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MOUSE-SPERMATID MICRONUCLEUS ASSAY ON THE CLASTOGENICITY OF X-RAYS AND CYCLOPHOSPHAMIDE

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ABSTRACT

Mouse-spermatid-micronucleus (Mus-SPMN) assay is one of the most relevant test systems for the clastogenicity of environmental pollutants in mammalian germ cells. A "Serial Fixation" of the X-irradiated cell samples (at 12 or 24 h intervals) was used to confirm the sensitive peak for micronuclei (MN) induction and to improve the efficiency of the test. The sensitive peak as revealed by the highest rate of MN in the early stage of spermatid between the treatment and fixation time was found to be 3.5 days. In order to determine the relative effectiveness of the Mus-SPMN test for chemical clastogenicity detection the X-ray and cyclophosphamide (CP) dose-response studies were carried out for comparison. X-ray dosages at 0, 0.5, 1, 2, 3, 4 and 5 Gy (whole body irradiation) induced 0.66, 2.45, 2.22, 4.44, 6.68, 7.00 and 13.34 MN/1000 spermatids respectively. Correlation coefficient and slope values were 0.92 and 0.022 respectively. The CP dose response curve was established when four groups of male mice were treated (ip injections) with 0, 30, 50 and 60 mg/kg of CP, 0.63, 7.33, 8.89 and 15.67 MN/1000 spermatids were induced respectively. The correlation coefficient and slope were 0.68 and 0.12, respectively. Roughly 3.5 ppm CP equals to 3.75 Gy of X-rays. These dose-related MN frequencies induced by clastogens could serve as a potential signal of the possible heritable mutations induced by the lower dosages of the same agent.

RESUMEN

El ensayo de micronúcleos en los espermátidas de ratón (SPMN-ratón) es uno de los sistemas de prueba más relevantes de la clastogenicidad producida por los contaminantes ambientales en las células germinales. La "fijación seriada" de las muestras de células tratadas con rayos X (a intervalos de 12 y 24 h) fue utilizada para conformar el pico sensible en la inducción de micronúcleos (MN) y tener mayor eficiencia en la prueba. El pico sensible, como lo revela la tasa más elevada de MN en el estado temprano de espermátidas entre el tratamiento y la fijación, ocurre a los 3.5 días. Para determinar la efectividad relativa de la prueba química de clastogenicidad de SPMN-ratón, se realizó la comparación de la dosis-respuesta de la CP con los rayos X. Las dosis de rayos X de 0, 0.5, 1, 2, 3, 4 y 5 Gy (en la irradiación total del cuerpo) indujeron 0.66, 2.45, 2.22, 4.44, 6.68, 7.00 y 13.34 MN/1000 espermátidas, respectivamente. El coeficiente de correlación y el valor de la pendiente fueron de 0.92 y 0.022, respectivamente. La curva de dosis respuesta con la CP se estableció con

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cuatro grupos de ratones machos (inyectados intraperitonealmente) con 0, 30, 50 y 60 mg/kg de CP que indujeron 0.63, 7.33, 8.89 y 15.67 MN/1000 espermátidas, respectivamente. El coeficiente de correlación y la pendiente fueron 0.68 y 0.12, respectivamente. Así, 3.5 ppm de CP fueron equivalentes a 3.75 Gy de rayos X. Estas frecuencias relacionadas con las dosis que produjeron los clastógenos pueden servir como una señal de potencia mutagénica provocada por dosis inferiores de los mismos agentes.

