

# Quantification of Apoptotic Testicular Germ Cells in Normal and Methoxyacetic Acid-Treated Mice as Determined by Flow Cytometry

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**ABSTRACT:** Several studies have reported the occurrence and significance of programmed cell death (apoptosis) of testicular germ cells in mammals. In those studies, apoptotic germ cells were identified by morphological criteria or by *in situ* end labeling (TUNEL) and were enumerated from histological sections by semi-quantitative and time-consuming techniques. In the present study, we have established a flow cytometric technique for quantification of TUNEL-positive cells in the mouse testis. Groups of five adult mice each received 0, 650, or 1300 mg/kg (IP) of methoxyacetic acid (MAA), and testes were collected 24 hours later. MAA is known to induce germ cell apoptosis in rodent testes. MAA induced a significant ( $P < 0.01$ ) dose-dependent decline in the percentage of pachytene spermatocytes (4C cells). DNA strand breaks generated by the activation of endogenous endonuclease in the apoptotic germ cells were detected by the *in situ* labeling of the 3'-OH termini with biotinylated dUTP in the presence of terminal deoxynucleotidyl transferase (TUNEL technique). Histologically, TUNEL-positive germ cells were observed in control testes, and the number of these cells was visibly increased following MAA exposure. As determined by flow cytometry, four cell populations contained TUNEL-positive cells: 1C cells (round spermatids), 2C cells (mainly spermat-

ogonia), S-ph cells (spermatogonial cells and preleptotene spermatocytes synthesizing DNA [the S-phase]), and 4C cells (primary spermatocytes). Analysis of the percentages of TUNEL-positive cells within each population yielded values of  $1.57 \pm 0.23\%$  for 1C cells,  $1.65 \pm 0.27\%$  for 2C cells,  $6.26 \pm 1.03\%$  for S-ph cells, and  $3.24 \pm 0.39\%$  for 4C cells. Hence, a substantial proportion of proliferating cells are undergoing apoptosis during normal spermatogenesis. The overall incidence of apoptotic cells among all testicular cells was around 2%. At 650 mg per kilogram of body weight, MAA induced a fourfold to eightfold increase ( $P < 0.001$ ) in the percentage of TUNEL-positive cells, compared with saline-treated controls, and, overall, 17% of testicular cells were apoptotic. This effect of MAA was most pronounced for S-ph and 4C cells, with 25–30% of cells being affected in each of those populations. At 1300 mg per kilogram of body weight, MAA had no further effect. These quantitative data demonstrate that 1) in the normal testis, it is mainly proliferating cells that undergo apoptosis, and 2) MAA induces primary spermatocyte loss by germ cell apoptosis.

Key words: Spermatogenesis, TUNEL.  
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The study of testicular germ cell apoptosis has become a subject of increasing interest for investigators working in the area of male reproductive endocrinology and toxicology (Darzynkiewicz et al, 1997). Although cohorts of spermatogonial stem cells enter the process of spermatogenesis, not all proliferating spermatogonia or their subsequent cell types survive and complete terminal differentiation. The presence of disintegrating germ cells at various stages of spermatogenesis of normal, healthy, and fertile individuals has been noticed in routine histological examination (Bartke, 1995). The loss of spermatogonial cells during normal spermatogenesis results in an estimated loss in the range of 25–75% of the theoretical

sperm production in the adult testis (Oakberg, 1956; Huckins, 1978; De Rooij and Lok, 1987). It has been shown that degeneration of rodent germ cells occurs via programmed cell death, or apoptosis (Allan et al, 1992; Tapanainen et al, 1993; Brinkworth et al, 1995; Henriksen et al, 1995; Sinha Hikim et al, 1995).

The active participation of the cell in its self-destruction is the characteristic feature of apoptosis. The cell triggers a series of events in tandem that lead to its degeneration and to the formation of apoptotic bodies that are subsequently engulfed by the neighboring cell without invoking inflammation (Wyllie, 1985, 1992; Arends et al, 1990; Tomei and Cope, 1991; Compton, 1992). The entire process of apoptosis is characterized by the following phenomena: increased intracellular calcium concentration, cell dehydration, increased lipid peroxidation, chromatin condensation originating at the nuclear periphery, activation of endonuclease that cleaves preferentially at internucleosomal DNA, proteolysis, and fragmentation of the nucleus and cell (Kerr et al, 1972; Wyllie et al, 1980, 1984; Wyllie, 1985, 1987, 1992, 1998). The presence of

