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DNA-Flow Cytometry of Defined Stages of Rat Seminiferous Epithelium: Effects of 3 Gy of High-Energy X-Irradiation

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Testes of adult Sprague-Dawley rats were irradiated locally by 3 Gy of 4 MeV X-rays produced by a linear accelerator. This type and dose of radiation gives an even distribution through the testis and selectively kills the proliferating spermatogonia. The seminiferous tubular cells were quantified by DNA flow cytometry at defined stages of the epithelial cycle at 7, 17, 22, 38, 52, and 80 days after irradiation. The flow cytometric technique was modified by using frozen instead of fresh samples. Freezing did not alter cell numbers when compared with fresh samples. At 7 days post-irradiation no significant changes were observed in any cell population by DNA flow cytometry, whereas histological analysis revealed a reduction in intermediate and type B spermatogonia. At 17 and 22 days post-irradiation, the number of cells at meiotic prophase (4C) was decreased, particularly in stages II–V of the cycle. In stages VII–VIII, cell numbers were 40 and 31%, and in stages IX–XIII, 24 and 43% of that in non-irradiated controls at 17 and 22 days, respectively. At 38 days after irradiation, both 4C and 1C (haploid) cells were decreased in number. The 4C cells were reduced to 24, 17, and 13% of that in non-irradiated controls in stages II–V, VII–VIII, and IX–XIII of the cycle, respectively. The corresponding numbers of 1C cells were 5, 17, and 4%. At 52 days after irradiation, 1C cells had declined to 38 and 19% of control values in stages II–V and IX–XIII, respectively. In stages II–V, 1C′ cells (haploid cells with condensed nuclei) declined to 28% of controls at 52 days. The present data provide a quantitative basis for the use of X-ray-irradiated rat testes as a model system in experiments pursuing interactions between Sertoli cells and spermatogenic cells.

Key words: testis, Sertoli, germ cell

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Irradiation of rat testis by a dose of 3 Gy destroys most of the cycling spermatogonia, but other spermatogenic cell types remain unaffected. Eventually, the irradiation-resistant noncycling spermatogonia repopulate the seminiferous epithelium (Dym and Clermont, 1970). This method has been used in studies of interactions between Sertoli cells

and spermatogenic cells, or localization of some phenomena in specific cell types of the seminiferous epithelium (Vihko et al, 1984; Pinon-Lataillade et al, 1988; Pineau et al, 1989; Kangasniemi et al, 1990a, 1990b).

Previously, a relatively low voltage (250 kV) irradiation was used, and the effect on the number of meiotic cells and spermatids in the rat was reported semi-quantitatively (Dym and Clermont, 1970). Pineau et al (1989) reported the effect of whole body neutron and gamma irradiation on spermatogenic cell number after acute and chronic irradiation. In the present study, linear accelerator-originated high voltage X-rays were used to produce a local homogeneous irradiation effect in the testis. The aim of this study was to quantitatively characterize the effect by combined DNA flow cytometry and morphometry.

We have previously performed DNA flow cytometric analyses of segments of seminiferous tubules at defined stages of the cycle to quantify cell numbers both in normal and pathologic testes (Toppari et al, 1985, 1986, 1988, 1989). Fresh samples were always used in these studies which caused limitations in designing the experiments. In toxicological studies of male reproduction, flow cytometry has proven to be a useful tool (Evenson et al, 1986a,b; Meistrich et al, 1978). In the present study, we investigated whether frozen samples can replace fresh samples.

Materials and Methods

Animals and Local Irradiation of Testes

Testes of Sprague-Dawley rats (3–4 months) were locally irradiated under light ether anesthesia by 3 Gy (calculated radiation dose in central target area, dose rate 2 Gy/min) of 4 MeV X-rays produced by a Clinac 4/100 linear accelerator (Varian, Palo Alto, CA). Focus-Skin-Distance (FSD) was 100 cm and field size was 5 × 5 cm.