INTRODUCTION

Among more than 200 short-term genotoxicity bioassays very few used chromosome damage in animal germ cells as end points (Waters *et al.* 1994) except several studies using radiation as a tool to develop the mouse-spermatid-micronucleus (Mus-SPMN) assay (Oakberg 1956, Oakberg and diMinno 1960 , Tsuchida and Uchida 1975, Adler 1977, Lahdetie and Parvinen 1981, Tates *et al.* 1983, Tates and de Boer 1984). In animal system, the Drosophila recessive mutation/ dominant lethal, the mouse dominant lethal, mouse bone marrow erythrocyte micronucleus assay, chromosome aberration and gene mutation in mammalian cell cultures were the well known assays that have a relatively sizable database. Database of the mouse heritable translocation test is rather small. Most of these animal assays are insensitive to mutagens or clastogens, cumbersome, and labor intensive. When mutation or chromosome damage is scored in the F_1 or F_2 generation in these animal assays, mutants or aberrant events are often lost through non-viable primary or secondary spermatocytes, spermatids or sperms which carry the defective genome. In the Mus-SPMN system, pachytene stage of the meiotic chromsome is used as the targets and determine the chromosome damage at the early spermatid stage to assess the micronuclei frequencies without going through the F_1 generation of the animal. Pachytene stage was presumably the most sensitive stage owing to the fact that crossing over process is involved in which four-strand chromatids under go breakage and fusion. Breaks that lead to the formation of micronuclei in the subsequent nuclear division can be the result failure of fusion due to interference of clastogens in the nucleus. Based on this assumption, the peak MN frequency should be induced at the pachytene stage and high MN frequency should appear in the early spermatids after a certain length of time when the chromosome breaks arrive at the end of meiosis and become micronuclei. This period of time is referred to as the recovery time. The recovery time can be easily determined by the conventional "Serial Fixation" technique. The technique involves the administering of an acute dose of treatment to a population of dividing cells and followed by fixing cell samples at intervals to capture the high peak of chromosome damage. The peak MN frequencies and the pachytene stage relationship was confirmed in the process of microsporogenesis in *Tradescantia* plant by radioisotope labeling of the meiotic stages (Taylor 1950) and serial fixation techniques (Ma *et al.* 1980). The stage duration scheme determined by radioisotope labeling matched closely with the first peak, pachytene stage 22 hr after treatment, and the second peak, pre-leptotene stage, 42 h after treatment. The process of microsporogenesis in plants is the counter part of spermatogenesis in animals. The microsporogenesis in plants is much shorter than spermatogenesis in mammals. The total length of meiosis in higher plants can be completed usually in less than 2 days while the spermatogenesis in the mouse may last 11–18 days (Oakberg 1956, Rough 1968). In the present study, the serial fixation technique was used in this mammalian system to ascertain the crucial recovery time for the improvement of the Mus-SPMN assay efficiency.

Recent report (Adler *et al.* 1996) on mammalian germ cell mutagen detecting bioassays, Mus-SPMN assay was used to determine the clastogenicity of acrylamide, epoxybutene, diepoxybutene, trophosphamide (Russo *et al.* 1994) and butadiene (Xiao and Tates 1995). There are two major advantages to use germ cells for genotoxicity detection. First, meiotic chromosomes are sensitive targets to clastogens. Second, chromosome damage or alteration of genes exhibited as an end point in germ cell assays may be considered as potential heritable changes.

After establishing the efficient recovery time (the sensitive peak MN frequency) the present study was designed to compare the clastogenicity of X-rays, a well-known physical agent, with CP, a common chemotherapeutic drug for cancer.

MATERIALS AND METHODS

The Pie Ball mouse line from the animal facility of Western Illinois University was used for this study. All the experimental mice were bred and maintained under the standard conditions according to the federal guidelines for animal care of U.S. Department of Health and Human Services (1985). The 9-11-week old males were selected for experimentation and randomly assigned to experimental groups. Three mice were

assigned to each of the experimental groups for control and treatments. Experimental animals were anaesthetized with ether in a 250-ml glass jar and the testicle tubules were obtained from one of the testes. The spermatogonia and various stages of spermatocytes as well as spermatids were released from tubules into a 2.2 % sodium citrate solution for preparation of smears on the slides. A modified and simplified procedure of the suspension procedure of Tates *et al.* (1983) was used for slide preparation. The cell sample on the slide was fixed in 100 % ethanol for 10 min and air-dried. Dried slides were then transferred into Sorensen's buffer solution for 15 min and stained with Giemsa. In general, 10 slides per animal were prepared and 5-6 of the well stained slides were selected for scoring of micronuclei frequencies (1000 spermatids per slide). Micronuclei which appeared purplish-pink, the same color as the nucleus proper, and clearly set apart from the nucleus were scored. A therapeutic X-ray machine (Standard X-ray Co., Springfield, MA) which was operated at 80 kVp, 4 mA, with 1 mm AL filter, 15 cm distance, and a dose rate of 0.6 Gy/min for all X-ray treatments was used in this study. Cyclophosphamide (Sigma Chemical Co., St Louis, MO) which is dissolved in distilled water and adjusted to pH 7 with phosphate buffer was used for intraperitoneal injection in the dose response study.