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DNA strand breaks in apoptotic germ cells can be detected *in situ* by labeling the 3'-OH termini in DNA breaks with biotinylated deoxyuridine triphosphate in the presence of exogenous terminal deoxynucleotidyl transferase, a procedure known as the TUNEL technique (Eastman and Barry, 1992; Gavrieli et al, 1992; Gorczyca et al, 1993). The studies thus far reported on testicular germ cell apoptosis have used classical histological examination of the *in situ* end-labeled testicular tissue to quantify apoptotic germ cells, which is a semi-quantitative, time-consuming, and painstaking procedure. Although a flow cytometric method to quantify apoptotic cells by TUNEL and by single cell gel electrophoresis technique (COMET) assay has already been applied to assess DNA strand breaks in abnormal human sperm (Gorczyca et al, 1993; Sailer et al, 1995; Aravindan et al, 1997), such a method has not been employed to identify the DNA breaks present in the induced or spontaneously occurring apoptotic testicular germ cells. The present study attempts to quantify the apoptotic testicular germ cells by flow cytometry. To standardize this technique, we chose to treat mice with methoxyacetic acid (MAA), which is known to induce testicular germ cell apoptosis (Foster et al, 1983, 1984; Creasy et al, 1985; Bartlett et al, 1988; Brinkworth et al, 1995).

## Materials and Methods

### Experimental Protocol

Eight-week-old C57/BL6 mice weighing  $30 \pm 2$  g were maintained under standard conditions of temperature, light, and humidity, with food and water provided *ad libitum*. Fifteen adult male mice were randomly assigned to three groups of five mice each. Each group of mice received a single intraperitoneal injection of 0 (saline), 650, or 1300 mg of MAA (Aldrich-Chemie, Steinheim, Germany) per kilogram of body weight. The MAA doses chosen were known to cause germ cell apoptosis in rodents (Foster et al, 1983, 1984; Creasy et al, 1985; Sharpe et al, 1993; Brinkworth et al, 1995). Twenty-four hours later, animals were decapitated after sedation with an overdose of carbon dioxide. The testes were removed, freed from fat, and weighed. One testis was used for flow cytometric analysis, and the other one was fixed in Carnoy's fluid for histological analysis.

### Preparation of Monocellular Suspension of Testicular Cells

Testicular cells were released from the seminiferous tubules (contralaterally to the epididymis to avoid the rete testis) in calcium- and magnesium-free phosphate-buffered saline (PBS) by mincing the tissue with fine curved scissors. The minced tissue was gently aspirated to disperse the cells and was washed with PBS (800 g for 10 minutes). The pellet was resuspended in 1 ml PBS and filtered through 100- $\mu$ m nylon mesh. The cells were fixed in 70% chilled ethanol and stored at 4°C until further analysis (Krishnamurthy et al, 1998).

### Propidium Iodide Staining of Testicular Cells to Determine the Percentages of Germ Cells by DNA Flow Cytometry

Testicular cells were stained with PI as previously described (Krishnamurthy et al, 1998). Briefly, an aliquot of 1–2 ml ethanol fixed testicular cells was washed twice with PBS and incubated in 300  $\mu$ l of 0.5% pepsin solution (prepared in 0.9% saline, pH 2.0) for 10 minutes at 37°C. After centrifugation, cells were stained with propidium iodide (PI) staining solution (25  $\mu$ g/ml PI, 40  $\mu$ g/ml RNase, and 0.3% Nonidet P-40 in PBS) for 20 minutes at room temperature. All chemicals were procured from Sigma Chemical Company (Deisenhofen, Germany), unless otherwise stated. The PI-stained cells were analyzed with a Coulter Flow Cytometer EPICS XL (Coulter, Krefeld, Germany) equipped with a 15-mW argon ion laser at an excitation wavelength of 488 nm. The fluorescence signals of the PI-stained germ cells were collected with a 620-band-pass filter (605–635 nm).