Animals were killed at 7, 17, 22, 38, 52, and 80 days after irradiation for DNA flow cytometric and morphologic analyses. Number of non-irradiated control animals was eight for both fresh and frozen specimens. Number of irradiated animals was four to five at 7, 17, 22, and 38 days post-irradiation, two at 52 days, and one at 80 days. For histological analyses, number of animals at each time point after irradiation was five to six.

Determination of the X-ray dose distribution was confirmed with TLD (Thermo Luminescent Dosimeter, litium fluoride TLD-pellet, Alnor, Turku, Finland) in water.

Seminiferous Tubule Preparations

Seminiferous tubule segments from stages II, VIII, and IX–XI of the seminiferous epithelial cycle, were prepared by using transillumination-assisted microdissection in phosphate-buffered saline (Parvinen and Vanha-Perttula, 1972). The length of each segment was 2 mm and three samples were analyzed at each stage. At 52 days post-irradiation, no transillumination pattern was seen since step 13–19 spermatids were reduced in number. The stage of cycle was identified by phase contrast microscopic examination of live cell squash preparations (Söderström and Parvinen, 1976; Toppari et al, 1985) from adjacent segments. At 52 days, samples were divided into two stage groups: II–V and IX–XIII.

Freezing of Seminiferous Tubular Segments

The isolated seminiferous tubule segments were frozen in styrofoam boxes to −80 C in 20 μl of phosphate-buffered saline containing 10% dimethylsulfoxide (DMSO). Samples were stored at −80 C for 1–60 days before analyses.

DNA Flow Cytometry

DNA flow cytometric analyses were performed as described previously (Toppari et al, 1989). The use of volume standard beads was modified. Tubule segments were treated with a detergent (0.3% Nonidet p-40, BDH, Poole, England) in phosphate-buffered saline containing 0.2% bovine serum albumin, followed by incubation with ribonuclease A (5 μg/ml, Sigma, St. Louis, MO) and staining with propidium iodide (25 μg/ml, Sigma). A FACScan flow cytometer (Becton-Dickinson, Mountain View, CA) was used with fluorescent particles (Fluorespheres, Coulter Electronics, Hialeh, FL) as volume standards. Instead of counting a constant number of standard particles, a total of 5000 fluorescent impulses were counted and calculated for volume standardization in each sample.

Step 1–14 spermatids form a haploid 1C peak; step 15–19 spermatids bind less propidium iodide producing the 1C′ peak which has been confirmed by sorting out the nuclei and examining them with a fluorescence and phase-contrast microscope (Toppari, 1986). The 2C peak contains somatic cells of seminiferous tubules (Sertoli cells), most spermatogonia, stage VII preleptotene spermatocytes, and secondary spermatocytes. Spermatogonia in their G_2/M phase of mitosis and primary spermatocytes form the 4C peak (Toppari et al, 1985).

Morphometric Analyses

Pieces of testes from both non-irradiated control animals and irradiated animals were fixed in Bouin's fluid, embedded in paraffin, sectioned at 5 μm and stained with the periodic acid-Schiff hematoxylin technique. Cells from right angle round cross-sections of seminiferous tubules were counted at stages II–V, VII–VIII, and IX–XIII of the cycle (five tubules per stage).

Statistical Analyses

Statistical analyses were made by a BMDP statistical program (Los Angeles, CA) using one-way analysis of variance and pairwise T-test together with Bonferroni corrections.