Statistical analyses

Micronuclei frequencies of 5–6 slides per mouse were pooled to derive the mean frequency for one mouse in the experimental group. The mean frequency of each experimental group which usually contained 3 mice was expressed in number of MNs per 1000 spermatids. Three to 5 data points per group were used for input into the statistical software "Systat" (Systat Co. IL) for statistical analyses. Descriptive analysis was applied to obtained means, and standard deviations. A one-way Analysis of Variance (ANOVA) test and Dunnett's t-test were performed to determine the significance (0.05) between the control and treated groups as well as the peak sensitivity from the groups of "Serial Fixation" experiment. The means of each of the data points were derived from 3 slides (1000 spermatids per slide) per mouse for the dose-response curve. Linear regression analysis for correlation coefficient, slope and intercept were obtained from the dose-response curve data.

Three series of experiments were conducted in this study. The experimental design and treatment and fixation procedures are described as follows:

Experiment for determination of the effective recovery time/peak sensitivity

In this study, 54 male mice were divided into 18 experimental groups and they were irradiated with 2 Gy of X-rays for whole body exposure except the control group. The meiotic spermatocytes were fixed at 12 h intervals for the second through the fifth day groups, and at 24 h intervals for the 5th through 14th day groups. Five–6 slides were scored from each of the treated mice and one slide from each of the control mice was scored to derive the means and standard errors.

Experiment for X-ray dose response and the CP dose response studies

Twenty one male mice were divided into 7 groups in the X-ray dose response series and 12 male mice were divided into 4 groups in the CP dose-response series. Animals were irradiated with X-rays at 0.5, 1, 2, 3, 4, 5 Gy whole body exposure except the control group. All experimental groups were given an appropriate recovery time which will be determined by the serial fixation study. Three slides were made from each of the 21 mice and a total of (3 slides of each of the treated mice were scored) 63,000 Golgi-phase round spermatids were scored to obtain 7 data points for the X-ray dose response curve. In the CP dose-response study, 3 mice for each of the 4 experimental groups (3 slides from each of the treated and control mice) were scored. A series of increasing doses of 30, 50 and 60 mg/kg were administered. While the control group received only saline solution. 36,000 spermatids were scored from 12 mice to establish the dose-response curve.

RESULTS AND DISCUSSION

The results of X-ray-irradiation and "Serial Fixation" experiment for determination of the most effective recovery time for the sensitive peak period of MN induction are given in **Table 1**. The sensitive peaks are also graphically illustrated in **Fig. 1**. Two peaks were found. The first peak of high radiosensitivity of the meiotic chromosomes appeared around 3.5 days and the second peak appeared around 10 days after treatment. The ANOVA test showed that there was a significant ($p < 0.001$) difference among experimental groups, and the results of Dunnet's t-test showed that the MN frequencies of these two peak groups were significantly ($p< 0.001$) different from the control group.

The duration of the meiotic stages of spermatogenesis and the stage sensitivity to clastogens has been topics of interest since the mid-50s (Oakberg 1956, Oakberg *et al.* 1960, Rugh 1968). Most of these earlier studies relied on cytological technique of serial sectioning of the developing seminiferous tubules and observation of the meiotic stages of spermatocytes for the stage develop-ment. Cell suspension and smear methods have been used since the late 70s (Adler 1977, Lahdetie and Parvinen 1981, Tates *et al.* 1983, Tates and de Boer 1984) to simplify the cytological procedure and improve the accuracy. Different recovery times were reported in the earlier studies. Based on the results of Oakberg's serial fixation study on the

TABLE I. FREQUENCY OF MICRONUCLEI IN SPERMATIDS OF MICE IRRADIATED WITH 2 GY AND CELL SAMPLES WERE FIXED AT 1/2 OR 1 DAY INTERVALS IN A SERIAL FIXATION EXPERIMENT