### In Situ End Labeling of Testicular Germ Cells for Flow Cytometric Analysis

The procedure employed for TUNEL was similar to the one described by Gorczyca et al (1993). Briefly, about 1–2 ml ethanol-fixed testicular cells was washed twice in PBS. The cells were treated with 300  $\mu$ l of 0.5% pepsin solution (prepared in 0.9% saline, pH 2.0) for 5 minutes at 37°C. The cells were washed twice in 1 ml PBS and resuspended in 50  $\mu$ l of TdT buffer containing 0.2 M potassium cacodylate, 2.5 mM Tris-HCl (pH 6.6), 2.5 mM CoCl<sub>2</sub>, 0.25 mg/ml bovine serum albumin, 5 units of terminal deoxynucleotidyl transferase (Promega, Madison, Wisconsin; Serva, Heidelberg, Germany), and 0.5 nM biotinylated deoxyuridine triphosphate (b-dUTP; Boehringer-Mannheim GmbH, Mannheim, Germany). Cells were incubated in this solution at 37°C for 30 minutes and then washed in PBS and resuspended in 100  $\mu$ l of a solution containing 4 $\times$ -concentrated saline-sodium citrate buffer, 2.5  $\mu$ g/ml fluoresceinated avidin (Boehringer-Mannheim), 0.1% Triton X-100, and 5% dry milk. The cells were incubated in this solution for 30 minutes at room temperature in the dark, washed in 1 ml of PBS containing 0.1% Triton X-100, and resuspended in 1 ml PBS containing 5  $\mu$ g/ml PI and 0.1% RNase. Control cells were processed as described but without TdT. The intensity of the green fluorescence of avidin-FITC-stained cells and of the red fluorescence of PI-stained cells was measured on the Coulter Flow Cytometer EPICS XL. The fluorescence signals of FITC were collected with a 525-band-pass filter (505–545 nm), and those of PI were collected at 620-band-pass filter (605–635 nm). A marker was set in the TUNEL histograms at the cut-off between background signals and positive staining, as determined from the control samples, and was applied to analysis of treated samples to quantify TUNEL-positive cells (see Fig. 3 for further details).

### In Situ End Labeling and Evaluation of Testicular Sections

Testicular tissue was fixed in Carnoy's fluid, dehydrated, embedded in paraffin wax according to routine procedures, and sectioned at 5  $\mu$ m. TUNEL was performed on the tissue according

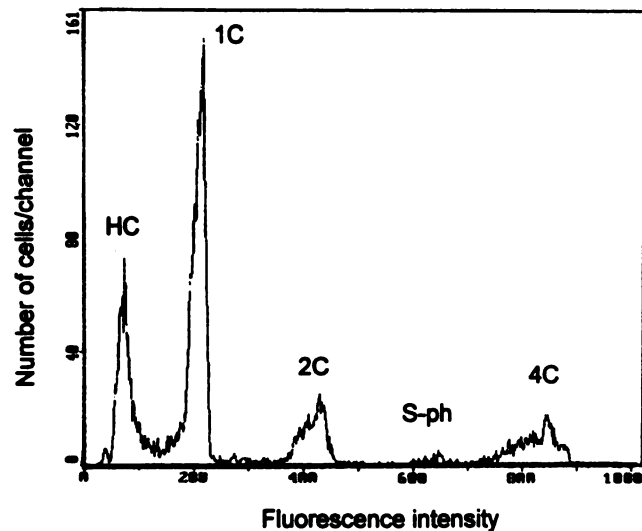


FIG. 1. Representative DNA content distribution flow cytogram of testicular germ cells from a control mouse. The five quantifiable populations were elongating and elongated spermatids (HC; H = hypostainability of compacted DNA during spermiogenesis), round spermatids (1C), spermatogonia and somatic cells (2C), and tetraploid cells comprising predominantly primary spermatocytes and G2-spermatogonia (4C). Spermatogonial cells synthesizing DNA and preleptotene spermatocytes appear between the peaks 2C and 4C and are represented as S-phase cells (S-ph).

to procedures described earlier (Gavrieli et al, 1992, Brinkworth et al, 1995). Briefly, sections were deparaffinized, rehydrated, washed for 10 minutes in running water, and rinsed in TdT buffer. The sections were incubated with a reaction mixture containing TdT buffer, 0.5 nM of biotin-16-deoxyuridine triphosphate (Boehringer-Mannheim), and 5 units of TdT (Promega; Serva) in a moist chamber for 1 hour at 37°C. The reaction was terminated by rinsing the slides thoroughly in Tris buffer. Extravidin-peroxidase complex and diaminobenzidine were used to visualize the biotinylation, and hematoxylin was used as a counterstain when appropriate. Control slides were processed in an identical manner, except that TdT was omitted. Spermatogenesis was evaluated according to the staging criteria described by Russell et al (1990). Since the classification of early stages is based almost entirely upon the morphology of the developing acrosome, which cannot be seen on hematoxylin-stained sections, assessment of these stages was made from longitudinal sections of the seminiferous tubules.

### Data Analysis

Statistical significances were assessed by one-way analysis of variance (ANOVA), followed by a Bonferroni test using Graph Pad Instat Software (Graph PAD, San Diego, California). The level of significance was set at 5%. Data are expressed as mean values  $\pm$  SEM.