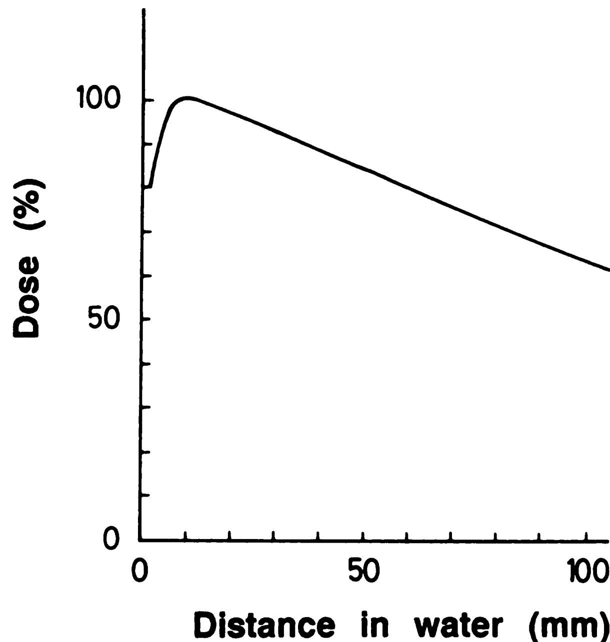

Fig. 1. The relative dose of irradiation at different distances (2.5, 5, 7.5, 10, 12.5, 17.5, 20, and 45 mm) in water measured by ThermoLuminescent Dosimeter. The dose was homogeneously 90–100% of the maximum between ca 5 and 40 mm from skin surface covering the space of the testis. All data points are accurately located on the curve with nondetectable standard deviations.

Results

The dose of irradiation in different parts of testis was homogeneous as revealed by measurement in the water model (Fig. 1). The maximal dose of irradiation was measured at the depth of 10 mm. The dose was 90–100% of the maximum between ca 5 and 40 mm from skin surface covering the area of the testis.

At 7 days post-irradiation, morphometric analyses showed that intermediate and type B spermatogonia had decreased to 3% of the non-irradiated control values in stages II–VI, and preleptotene spermatocytes of stages VII–VIII and leptotene and zygotene spermatocytes in stages IX–XIII were reduced to 13 and 56% of control values, respectively (Table 1, Fig. 2b). However, no statistically significant differences were found in the numbers of any cell types by flow cytometry (Fig. 3).

At 17 days post-irradiation, flow cytometric analyses showed that the 4C cells were reduced to 22, 40, and 24% of control values in stages II, VIII, and IX–XI, respectively (Fig. 3). Morphological analyses showed low numbers of spermatogonia (intermediate and type B), preleptotene- and leptotene spermatocytes, and pachytene spermatocytes (stages II–VIII, Table 1, Fig. 2c).

At 22 days, 4C cells were reduced to 25, 31, and 43% of control values in stages II, VIII, and IX–XI, respectively as analyzed by flow cytometry (Fig. 3). At 38 days, both the 4C and 1C cells showed low numbers: the 4C cells were reduced to 24, 17, and 13% of control values, and the 1C cells to 5, 17, and 4% in stages II, VIII, and IX–XI, respectively. Morphometric analyses gave similar results: pachytene spermatocytes and round spermatids showed low numbers (Table 1, Fig. 2d).

TABLE 1. *Number of spermatogenic cells after irradiation**

Stage	Cell type†	7 d	17 d	38 d	52 d
II–VI	I + B	3§	18‡	71	80
	ps	104	8§	6§	49‡
	rs	106	99	7§	23§
VII–VIII	pl	13§	23§	79	76
	ps	104	9§	1§	52§
	rs	103	104	41§	18§
IX–XIII	zs + ls	56	4§	60	86
	ps	101	43	15§	67‡
	rs	105	111	58§	27§

*Cell numbers are presented as percent of non-irradiated control (mean, n = 5) at 7, 17, 38, and 52 days post-irradiation. Cells from right angle round cross-sections of five rat seminiferous tubules per stage were counted.

†I = intermediate spermatogonia
B = type B spermatogonia
ps = pachytene spermatocytes
rs = round spermatids
pl = preleptotene spermatocytes
zs = zygotene spermatocytes
ls = leptotene spermatocytes

‡,§ Statistically significant differences as compared with non-irradiated control (§$p < 0.01$; ‡$p < 0.05$).

