Response of murine spermatocytes to the metaphase-arresting effect of several mitotic arrestants[1]

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Summary. Three mitotic arrestants, colchicine, Colcemid and vinblastine, were evaluated for their effects on producing meiotic arrest in the laboratory mouse. Colchicine is the most effective and its effect is both dose- and time-dependent. Treatment of mice with 40 mg/kg of colchicine for 3 h resulted in drastic improvement of the yield and quality of M-II figures. This treatment did not appear to increase the aneuploidy frequency in M-II's.
Key words. Mitotic arrestants; spermatocyte; chromosomes; meiotic metaphase accumulation.

Cytogenetic investigations regarding genotoxic effects of environmental mutagens on murine spermatocytes may employ two stages whose chromosomal morphology is favorable for analyses: diakinesis/first meiotic metaphase (M–I) and second meiotic metaphase (M–II). M–I has been the cell type most frequently used, since such cells are usually present in sufficient numbers in testicular preparations; and structural aberrations, particularly translocations, can be easily detected in these cells. However, M–I analyses present some shortcomings because mutagen-induced numerical chromosomal aberrations resulting from meiotic nondisjunction cannot be detected. Such aberrations can only be detected in M–II cells. Since numerical chromosomal aberrations comprise the major type of chromosomal disorder found in human newborns[2], the use of M–I figure for assessing information on reproductive risk is circumscribed unless M–II analyses are concurrently performed.

Few investigators have used both M–I and M–II figures to analyze genotoxic effects of mutagens on germinal cells. This is partly due to technical difficulties in obtaining suitable quality and quantity of M–II cells in testicular preparations. Apparently, the standard procedure of Evans et al.[3] for making testicular preparations produced varying qualities of M–II spreads in different strains of mice. While considerable numbers of M–II's with good quality could be obtained in some strains, including the NMRI[4] and the Q strains[5], acceptable M–II figures were deficient in testicular preparations of other strains, including the Swiss and the C3H. Various mitotic arrestants have been used to pretreat the animals before sacrifice in an attempt to increase the yield and quality of M–II spreads. However, while some investigators experienced success, most experienced frustrations. Therefore, it is important to conduct a systematic study to determine the most effective meiotic arrestant to be used, and the optimal concentration and exposure duration of such treatment.

In the present study, three mitotic arrestants: cholchicine, Colcemid and vinblastine were evaluated for their effectiveness in arresting meiotic metaphases (both M–I and M–II) in the Swiss strain, notoriously known for its lack of good quantity and quality of M–II figures. Further, the dose-response and time-response of meiotic accumulation following colchicine treatment were determined so that the optimal concentration and exposure duration could be established.

Materials and methods. Young, mature laboratory mice of the Swiss strain (2–3 months of age weighing approximately 30 g) were used throughout this investigation. Three mitotic arrestants, colchicine, Colcemid and vinblastine, were evaluated for their effectiveness in arresting meiotic metaphases (M–I and M–II). These compounds were given to the animals by i.p. injections, and physiological saline was used as the solvent. Untreated animals or animals treated with saline alone served as controls.

In the first experiment, designed to demonstrate the dose-response of metaphase accumulation following colchicine treatment, three doses of colchicine (2, 20 and 40 mg/kg) were employed. These doses were chosen to encompass the range used by various investigators. All animals (three for each dose and three for the saline-treated control) were sacrificed 3 h after injection. Their testes were removed and single cell suspensions of testicular cells were made by passing the tissue through a wire screen in 2.2% sodium citrate solution, according to the method of Brewen and Preston[6]. The suspended cells were then treated with a hypotonic solution (1% sodium citrate) for 15 min, centrifuged at approximately 400 × g for 10 min, and fixed in methanol-acetic acid (3:1) mixture. Conventional air-dried preparations were then made for subsequent staining in 4% Giemsa in 0.01 M phosphate buffer for approximately 10 min.

The frequencies of M–I and M–II figures in the resulting preparations were determined by the percentages of such figures in at least 4000 interphase cells (excluding spermheads) counted for each sample. All nuclei, including occasional spermatogonial mitoses, were counted as interphases and all M–I and M–II figures, regardless of their quality, were scored.

In the second experiment, designed to determine the time-response of metaphase accumulation following colchicine treatment, animals were treated with 40 mg/kg of colchicine and sacrificed at 1,2 and 3 h after injection. Again, three animals were used for each time period and three animals treated with saline alone and sacrificed at 3 h post-injection served as controls. Testicular preparations were made and the frequencies of M–I and M–II figures in these preparations were determined as described above.