## Results

Administration of MAA did not affect testicular weight (data not shown). Figure 1 depicts the frequency distri-

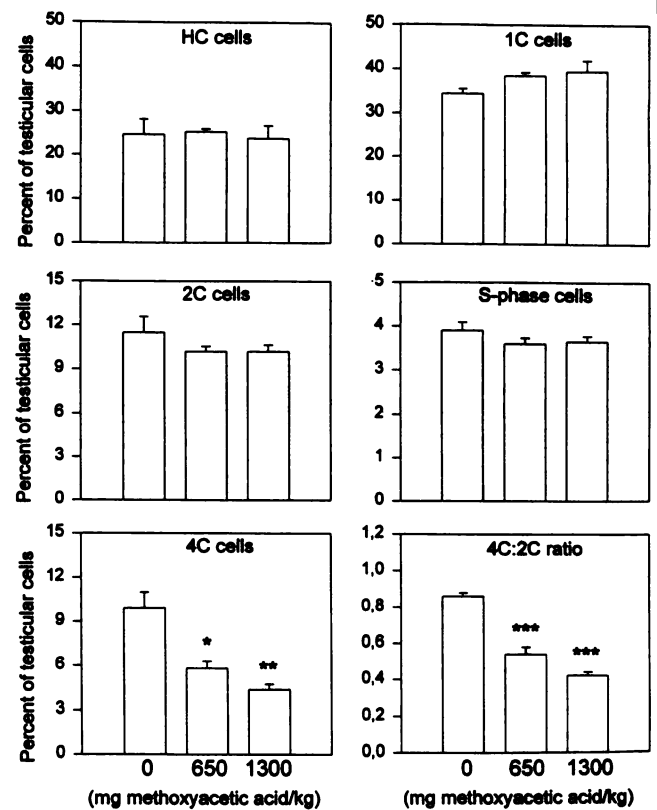


FIG. 2. Effects of methoxyacetic acid treatment on testicular cell populations in the mouse. Data are mean values  $\pm$  SEM of five animals/group. \*  $P < 0.05$  vs. control (0 mg/kg); \*\*  $P < 0.01$  vs. control; \*\*\*  $P < 0.001$  vs. control.

bution histogram of the pepsin-treated, PI-stained testicular cells as determined by DNA flow cytometry. The five quantifiable populations were as follows: elongating and elongated spermatids (HC; H = hypostainability of compacted DNA during spermiogenesis), round spermatids (1C), spermatogonia and somatic cells (2C), and tetraploid cells predominantly comprising primary spermatocytes and G2 spermatogonia (4C). Spermatogonial cells and preleptotene spermatocytes synthesizing DNA appear between the peaks 2C and 4C and are represented as S-ph cells. The proportion of somatic cells (Sertoli cells, Leydig cells, and other nongerm cells) has been shown to comprise less than 3% of total testicular cells in the mouse testis (Clausen et al, 1978; Suter et al, 1997). Although administration of MAA resulted in a slight decrease in the proportion of 2C cells and S-ph cells, a significant ( $P < 0.01$ ) and dose-dependent decline was observed in the 4C population (Fig. 2), compared with the saline-treated control group. Consequently, the 4C:2C ratio dropped significantly ( $P < 0.001$ ).

Figure 3 depicts the representative TUNEL data from the mice treated with 0, 650, and 1300 mg of MAA per kilogram of body weight as determined by flow cytometry. Figure 3a, f, and k shows the frequency histograms

