

Flow cytometric evaluation on the age-dependent changes of testicular DNA contents in rats

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An age-dependent cellular change of DNA contents in the testis of Sprague-Dawley rats was investigated by flow-cytometric method. Testicular cell suspensions at the age of 4, 5, 6, 7, 8, 10, 12, 16 and 26 weeks were prepared and stained with propidium iodide. The relative proportions in the number of mature and immature haploid (1n), diploid (2n), S-phase and tetraploid (4n) cells were calculated. The proportion in the number of mature haploid cells was sharply increased to the age of 10 weeks (about 38%), thereafter increased slightly to the level of 42% at the age of 26 weeks. The proportion of immature haploid cells was dramatically increased to the age of 6 weeks, then maintained at the level of 20 to 30% thereafter. The proportion of diploid cells was 64% at the age of 4 weeks, then decreased gradually through the age of 26 weeks. The proportion of S-phase cells was increased to the age of 4 weeks, then maintained at a plateau level to the age of 26 weeks. The proportion of tetraploid cells were about 26% at the age of 4 weeks, then decreased gradually to the age of 26 weeks. These results suggest that the proportions of testicular cells may depend on the age of the rat and that the flow cytometric method may be useful in the evaluation of the spermatogenic status with regard to accuracy and sensitivity.

Key words: flow cytometry, SD rats, testis, DNA contents

Introduction

Assessment of a chemical or physical agent on reproductive functions is of paramount important when it may interfere with the ability of individuals to produce normal progeny [12]. Several methods have been used for the evaluation of a chemical on testicular damage. They include

mating and pregnancy outcome, sperm production and motility, and histopathology, etc [11]. These methods are, however, subjective and time-consuming [11]. Recently, flow cytometry (FCM) method has been used as a useful investigative tool in a wide range of disciplines including spermatogenic analysis [10]. 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 cell sampling [4]. It also provides quantitative values for evaluating different cell types on the basis of their DNA ploidy/stainability level [8].

Some mutagenic and/or cytotoxic chemicals have been known to exhibit stage-specific effects on germ cells or on reproductive maturation. The effects of these chemicals on developmental changes in the growing mammalian testis can be evaluated by FCM. Using FCM technique, we report the relative proportion of propidium iodide (PI)-stained testicular cells of Sprague-Dawley (SD) rats aged from 4 to 26 weeks old.

The results obtained in this study may support the usefulness of FCM with regard to accuracy and sensitivity in the evaluation of the spermatogenic status in normal and disturbed situation in rats.

Materials and Methods

Experimental Animals

Male SD rats aged from 4 to 26 weeks old were obtained from the laboratory animal resources of Korea Food and Drug Administration (KFDA). The animals were kept in plastic cages and fed with pelleted food and tap water *ad libitum*. Animal facilities were maintained at the temperature of $21\pm 2^{\circ}\text{C}$, the relative humidity of 60%, and a 12-h light/dark cycle. The animals were divided into nine experimental groups dependent on the age of rats (4, 5, 6, 7, 8, 10, 12, 16 and 26 weeks old). Each group contained 5 male rats.

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Sample Preparation

Five animals were killed by cervical dislocation at the age of 4, 5, 6, 7, 8, 10, 12, 16, and 26 weeks. Both right and left testes were surgically excised and testicular cell solutions were prepared for determining the relative proportions of haploid, diploid, S-phase and tetraploid cells using FCM technique. The preparation of testicular sample was performed by the following three steps. The stock solution containing 0.5 mM Tris, 3.4 mM trisodiumcitrate, 0.1% nonidet P-40 (NP-40), and 1.5 mM spermine tetrahydrochloride was prepared. First, clean nuclei were obtained by treatment of solution A (pH 7.6) containing 1,000 ml of the stock solution and 30 mg of trypsin. The trypsinization by solution A increased the fluorescence of nuclei with dense chromatin, presumably by splitting some chromosomal proteins. Second, RNase treatment with solution B (pH 7.6), containing 1,000 ml of stock solution, 500 mg of trypsin inhibitor, and 100 mg of RNase A, prevented dye binding to double-stranded RNA. Third, the use of solution C (pH 7.6), containing 1,000 ml of stock solution, 416 mg of propidium iodide, and 1160 mg of spermine tertahydrochloride, increased optimal stability. In brief, after removal of fat and connective tissue, testes were stored in citrate buffer (250 mM Sucrose, 40 mM Trisodium citrate · H₂O, 5% DMSO, pH 7.6) at -80°C in polypropylene tubes (52 × 17 mm tubes with screw cap, Wheaton, Millville, N.J. USA) until use. Each sample was thawed, decapsu-