Expt. Group (recovery time/day)	MN frequencies MN/spermatids scored I [b]	II [b]	III [b]	MN/1000 spermatids Mean +/- S.E.		Remarks (significance)[a] p
Control	1/1000	0/1000	1/1000	0.67	0.34	
0	5/5005	13/6019	7/6000	1.46	0.16	
2	14/5011	15/5010	19/6080	2.86	0.56	
2.5	24/5023	20/5000	17/5000	4.06	0.21	
3	34/5020	17/5017	35/5000	5.73	0.52	
3.5	46/5081	76/5060	66/5060	12.33	0.79	< 0.001
4	40/5031	36/5000	26/5000	6.80	0.37	
4.5	56/6309	21/5000	30/5000	6.33	0.60	
5	45/5000	25/5000	38/5000	7.20	0.52	< 0.038
6	51/5000	25/5000	50/5000	8.40	0.76	
7	34/5000	23/5000	24/5000	5.40	0.31	
8	55/5000	32/5000	38/5000	8.33	0.62	< 0.01
9	17/5000	38/5000	34/5000	5.93	0.57	
10	44/5000	50/5000	99/5000	12.67	1.47	< 0.001
11	26/5000	26/5000	32/5000	5.60	0.17	
12	25/5000	31/5000	25/5000	5.40	0.17	
13	30/5000	23/5000	40/5000	6.60	0.61	
14	37/5000	22/5000	63/6000	7.43	0.79	< 0.029

[a]P values of significance are derived from the Dunnett's t-test.
[b]Roman numerals denote 3 trials with 17 recovery time groups. Five mice were used in each of the X-irradiated 17 groups and one mouse was used in each of the 3 trials.

stage durations of spermatognesis of the mouse, the recovery time should be around 9 days if the acute X-ray treatment was applied at pachytene of the prophase I and MN were observed in the early spermatids (Oakberg 1956). Early spermatids are well suited for MN scoring because they are the products of the second round of nuclear division at the end of meiosis. According to stage duration scheme which was established by Rough (1968), Tates *et al.* (1983) and Tates and de Boer (1984), one peak found in the mouse spermatids was on about the 2nd day (49 h after treatment). Another result of Oakberg's (1957) study using radioisotope labeling approach gave a 2.2 day recovery time. The 2 day and 14 day recovery times were reported in acrylamide treatment (Russo *et al.* 1994), and 14th day peak was found in diepoxybutene treatment (Russo *et al.* 1994) and 16th day peak was found in trophosphamide treatment (Russo *et al.* 1994). The 15th day peak was also reported in butadiene treatment (Xiao and Tates 1995). These discrepancies in the recovery time in the Mus-SPMN assay are expected because the early spermatids in seminiferous tubules in a testis are not synchronized as in the case of the early tetrads of *Tradescantia* plants. Although the last stage of spermatogenesis in the mouse should end up in four-cell stage but the four daughter cells at the end of second division are not clustered in the form of a tetrad as in the case of *Tradescantia*. The early tetrad in *Tradescantia* has its own envelope that keeps the four cells intact for MN scoring. The high efficiency of the Trad-MCN assay is attributed mainly to the synchrony of the pachytene and tetrad stages in meiosis and the ease in choosing the appropriate flower buds that contain mainly tetrads, while the early spermatids in mouse are not easily selected from different tubules.

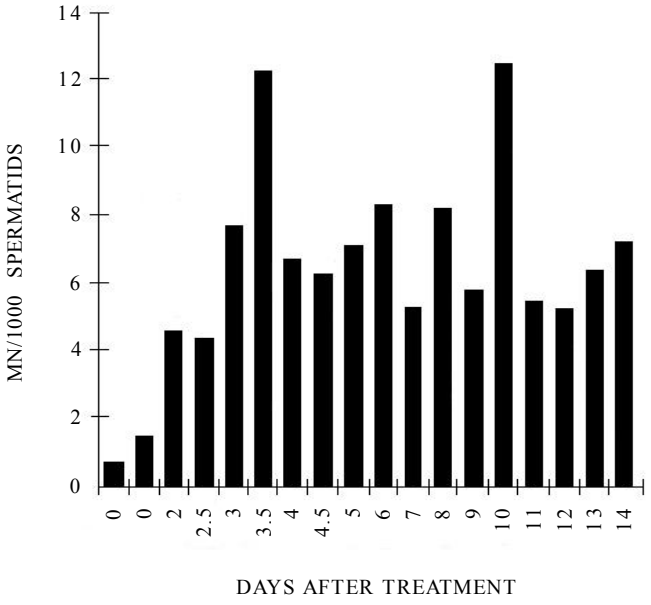

Fig. 1. Bar graph showing the peak MN frequencies obtained from the serial fixation experiment