In the third experiment, designed to compare the effectiveness of colchicine with that of Colcemid and vinblastine in arresting metaphases, animals were treated with 10^{-4} M of each agent. This molar concentration was equivalent to 40 mg/kg of colchicine, 37 mg/kg of Colcemid and 90 mg/kg of vinblastine. Animals (three for each treatment condition) were sacrificed 2 h after injection, and the frequencies of M–I and M–II figures in the testicular preparations were determined.

In the fourth experiment, designed to compare the yield of acceptable M–I and M–II figures between testicular preparations of colchicine-treated and untreated mice, four animals were injected with 40 mg/kg of colchicine 3 h before sacrifice and three animals were sacrificed without colchicine treatment. The number of M–I and M–II figures acceptable for chromosome analysis in one of the slide preparations from each animal was counted. The cell density in each slide was kept approximately the same.

Number of acceptable M–I and M–II figures per air-dried slide from testicular preparations of mice with or without colchicine pretreatment (40 mg/kg by i.p. injection, 3 h before sacrifice)

Animal	Treatment	Number of M–I	Number of M–II
1	Colchicine	1276	1000
2	Colchicine	344	604
3	Colchicine	656	868
4	Colchicine	732	1032
Average		752	876
5	Control	176	16
6	Control	80	24
7	Control	32	16
Average		96	18.67

In the final experiment, designed to estimate the incidence of aneuploidy in M II's of Swiss mice, and to determine if the 3-h colchicine treatment increases the frequency of aneuploidy, 1000 M–II's from 10 colchicine-treated animals (100 cells per animal) were subject to chromosome counting. Since the aneuploidy frequency in M–II's of untreated Swiss mice was difficult to establish due to the paucity of such cells, the frequency of aneuploidy in treated animals was compared to the reported average frequency of 0.38% in 13 other strains of mice[7].

Results. Experiment 1. Figure 1 shows the frequencies of M–I and M–II figures in mouse testicular preparations, following a 3-h treatment in vivo with various doses of colchicine. At 2 mg/kg, the percentage of M–I figures in treated animals was not different from that of the controls, but the frequency of M–II figures was increased by approximately two-fold. However, the quality of these M–II figures in terms of suitability for chromosome counting was not improved (fig. 3b). At 20 mg/kg, the frequency of M–I figures in treated animals was slightly elevated, and a 3-fold increase in the frequency of M–II figures was observed. The quality of these M–II figures was improved but still not optimal (fig. 3c). At 40 mg/kg, the frequencies of both the M–I and M–II figures were drastically increased to four times of the control value. A low magnification field which shows accumulation of such figures is shown in figure 3a. Most significantly, the quality of the M–II figures was excellent for chromosome counting (fig. 3, d and e). Although some of the M–I figures showed highly contracted chromosomes, those with optimal chromosomal morphology were always abundant. Therefore, this experiment showed that in order to dramatically improve the yield and quality of M–II figures, animals should be treated with a concentration of colchicine that is as high as 40 mg/kg.

Experiment 2. Figure 2 illustrates the frequencies of M–I and M–II figures in animals treated with 40 mg/kg of colchicine for 1, 2 and 3 h before sacrifice. At the end of 1-h colchicine treatment, a nearly 2-fold increase in the frequency of M–I figures and 2- to 3-fold increase in the frequency of M–II figures were noted. However, the quality of the M–II figures was still poor. A dramatic increase in the frequency of M–II figures was observed after 2 and 3 h of colchicine treatment. Not only did the M–II frequency increase to 4-fold of the control level, the quality of the M–II figures was also drastically improved. The 3-h colchicine treatment condensed the metaphase chromosomes more than the 2-h treatment; therefore, the former treatment was preferred if chromosome counting was desired. The increase in the frequency of M–I figures was slower than that of the M–II's during the first 2 h of colchicine treatment, but during the 3rd h, the frequency of M–I figures increased rapidly to a level that was approximately four times of the control value. From this experiment, we concluded that the optimal duration of colchicine treatment was between 2 and 3 h.

Experiment 3. The relative frequencies of M–I and M–II figures in animals treated with 10^{-4} M of colchicine, Colcemid and vinblastine for 2 h were shown in figure 2. While all three mitotic arrestants at this dose caused accumulation of meiotic metaphases, colchicine was the most effective in increasing the yield of M–II spreads. Both colchicine and Colcemid improved the quality of M–II spreads by contracting the chromosomes, but vinblastine failed to exert the same effect. Therefore, this experiment demonstrated that among the 3 mitotic arrestants, colchicine is the most effective in arresting meiotic metaphases.

