage are not affected, (iii) meiotic division of secondary spermatocyte is suppressed but (iv) proliferation of spermatogonia and production of spermatocytes are little affected at least for a week. Levin et al. (1988) reported that sperm output through the vas deferens continued for 32 days after a single dose of NB, but their result cannot be compared with our finding which was obtained by observation of the testis.

The one-parametric flow cytometry of pepsin-treated testicular cells is incapable of distinguishing between spermatids and mature sperms within a haploid group. It is also not possible to distinguish spermatogonia, secondary spermatocytes and Sertoli cells, as well as Leydig and interstitial cells, within a diploid cell compartment. To identify and differentiate various cell types within each ploidy compartment, the techniques of multiparametric flow cytometry are required (Hittmair et al., 1992), and it has been proved to be possible using acridine orange (Evenson and Melamed, 1983; Janca et al., 1986), anti-BrdU antibody (Clausen et al., 1992; Meistrich et al., 1994) or anti-vimentin antibody (Hittmair et al., 1994). Cell kinetic analysis of spermatogenesis is insufficient as a means of toxicity testing, but the effect of abnormal chromatin condensation (Doibrinski et al., 1994) or sperm viability and number (Takizawa et al., 1995) on

![Fig. 6. Ratios of testicular cells after recovery periods (0 week : ; 2 weeks : ; 4 weeks : ) were compared with the value of control animals given corn oil for 2 weeks ( ). The columns with * and ** ( , , and ) were significantly different from control ( ) (* : P < 0.05, ** : P < 0.01). The columns with ### ( and ) were significantly different from (P < 0.01).]
fertility can also be assessed by flow cytometry.
An absolute number of cells in each ploidy compartment, not to say of a whole testis, cannot be counted by any means. As a practical alternative, we measured a relative number of cells and, by making reference to the testicular weight, we made a semi-quantitative judgment as to the increase or decrease of a total cell number. At the base of our judgment, there is an assumption that the weight will grossly reflect a total number of cells, although this depends on various histopathological changes such as edema, fibrosis, hemorrhage, etc. For that reason, a histological survey of materials will remain an important procedure that complements quantitative methods of analysis. With all of these limitations, flow cytometry provides an efficient, objective and quantitative means for the evaluation of male reproductive toxicity.

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REFERENCES