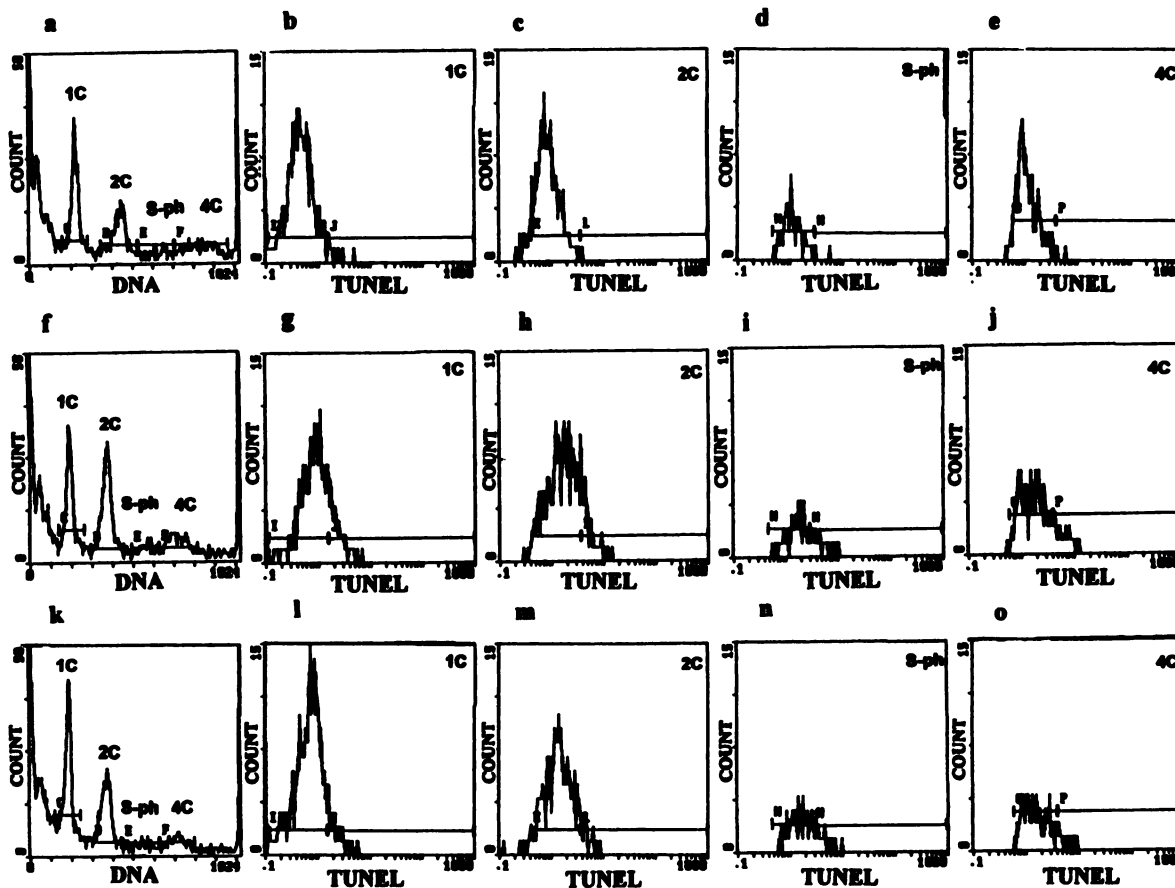


FIG. 3. Representative DNA histograms of the testicular germ cells (a,f, and k) and frequency histograms of the *in situ* end-labeled (TUNEL) germ cells (b-e, g-j, l-o) of mice treated with 0 (upper panels), 650 (middle panels), and 1300 mg (bottom panels) of methoxyacetic acid. Panels a, f, and k show the representative frequency histograms of the PI-bound germ cell distribution for each treatment group. The markers set on these histograms were retained from the control (i.e., no enzyme) samples to quantify TUNEL-positive cells. The cells falling in the markers J, L, N, and P of panels b-e, g-j, and l-o were considered TUNEL-positive cells.

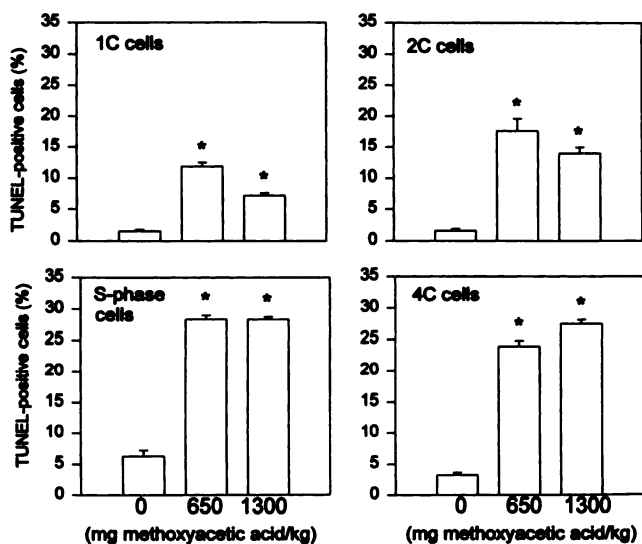


FIG. 4. The percentage of TUNEL-positive cells within each population of testicular germ cells in mice treated with 0, 650, and 1300 mg of methoxyacetic acid. Data are mean values  $\pm$  SEM of five animals/group. \*  $P < 0.001$  vs. control (0 mg/kg).

of the propidium iodide-counterstained germ cell distribution of mice treated with 0, 650, and 1300 mg MAA, respectively. Figure 3b-e, g-j, and l-o show the corresponding green fluorescence histograms (obtained by gating the different germ cell populations according to DNA content; see Fig. 3a, f, and k) of TUNEL cells of the 0, 650, and 1300 mg of MAA-treated mice, respectively. The markers set on these histograms were retained from the control (i.e., no enzyme) samples to quantify TUNEL-positive cells. The cells falling in the markers J, L, N, and P of Figure 3b-e, g-j, and l-o were considered TUNEL-positive cells. Figure 4, derived from Figure 3b-e, g-j, and l-o, shows the percentage of different types of TUNEL-positive germ cells of the mice treated with 0, 650, and 1300 mg of MAA.