lated and minced with surgical scissors, and treated for 30 min at room temperature with citrate buffer under a gentle magnetic stirring. Staining was done by a stepwise addition of the staining solutions. Solution A (1800 µl) was added to 200 µl of cell suspension (2×10^6 cells) in citrate buffer filtered through a polypropylene filter with 149-µm pore size (Spectrum Laboratories, Inc.) in order to discard tissue debris and the solutions were mixed gently. After standing for 10 min at room temperature and inverting the test tubes 2-3 times, 1500 µl of solution B was added to the cell solutions and again mixed gently. Then after standing for 10 min at room temperature, 1000 µl of ice-cold solution C was added to the cell solutions. The cell solutions were mixed and filtered through a 60-µm nylon filter (Spectrum Laboratories, Inc.) into tubes wrapped in aluminum foil for light protection of the propidium iodide. After addition of solution C, the samples were kept in an ice bath for 30 min to 3 hours until analysis.

Flow cytometry

The DNA contents of the dispersed testicular cells were measured by FCM (Coulter Epics XL, Coulter Corp., USA) which was 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

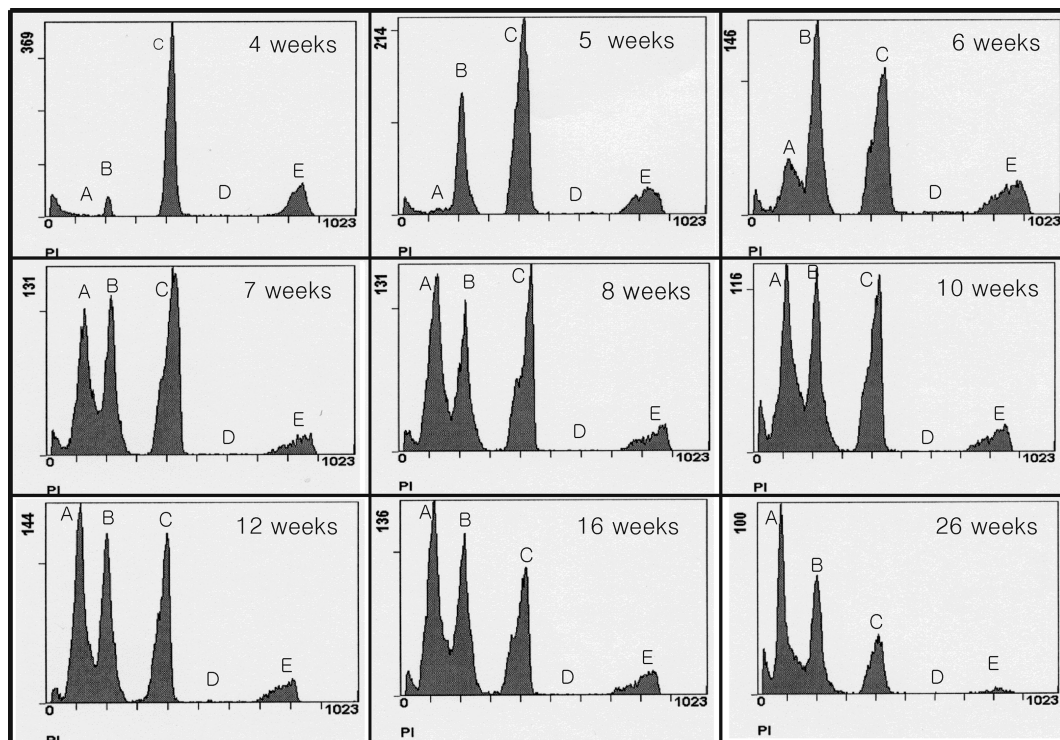


Fig. 1. Representative histograms driven from PI-stained testicular cells sampled at 4, 5, 6, 7, 8, 10, 12, 16 and 26 weeks age. A, B, C, D and E peaks represent mature haploid, immature haploid, diploid, S-phase and tetraploid cells, respectively.