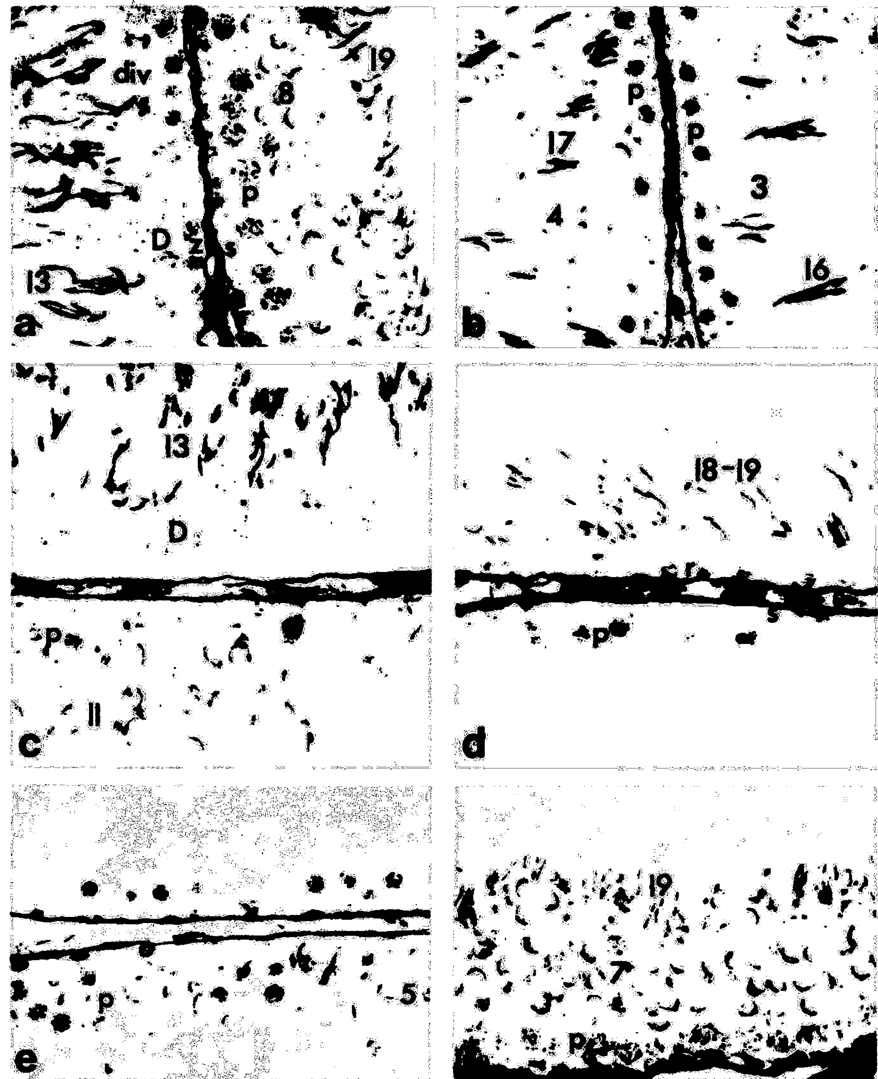

Fig. 2. Light micrographs of PAS-hematoxylin stained sections of the testis of non-irradiated control (a) and irradiated rat at 7 (b), 17 (c), 38 (d), 52 (e) and 80 (f) days after irradiation. In the control section (a), left side is from stages XIII (lower part) and XIV (upper part), and right side from stage VIII of the seminiferous epithelial cycle. Spermatogonia and preleptotene spermatocytes (r) lay on the basement membrane of the tubules between Sertoli cells (s). Leptotene, zygotene (z), pachytene (p), diplotene (D), and dividing (div) spermatocytes form the next layer, above which round and elongating spermatids (indicated by arabic numerals 3–19) are located. At 7 days post-irradiation (b), the basement membrane of the seminiferous epithelium was devoid of spermatogonia and early primary spermatocytes, whereas pachytene spermatocytes and spermatids were present in the epithelium as shown in these stage III (right side) and IV (left side) segments. At 17 days (c), the absence of most primary spermatocytes was evident. In stage XI (lower part), leptotene spermatocytes were completely absent and the number of pachytene spermatocytes was reduced. In stage XIII (upper part), zygotene spermatocytes were absent, whereas many diplotene spermatocytes were still present, indicating that the gap in spermatogenesis extended to stage XI pachytene spermatocytes at this timepoint. At 38 days (d), pachytene spermatocytes and round spermatids were depleted, whereas spermatogonia and early spermatocytes were repopulated. At 52 days (e), all spermatids were decreased in number in irradiated animals, whereas meiotic cells had recovered. At 80 days post-irradiation, a total recovery in the irradiated testes was observed. × 310.