Experiment 4. The table shows the number of acceptable M–I and M–II figures in each slide preparation from mice treated with or without colchicine (40 mg/kg) for 3 h. In colchicine-treated animals, the average number of M–I and M–II figures acceptable for chromosome analysis per slide was 752 and 876 respectively. In untreated animals, the average number of M–I and M–II figures per slide was only 96 and 18 respectively. Therefore, the colchicine treatment produced an approximately 8-fold increase in the frequency of acceptable M–I figures and 50-fold increase in the frequency of acceptable M–II figures. These data demonstrated that colchicine treatment improves the yield of both M–I and M–II figures that are suitable for cytogenetic studies.

Experiment 5. In the 1000 M–II figures from 10 Swiss mice that were pretreated with colchicine, the aneuploidy frequency was

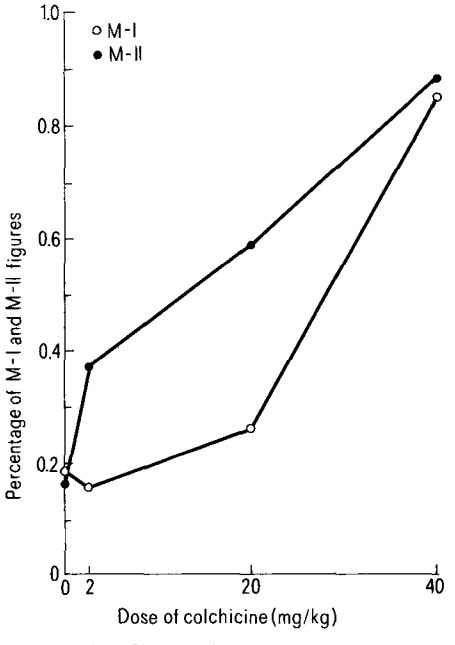

Figure 1. Frequencies of M–I and M–II figures in air-dried preparations of suspended testicular cells from mice treated in vivo with various doses of colchicine for 3 h. Each data point represents the average value of three mice, totalling more than 13,000 nuclei (excluding sperm heads).

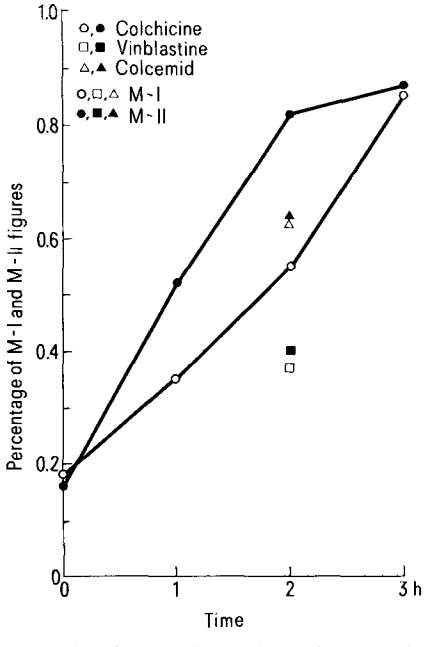

Figure 2. Frequencies of M–I and M–II figures in air-dried preparations of suspended testicular cells from mice treated in vivo with 40 mg/kg of colchicine for 1, 2 and 3 h. Each data point represents the average value of three mice, totalling more than 13,000 nuclei (excluding sperm heads).

0.4% (estimated by doubling the frequency of hyperhaploidy). Since this frequency was not different from that reported previously in other strains of mice, 0.38%, it was apparent that the colchicine treatment for the duration prescribed did not increase the aneuploidy frequency in M–II's.

Discussion. For decades, colchicine, Colcemid and vinblastine have been used by investigators to arrest mitoses in mammalian cell cultures for cytogenetic studies. Generally, the concentrations used are approximately 0.5 µg/ml for colchicine, 0.05 µg/ml for Colcemid, and 0.09 µg/ml for vinblastine, equivalent to approximately 10^{-6} M, 10^{-7} M, and 10^{-7} M respectively. Exposure of cultured cells to these mitotic arrestants for only 1 h results in the accumulation of metaphases and contraction of chromosomes. However, when a similar dose, or a dose several times higher, was administered to mice in vivo, M–II figures generally failed to be arrested within 3 h. The dose-response curve reported here emphatically showed that to achieve a drastic accumulation of M–II's, a dose of colchicine 100 times higher than that used in cell cultures must be employed. In retrospect, the reason that many investigators failed to obtain sufficient M–II figures for chromosomal analysis was due to the insufficient concentrations of colchicine or Colcemid used.