The "Serial Fixation" technique used in this study focused on the early spermatids among cells of various

TABLE II. RESULTS OF X-RAY DOSE-RESPONSE STUDY WITH 6 INCREASING DOSAGE OF X-RAYS VERSUS MICRONUCLEI FREQUENCIES IN THE EARLY SPERMATIDS OF THE MOUSE UNDER THE 3.5 DAY RECOVERY TIME

X-ray doses (Gy)	MN/1000 spermatids Data collected in 3 repeated trials			MN/1000 spermatids Mean +/- S.E.	
	I [a]	II [a]	III [a]		
Control	1.00	0.00	1.00	0.66	0.34
0.5	4.00	1.67	1.67	2.45	0.43
1.0	2.33	3.00	1.33	2.22	0.28
2.0	3.00	5.33	5.00	4.44	0.42
3.0	5.00	8.67	6.67	6.78	0.61
4.0	8.67	6.67	5.67	7.00	0.51
5.0	11.67	13.67	14.67	13.34	0.51

[a]Roman numerals denote 3 repeated trials of this experiment with 3 mice used in each of the X-irradiated dosage groups. One mouse was used in each trial of the control groups (1000 spermatids were scored from each animal)

stages of spermatogenesis that were released from seminiferous tubules on the slides for scoring of MN frequencies after X-irradiation of the animal. Assume that the peak MN frequency was the results of X-ray damage to the DNA at the pachytene stage. The recovery times used by the earlier studies of Oakberg (1956) and Tates (1983) were close to the 3.5 day recovery time obtained in this study. The peak MN frequecies induced by neutrons (Tates 1983) were in the 3rd and 6th days after treatment. Collins (1992) chose 13.5 day recovery time for his study could coincide roughly to the second sensitive peak (10 day) found in the present study. Based on the stage durations found by Oakberg (1956) these two peaks found in his serial fixation study should coincide with the pachytene stage for the first peak (3.5 days after treatment) and around the zygotene stage for the second peak (10 days after treatment). These results are in agreement with Tsuchida and Uchida's findings on the duration of the meiotic stages (1975). The recovery time used by Russo *et al.* (1992) in adriamycin study and the stage duration published by Rough (1968) roughly matched the first peak obtained in this study.