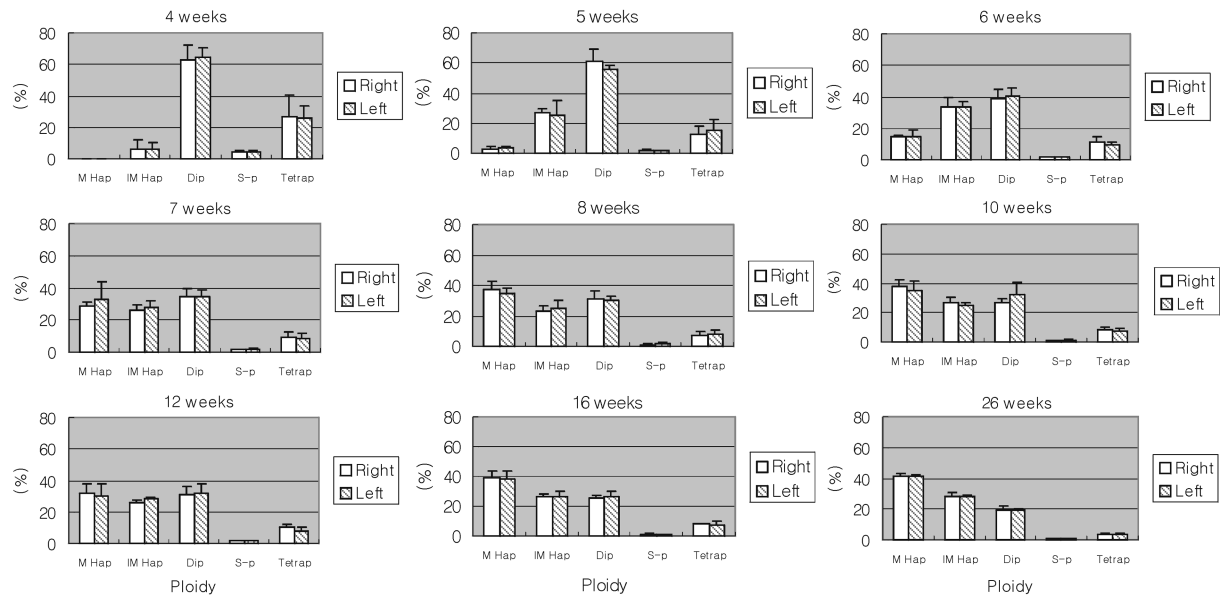


Fig. 2. Age-dependent percentages of mature haploid(M Hap), immature haploid(IM Hap), diploid(Dip), S-phase(S-p) and tetraploid(Tetrap) cells present in testis from prepubertal to adult male SD rats.

stain absorbed and so directly related to the DNA content of each cell. A total of 2×10^4 events was accumulated for each histogram. The histograms were analysed with the curve-integration routines provided by the Coulter Multi-parameter 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.

Results

Testicular cells obtained from rats aging 4, 5, 6, 7, 8, 10, 12, 16 and 26 weeks old were placed in suspension, stained with PI, and measured by flow cytometry. Fig. 1 displays representative frequencies showing changes in the proportion in the number of 1n (mature and immature haploid), 2n (diploid), and 4n (tetraploid) cells in the testis of rat. Testes of 4 weeks old rats exhibited two major peaks [2n cells (64%) and 4n cells (26%)] and one minor peak [1n cells (6%)]. In 5 weeks old rats, three definite populations (1n, 2n, and 4n) were observed. In 6 weeks old rats, testicular samples exhibited two distinct peaks within the 1n cell population consisting of round/elongating spermatids and elongated spermatids. In rats older than 6 weeks, the proportion of elongated spermatids increased steadily.

The ratios of the four testicular populations at each time point were shown in Fig. 2. The initial appearance of 1n cells (immature haploid) in 4 weeks old rats was coincident with the maximal number of the 4n cells. The proportion in the number of mature haploid cells was sharply increased to the age of 10 weeks (about 38%), thereafter

increased slightly to the level of 42% at the age of 26 week except for 12 weeks old rats. The proportion of immature haploid cells was dramatically increased to 33% at the age of 6 weeks, then maintained at the level between 20 and 30% thereafter. The proportion of diploid cells was 64% at the age of 4 weeks, then decreased gradually through the age of 26 weeks. The proportion of S-phase cells was also high at the age of 4 weeks, then maintained at a plateau level to the age of 26 weeks. The appearance rates of 4n cells showed a similar pattern to those of 2n cells. The proportion of tetraploid cells were about 26% at the age of 4 weeks, then decreased gradually to the age of 26 weeks.