As determined by flow cytometry, four cell populations contained TUNEL-positive cells: 1C, 2C, S-ph, and 4C cells. Analysis of the percentage of TUNEL-positive cells within each population yielded values of  $1.57 \pm 0.23\%$  for 1C,  $1.65 \pm 0.27\%$  for 2C,  $6.26 \pm 1.03\%$  for S-ph, and  $3.24 \pm 0.39\%$  for 4C cells (Fig. 4). Hence, a sub-

Table 1. TUNEL-positive cells as percentages of all testicular cells with respect to the appearance of each segment of the cell population based on DNA content in the testis of control and methoxyacetic acid-treated mice (MAA)

MAA (dose)	Cell population				
	1C cells	2C cells	S-phase	4C cells	Total*
0 mg/kg	0.65 ± 0.02	0.44 ± 0.03	0.58 ± 0.03	0.60 ± 0.03	2.3
650 mg/kg	4.48 ± 0.16†	6.02 ± 0.40†	3.17 ± 0.11†	3.84 ± 0.11†	17.5
1300 mg/kg	3.18 ± 0.04†	4.25 ± 0.13†	3.06 ± 0.14†	3.30 ± 0.08†	13.8

Mean ± SEM; n = 5/group.

\* Total value represents the percentage of testicular cells undergoing apoptosis.

† P < 0.001 vs. 0 mg/kg.

stantial proportion of proliferating cells is undergoing apoptosis during normal spermatogenesis. The overall incidence of apoptotic cells in all testicular cells was 2.3% (Table 1). At 650 mg/kg, MAA induced a fourfold to eightfold increase ( $P < 0.001$ ) in the frequency of TUNEL-positive cells, compared with their appearance in saline-treated controls (Fig. 4). This effect of MAA was most pronounced for S-ph and 4C cells, with 25–30% of cells affected in each population (Fig. 4). Following MAA treatment, 17% of testicular cells were apoptotic (Table 1). No further increase of apoptosis was observed in mice treated with 1300 mg of MAA, compared with those treated with 650 mg. Also, a significant ( $P < 0.05$ ) decline in the mean channel number of the fluorescence intensity of the PI-bound testicular cells was observed in MAA-treated mice (Table 2).

Histological analysis revealed TUNEL-positive germ cells in the control testis (Fig. 5a). These cells comprised late spermatocytes in the division phase, secondary spermatocytes (Fig. 5c), and early round spermatids. TUNEL-positive cells were also encountered at the base of the germinal epithelium, but it could not be decided unequivocally whether these cells were spermatogonia or early spermatocytes, except for occasional dividing cells, which were considered spermatogonia. Administration of MAA caused a massive accumulation of TUNEL-positive germ cells (Fig. 5b). These cells were predominantly spermatocytes (Fig. 5d). A few early round spermatids were also found to be TUNEL-positive. TUNEL-positive somatic cells were not encountered in the present study.

Table 2. Mean channel number of fluorescence intensity of propidium iodid-bound testicular cells from mice treated with methoxyacetic acid (MAA)

MAA (dose)	1C cells	2C cells	S-phase	4C cells
0 mg/kg	241 ± 7	422 ± 10	601 ± 10	811 ± 20
650 mg/kg	193 ± 1*	373 ± 2*	559 ± 3*	730 ± 3*
1300 mg/kg	185 ± 1*	366 ± 1*	552 ± 2*	721 ± 1*

Mean ± SEM; n = 5/group.