At 52 days post-irradiation, the number of 1C cells was decreased to 38 and 19% of control values in stages II–VI and IX–XIII, respectively (Fig. 3). The numbers of 1C′ cells was reduced to 28% of control value in stages II–VI (data not shown). Morphometric studies confirmed low pachytene spermatocyte and spermatid numbers (Table 1, Fig. 2e).

One animal was analyzed by flow cytometry at 80 days post-irradiation, and normal cell numbers were measured in all stages of the cycle (Figs. 3, 2f). Also the transillumination pattern was normal. Cell numbers from fresh and frozen specimens of non-irradiated animals did not differ significantly from each other (Fig. 3).

Discussion

The present data are in good accord with our previous results of cell numbers in seminiferous tubules after irradiation by a 250 kV X-ray generator (Toppari et al, 1988). However, dosage in different parts of testes is more homogenous when a 4 MeV linear accelerator is used to produce irradiation as compared with an X-ray generator; the present dose distribution analyses showed a maximal dose in the approximate center of the testis, and irradiation effect was homogenous throughout the organ. The maximal dose was at skin when 250 kV voltage was used.

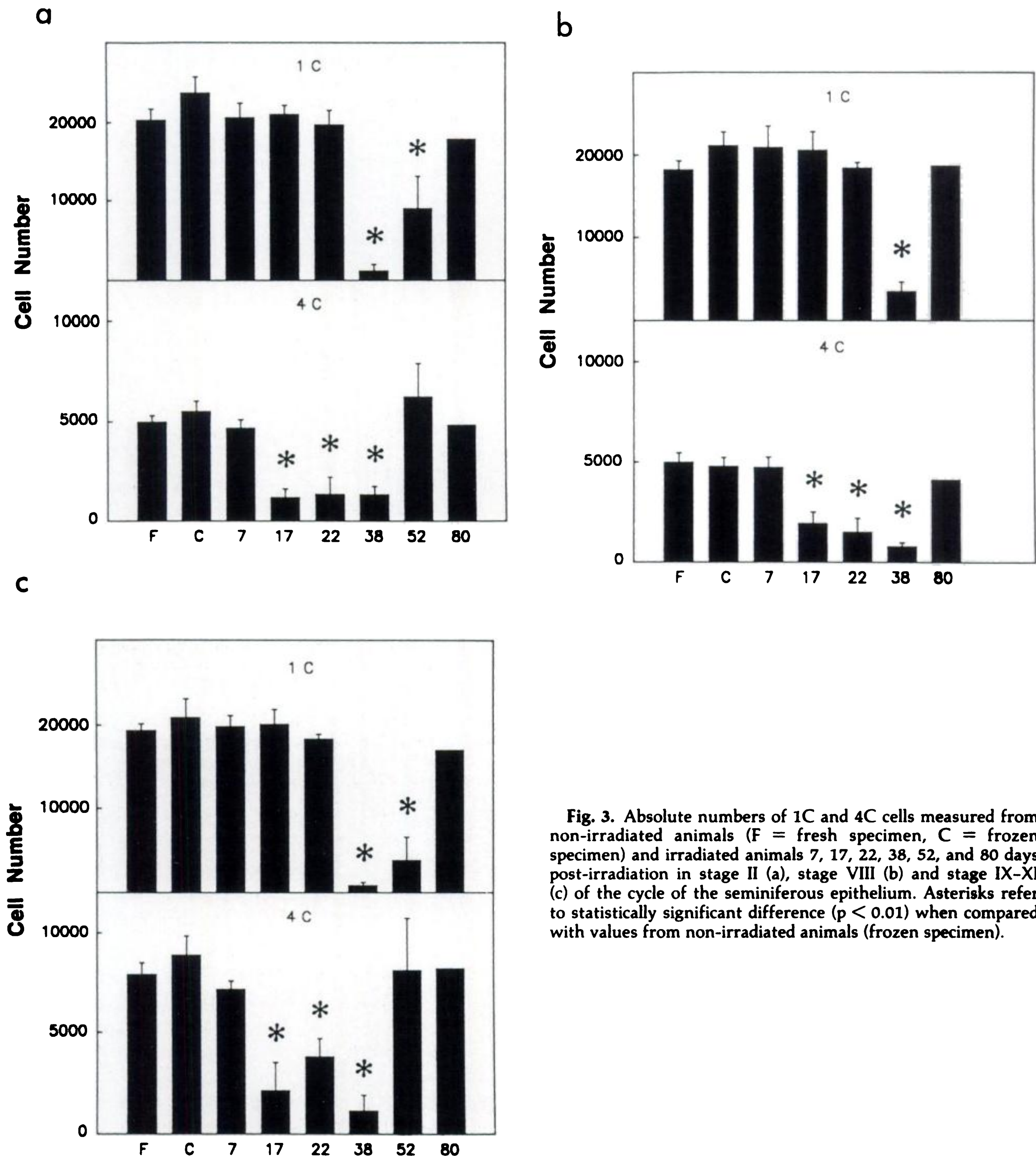

Fig. 3. Absolute numbers of 1C and 4C cells measured from non-irradiated animals (F = fresh specimen, C = frozen specimen) and irradiated animals 7, 17, 22, 38, 52, and 80 days post-irradiation in stage II (a), stage VIII (b) and stage IX–XI (c) of the cycle of the seminiferous epithelium. Asterisks refer to statistically significant difference ($p < 0.01$) when compared with values from non-irradiated animals (frozen specimen).

Pineau et al (1989) used whole body irradiation to destroy differentiating spermatogonia (0.99 Gy of neutron and 0.24 Gy of gamma-rays). Cell numbers after irradiation declined in the same manner as in our study, but the recovery from the irradiation effect was slower, suggesting that the present method may cause more selective destruction of specific spermatogenic cells. The systemic effects of irradiation are also avoided when irradiation is given locally.