Discussion

Several variables including seminiferous tubule diameter, testicular biopsy score, and tubular fertility have been used to assess the status of the testis. Recently, flow cytometry has been reported to be the most sensitive method [11]. In this study, we identified the various cell types occurring in the testes of SD rats aged from 4 weeks to 26 weeks old using FCM. Especially, the preparation of samples was performed by a integrated set of methods [3]. FCM analysis was performed on unfixed material to avoid a potentially selective cell loss caused by centrifugation step. Clumping and staining artifacts caused by fixatives were avoided. In addition, samples could be long-term stored by freezing(-70°C) in a citrate buffer with dimethyl sulfoxide (DMSO).

In this study, the three major phases including haploid cells (1n, round, elongating and elongated spermatids,

spermatozoa), diploid cells (2n, spermatogonial cells, secondary spermatocyte, Sertoli cells, Leydig cells) and tetraploid cells (4n, mostly primary spermatocyte) could be distinguished clearly by comparing fluorescent properties of propidium iodide-stained testicular cell populations [7]. Of these three phases, the haploid (1n) region was splitted into two peaks because of different stainability of elongated and round/elongating spermatids. This may reflect progressive condensation of chromatin structure. The nuclear packaging is known to reduce the number of DNA sites available for fluorochrome binding, thus resulting in an apparently sub-haploid DNA content [9]. The appearance of round spermatids in 4 weeks old rats marked the beginning of the first round of spermiogenesis, which continued to 4~5 weeks and was completed by 5 weeks when elongated spermatids were first detected (Fig. 1 & 2). In 4 to 8 weeks old rats, a dramatic change was occurred in the cell ratios in 1n, 2n and 4n testicular populations (Fig. 2). Briefly, mature haploid cells increased steadily through 26 weeks and immature haploid cells also did steadily through 6 weeks, then reaching to an plateau level at the age of 7 weeks to 26 weeks. In distribution of 2n cells, the relative proportion was the highest at the age of 4 weeks, but steadily decreased through 26 weeks. In this study, a decrease in 2n cell population and an accompanying increase in 1n cells population may result from the meiosis of secondary spermatocytes. There was a report that the proliferation of Sertoli cells supporting the development of germ cells may stop at the age of 12 days and the mitotic division of spermatogonia may occur at a relatively slow rate between the age of 13 and 84 days [12]. The appearance rates of 4n cells showed a similar pattern to those of 2n cells. Namely, the distributions of 4n and 2n cell populations were the highest with 26% and 64%, respectively, in 4 weeks old rats, but thereafter steadily decreased through 26 weeks (4% and 20%, respectively). The increases may result from accumulation of primary or secondary spermatocytes before the onset of the first or secondary meiotic division, and the decreases may result from a reduction in spermatogonia/preleptotene stages or spermatocytes. In the present study, collagenase was not used to liberate testicular cells; therefore, it is assumed that 2n and 4n cell populations contain primarily seminiferous epithelial cells which are more easily liberated by mechanical disruption than are somatic interstitial cells. Janca *et al* [5] reported that round and elongated spermatids appear at the age of 18 and 30 days, respectively, in mouse but an adult pattern occurs after 38 days old. Clausen *et al.* [1] said that the frequencies of 1n, 2n and 4n cell populations reach to adult levels at the age of 48 days in mouse and at the age of 40 days in rats. However, in the present study, the cell proportions were not increased to an adult level until 8 weeks (Fig. 1 & 2). These diverse results in testicular cell maturation might be related to the some differences in animal

strains, tools for observation, and treatment processes for analysis.

In this study, the kinetics of cellular changes in PI-stained testicular cells of growing SD rats was characterized by FCM. Because of the heterogeneity of cell classes involved in spermatogenesis, a detail assessment of changes in cell cycle may not be possible by means of DNA frequency distribution patterns alone. However, our work provides an basis for current studies evaluating the effects of exposure to chemical agents during different stages of reproductive development.

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