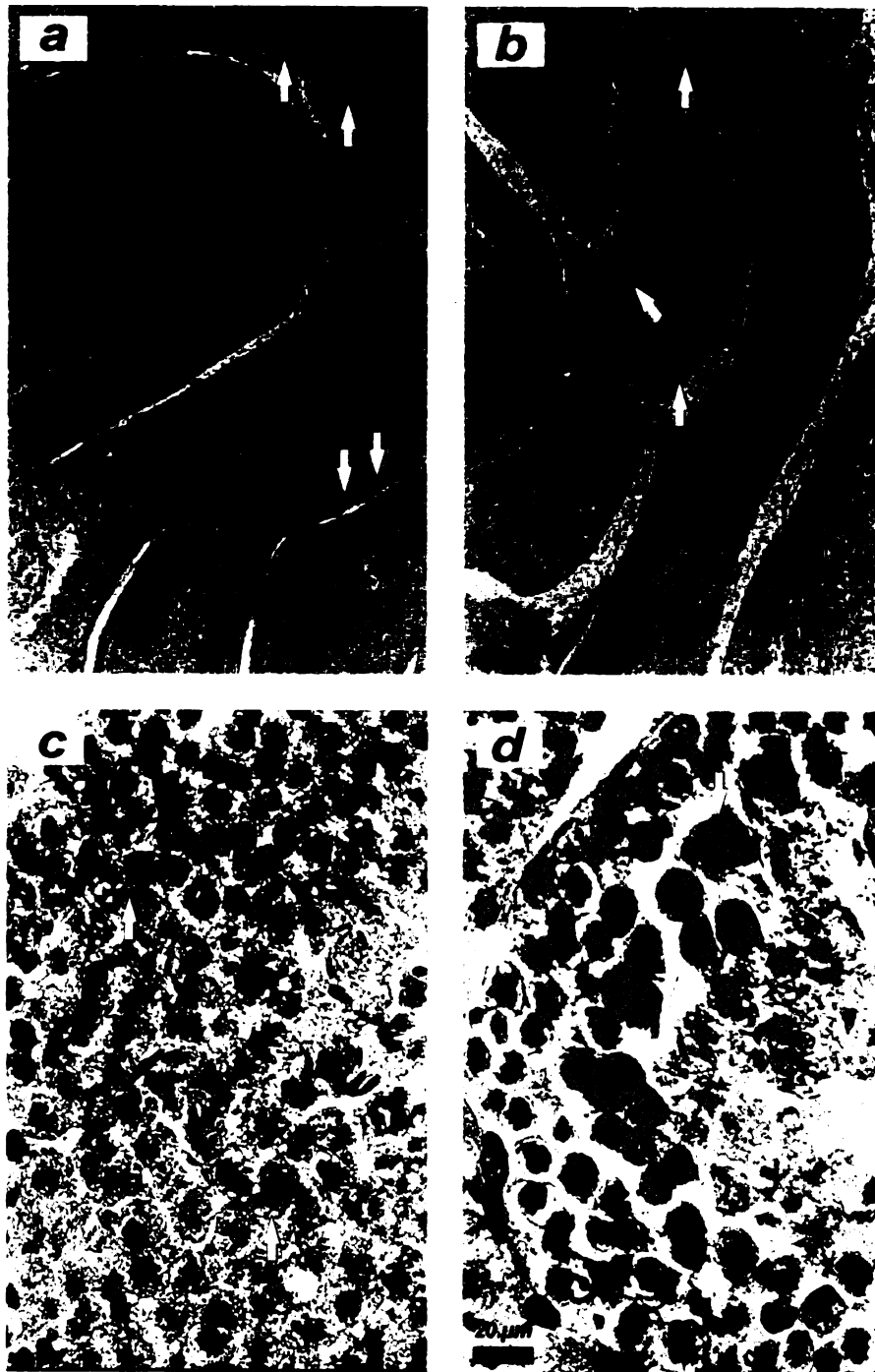
\* P < 0.05 vs. 0 mg/kg.

## Discussion

Although the phenomenon of testicular apoptosis is established beyond doubt, quantitative data on the incidence and abundance of affected testicular cells is lacking. In the present study, a flow cytometric technique was established to quantitate the proportion of apoptotic germ cells in control as well as in experimental mice. Our data indicate that at any given time, approximately 2% of testicular cells, on average, undergo DNA fragmentation, a sign of apoptosis, in the mouse testis. Only about one-fourth of the apoptotic germ cells were found to be from the nondividing population. However, analysis of apoptotic cells in each cell population revealed a much higher frequency of apoptosis, i.e., around 6% of S-ph cells and 3% of 4C cells, compared with 1.5% of either 1C or 2C cells. These cell populations comprise mainly spermatogonia preparing for mitotic divisions and spermatocytes during or after S-phase of the cell cycle. Thus, it appears that actively dividing germ cells are subject to apoptosis at a substantial rate and that apoptosis represents an important mechanism for the control of germ cell numbers being produced. Indeed, inhibition of germ cell apoptosis in mice led to considerable overpopulation of early germ cells and caused infertility (Furuchi et al, 1996; Rodriguez et al, 1997).

The flow cytometric method for analysis of testicular apoptotic germ cells described in the present study is rapid, quantitative, specific, and sensitive. The TUNEL-positive types of germ cells detected by histological and flow cytometric analysis were comparable. In the intact testis, apoptotic cells are not distributed uniformly throughout the testicular section (see Fig. 5 in present study; Brinkworth et al, 1995, 1997; Sinha Hikim et al, 1997) and vary among tubules of the same stage within a given animal or even within individual tubules (Clark et al, 1997). Hence, reliable and representative histological quantification of TUNEL-positive germ cells necessitates extensive sampling and analysis.