Our finding that a large dose of colchicine is required for improving the yield and quality of M–II spreads is in agreement with that of Beatty et al.[7] who used a similar dose of colchicine (0.3 ml of 0.5% colchicine per mouse, equivalent to 50 mg/kg for a 30-g mouse) to obtain M–II figures in 13 strains of mice. The same treatment was also used by Paccherotti et al.[8] and Russo et al.[9] to obtain M–II figures in C57BL/Cn3× C3H/Cn3 F1 hybrid mice. Therefore, the colchicine treatment seems to be effective regardless of the strain of the mouse used. It is of interest to note that although Beatty et al.[7] developed the procedure for enhancing the yield of M–II figures nearly 10 years ago, very few cytogeneticists followed or even were aware of this procedure. Apparently, Paccherotti and her colleagues were the only investigators who adopted the procedure to study mutagen-induced aneuploidy and chromosomal aberrations. The present investigation, in which the dose- and time-response of colchicine effect were systematically examined, reaffirms the applicability of the Beatty's procedure.

The relative ineffectiveness of vinblastine to arrest meiotic metaphases was unexpected, because in the mouse bone marrow in vivo, vinblastine is a much more potent mitotic arrestant than Colcemid[10]. A likely explanation is that vinblastine is a larger molecule than colchicine or Colcemid; (mol. wt = 909 for vinblastine, 399 for colchicine and 371 for Colcemid), therefore, a longer time period is required for vinblastine to cross the blood testis barrier and reach the spermatocytes.

The simple procedure for obtaining excellent M–II figures in mice should prove useful in studies of mutagen-induced aneuploidy. The same procedure has also been proven applicable to Chinese hamsters. Since colchicine treatment also increases the yield of M–I spreads in the same preparations, with this procedure, both M–I and M–II figures can be concurrently analyzed to determine, respectively, the clastogenic and aneuploidy-producing potential of mutagens in murine germ cells.

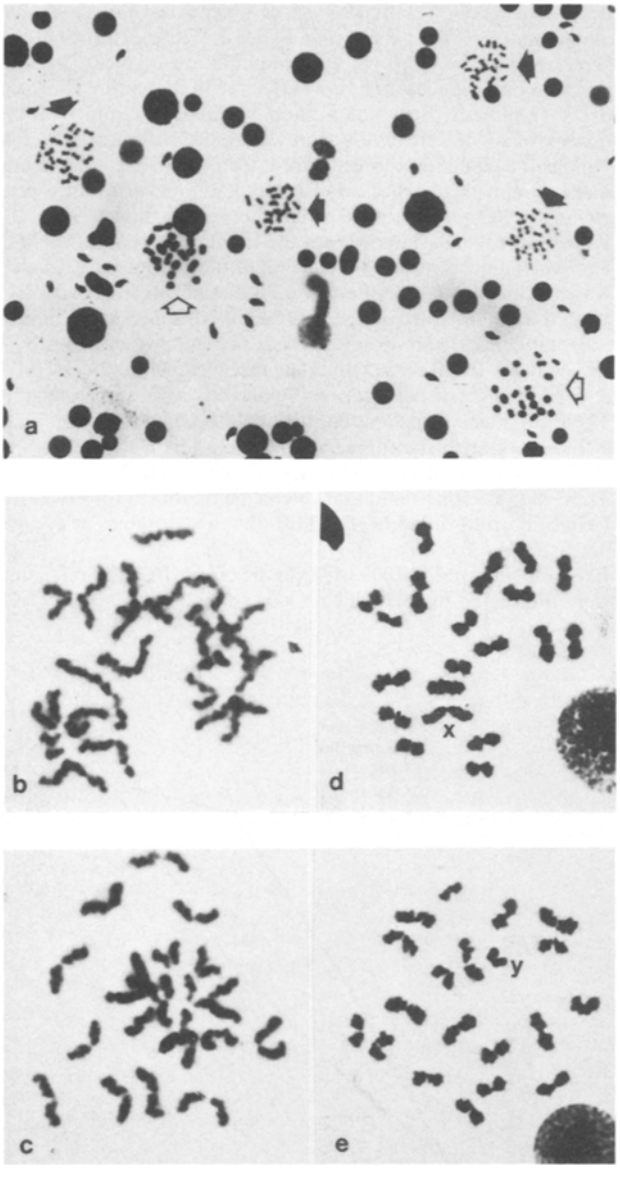

Figure 3. *a* A low magnification field showing an accumulation of M–I (white arrowheads) and M–II (dark arrowheads) spreads in a mouse testicular preparation. The animal was injected i.p. with colchicine (40 mg/kg), 3 h before sacrifice. ×160. *b* and *c* M–II figures representing the majority of such spreads from an untreated Swiss mouse. Such spreads do not allow accurate counting of chromosome numbers. ×1500. *d* and *e* M–II figures from an mouse that was treated with 40 mg/kg of colchicine for 3 h before sacrifice. Such spreads are suitable for analyses of chromosomal aneuploidy and breakage. The X and Y chromosomes can be distinguished from the autosomes due to their heteropycnotic characteristics. ×1500.

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