Chemicals have also been used to generate 'gaps' in the seminiferous epithelium. Methoxy acetid acid (MAA) destroys spermatogenic cells (Bartlett et al, 1988). This treatment resulted in the selective depletion of pachytene, and later spermatocytes, from seminiferous tubules at all stages other than VIII–XI of the epithelial cycle. After treatment, mainly round and elongated spermatids were decreased in number in the majority of seminiferous tubules. However, no changes in spermatogonial number were reported. All cytotoxic drugs may have systemic effects and longer exposure times than irradiation, which may confound the results. MAA may prove useful in the study of paracrine interactions *in vivo* considering pachytene primary spermatocytes. However, all spermatogenic cell types can be selectively depleted by using local irradiation of testes.

When results of morphologic and flow cytometric studies are compared, there seems to be a tendency to get greater cell numbers by flow cytometric analyses. This might be a consequence of the microdissection method used in the isolation of flow cytometric samples, since a proper stage identification may cause a bias to select the best-maintained tubules for analysis. It is, therefore, recommended to use combined flow cytometric and morphologic analyses in quantitative studies of spermatogenesis. This is particularly necessary in the assessment of the 2C cells, because those are influenced by both Sertoli cells and spermatogonia. Morphometry is therefore much more sensitive in detection of changes in the spermatogonia population, whereas the number of spermatids (1C′: steps 15–19; 1C: steps 1–14) and primary spermatocytes (main part of the 4C group) can be determined fairly accurately by DNA flow cytometry.

Freezing and storing the seminiferous tubular specimen for DNA flow cytometric analyses proved to be a practical technique in quantitative analyses of spermatogenic cell numbers. Freezing did not affect the cell numbers or stainability, as evaluated by relative fluorescence intensities. This study provides a quantitative basis for the interpretation of results obtained in the studies of testicular cell interactions using locally irradiated rat testis model.

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