The objective of the present investigation was to establish a flow cytometric technique to quantify apoptotic



**FIG. 5.** Micrographs from mouse testis depicting *in situ* end-labeled (TUNEL) germ cells from control animals (**a, c**) and from animals exposed to 650 mg/kg (**b**) and 1300 mg/kg (**d**) of methoxyacetic acid. TUNEL-positive germ cells are identified by brownish-colored product (diaminobenzidine). (**a,b**) Low-power micrographs of sections without counterstain. Arrows highlight TUNEL-positive germ cells. Asterisk in **b** indicates a tubule section containing numerous TUNEL-positive cells in the basal region. (**c,d**) Sections were counterstained with hematoxylin. (**c**) Arrows point to a dividing TUNEL-positive spermatocyte in stage XII (left) and to a secondary TUNEL-positive spermatocyte (right). (**d**) TUNEL-positive dividing spermatocytes in stage XII following treatment with methoxyacetic acid. Note that most of the late spermatocytes are apoptotic. The arrow highlights a secondary spermatocyte in the process of cell separation.

germ cells in control and experimental animals. Therefore, adult male mice were treated with MAA, which is known to selectively and rapidly induce testicular germ cell death at specific stages (Hardin, 1983; Moss et al, 1985; Foster et al, 1987). Methoxyacetic acid is an ethylene glycol, has a broad spectrum of industrial applications, and has been implicated as a testicular toxicant. In the present investigation, MAA reduced the population of primary spermatocytes (4C cells) by about 50% within 24 hours (Fig. 2). This observation is in good agreement with earlier studies in which MAA has been shown to selectively destroy spermatocytes, both *in vivo* and *in vitro* (Bartlett et al, 1988; Allenby et al, 1991; Mc Laren et al, 1993; Sharpe et al, 1993). Although this decline was dose-dependent, the effects of 650 and 1300 mg of MAA per kilogram of body weight were not significantly different, suggesting that 650 mg provides a maximal dose for eliciting the initial effects. This dose was based on data obtained from rats, and a further group receiving twice the dose was included, since the effects of MAA on mouse spermatogenesis have not been reported. However, administration of a related compound, ethylene glycol monomethyl ether, to mice over a dose range of 500–1500 mg per kilogram of body weight produced a similar spermatogenic lesion, i.e., both late spermatocytes and early spermatids were affected (Anderson et al, 1987). In the present study, spermatid numbers were not changed, but TUNEL-positive spermatids were observed.

The criteria described by Darzynkiewicz et al (1997) for somatic cells were used to identify apoptotic cells in the testis, i.e., objects with a fractional DNA content that was not less than 10–20% of that of intact G1 cells were considered in order to prevent underestimation of apoptosis. Treatment of mice with higher doses of MAA (1300 mg) did not result in any significant increase in the percentage of different types of TUNEL-positive germ cells. This may be due to further decline in the percentage of 4C cells compared with that associated with the 650-mg dose of MAA. It could also be due to a significantly higher loss of fractional DNA content compared with that resulting from the 650-mg dose of MAA, and such degenerating apoptotic cells may not be quantified by flow cytometry. It should also be noted that the percentage of TUNEL-positive 4C cells observed reflects a previous loss of about 50% of the 4C cells, unlike the percentage of other types of germ cells (1C, 2C, and S-ph), which was unaffected by any such loss. The use of linear, rather than logarithmic, scale was preferred, as linear scaling provides a better assurance that cells with minimal DNA content can be excluded from the analysis (Darzynkiewicz et al, 1997). As there were no TUNEL-positive somatic cells (Leydig, Sertoli, or peritubular myoid cells) observed in the histological examination of the present study or in studies reported earlier (Brinkworth et al,

1995; Clark et al, 1997), we conclude that the percentage of TUNEL-positive 2C cells mainly comprises spermatogonia.

The killing of spermatocytes in MAA-treated rats (Brinkworth et al, 1995) and in cultured rat seminiferous tubules treated with MAA (Li et al, 1996) has been shown to occur by apoptosis. Apoptosis of different types of testicular germ cells of mice treated with MAA was identified in the present study by labeling the 3'-OH termini of the fragmented DNA, beginning 24 hours after MAA treatment. The degeneration of testicular germ cells by apoptosis in the normal as well as the MAA-treated adult male mouse was identified and quantified with flow cytometry (Fig. 4). The PI counterstaining indicated four types of germ cell populations in the TUNEL-stained testicular cells: 1C, 2C, S-ph, and 4C. The quantification of the TUNEL-positive HC population was considered inappropriate because the overlapping of green signals from the PI-bound cell debris appears in the same channels as that of HC cells. The significant decrease in the mean channel number of the fluorescence intensity of the PI-bound germ cells of 650 and 1300 mg MAA-treated mice, compared with those in saline-treated control mice, further indicates loss of DNA strands from the nucleus in the apoptotic cells (Table 2).

In summary, the present investigation provides quantitative data on the incidence of apoptosis in mouse testis, both normally and following toxin-induced loss of germ cells. The results show that apoptosis is most pronounced in proliferating cells and that MAA affects primary spermatocytes through apoptosis.

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