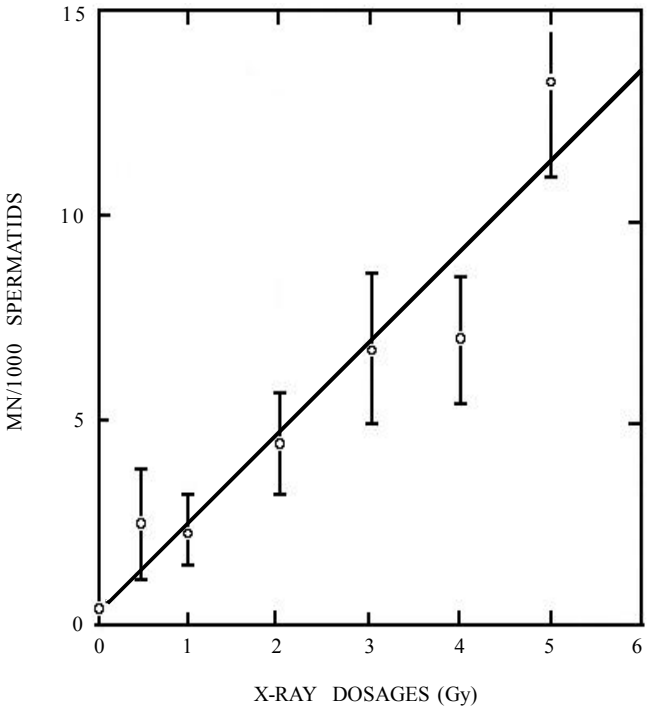

Fig. 2. The X-ray dose-response curve

The results of the X-ray dose-response study are given in **table 2** and the dose response curve is shown in **Fig. 2**. Linear regression analysis yielded the correlation coefficient of 0.92 and the slope value of 0.022. The X-ray dose-response curve established in an earlier study with Trad-MCN assay (Ma *et al.* 1980) yielded a correlation coefficient of 0.98 and the slope value of 2.05. Based on the slope values of these two assays, sensitivity of the Mus-SPMN assay is about 100 times lower than that of the Trad-MCN assay. In terms of the simplicity of experimental procedure, only 300 tetrads are required to establish a MCN/100 data point in the Trad-MCN assay under a 400X magnification, compare to scoring 1000 spermatids under 1000X magnification in the Mus-SPMN assay in order to established a MN/1000 data point. Further advantages in the Trad-MCN system are that the scoring of micronuclei frequency takes place in

TABLE III. RESULT OF CYCLOPHOSPHAMIDE DOSE-RESPONSE STUDY WITH 4 INCREASING DOSAGES VERSUS MICRONUCLEI FREQUENCIES IN THE EARLY SPERMATIDS OF THE MOUSE UNDER THE 3.5 DAY RECOVERY TIME

Expt. Group	Dosage mg/kg	Sample N	MN/1000 spermatids Mean +/- S.E.	
Control	0	9	0.63	0.17
Treated – 1	30	9	7.33	0.79
Treated – 2	50	9	8.89	0.56
Treated – 3	60	9	15.67	1.08

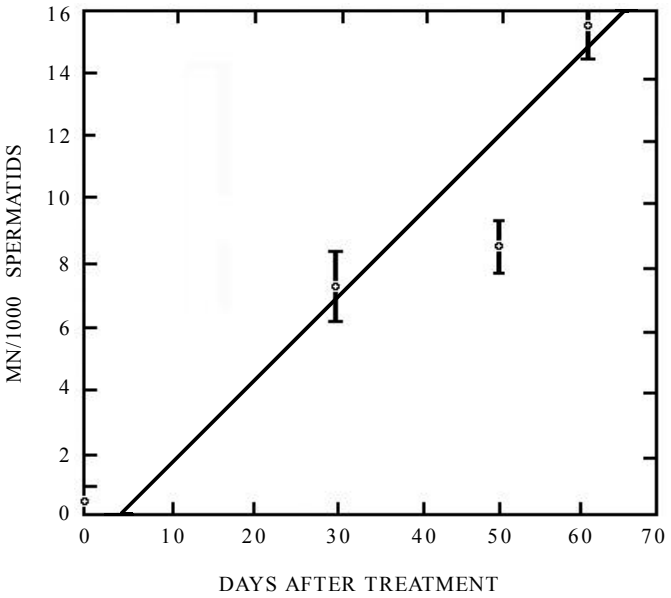

Fig. 3. The cyclophosphamide dose-response curve

the basic units of tetrads while the tetrad status in the Mus-SPMN could not be maintained for micronuclei scoring.

Results of the CP dose response study are given in **table 3**, and the dose-response curve is shown in **Fig. 3**. The correlation coefficient, slope values are 0.68, and 0.12 respectively. The sensitivity of the meiotic spermatocytes to CP judged by the slopes of the dose-response curve is about 6 times greater than to the X-rays. The relative effectiveness of this chemical compared with X-rays is about 33mg/kg (33 ppm) to 3.75 Gy of X-rays.

In conclusion, the Mus-SPMN assay is a relatively efficient bioassay for detection of clastogenic effects directly on germ cells of mammals and gives potential signal of heritable mutations and chromosome anomalies in future generations. Two peaks were obtained from the "Serial Fixation" study, i.e. the 3.5 day peak is the results of clastogenic effect on or around pachytene stage and the 10 day peak shows the effect on pre-meiotic cell. The short recovery time (3.5 days) should be used to detect the chromosome damage. This will not only make Mus-SPMN assay a truly short-term test but also reduce the unnecesary variance which is often associated with long recovery time.

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