

## MOUSE-ERYTHROCYTE MICRONUCLEUS (Mus-EMN) ASSAY ON THE CLASTOGENICITY OF INDUSTRIAL WASTEWATER

Te-Hsiu MA<sup>1</sup>, Xiaodong ZHOU<sup>1</sup>, G. Flavia LOARCA<sup>2</sup>, Gemma G. ARREOLA<sup>2</sup> and Salvador U. LECONA<sup>2</sup>

<sup>1</sup>Laboratory of Environmental Mutagenesis, Department of Biological Sciences, Western Illinois University, Macomb, IL, 61455 USA.

<sup>2</sup>Centro de Estudios Académicos sobre Contaminación Ambiental, Universidad Autónoma de Querétaro, Querétaro, Qro., México.

(Recibido enero 1995, aceptado julio 1995)

Keywords: mouse, erythrocytes, micronucleus, clastogenicity, wastewater

### ABSTRACT

The Mouse-Erythrocyte-Micronucleus (Mus-EMN) assay is a modified mouse-micronucleus test which conventionally scores the micronucleus (MN) frequencies in bone marrow polychromatic erythrocytes (PCE). In the Mus-EMN assay, MN frequency is scored in mature normochromatic erythrocytes (NCE) in the circulating blood obtained from the tail or the eye socket of the mouse. This simplified *in vivo* Mus-EMN assay was used to monitor the chronic clastogenicity of industrial wastewater throughout one year in the current study. Twenty four young (2 month old) CD-1 white mice were divided into 3 groups of 8, with 4 males and 4 females in each group, and caged individually for this study. Two treated groups of animals were fed with industrial wastewater collected weekly from the down stream of the Arena Canal (wastewater disposal system) from the Benito Juárez Industrial District of the Querétaro City of Mexico. In order to reduce the toxicity, wastewater samples were diluted with tapwater at 1:5 ratio (wastewater: tapwater) for treated group 1 and a 1:2 ratio for treated group 2. Animals of the control group drank the tapwater. Red blood samples were collected monthly from the tail and blood smears were double stained with hematoxylin and Giemsa, and about 10,000 mature red blood cells were scored from each of the 8 slides of the experimental groups to derive the means and standard errors. Results of this year-round study showed a significant increase in MN frequencies (5.08 - 9.80 MN/1000 cells) in the treated groups during the months of October through January of the following year, the dry season of this area of Mexico. The MN frequencies of the treated mice declined to the control level (1.29 - 3.20 MN/1000 cells) after 6-7 months of continuous exposure. Results of this study indicate that the Mus-EMN assay is adequate for chronic clastogenicity tests of water pollutants with the maximum time limit around 6 months which is about 20% of the youthful life of the mouse.

### RESUMEN

El ensayo de Micronúcleos (MN) en Eritrocitos de Ratón, es una prueba modificada que registra convencionalmente las frecuencias de micronúcleos (MN) en los eritrocitos policromáticos (EPC) de la médula ósea. En esta prueba se registra la frecuencia de MN en eritrocitos normocromáticos (ENC) maduros de la sangre circulante obtenida de la cola o de la cavidad del ojo de ratón. Esta prueba simplificada *in vivo* fue usada como monitor de la clastogenicidad crónica de las aguas de desecho industrial a través de todo el año. Veinticuatro ratones blancos CD-1 jóvenes de 2 meses, se dividieron en 3 grupos de 8 con 4 machos y 4 hembras y fueron enjaulados individualmente para este estudio. Dos de los grupos de animales fueron alimentados con agua de desecho industrial colectada semanalmente de la corriente baja del Canal Arena (sistema de eliminación de agua de desecho) del Distrito Industrial Benito Juárez de la Ciudad de Querétaro de México. Para reducir la toxicidad, las muestras de agua residual fueron diluidas con agua corriente en proporción 1:5 (agua de desecho: agua corriente) para el grupo tratado 1 y de 1:2 para el grupo tratado 2. Los animales del grupo testigo bebieron agua corriente. Las muestras de sangre fueron colectadas mensualmente de la cola y los frotis fueron teñidos doble con hematoxilina y Giemsa y alrededor de 10,000 células rojas maduras de la sangre se registraron de cada 8 preparaciones de los grupos experimentales para obtener las medias y los errores estándar. Los resultados de este estudio de todo el año mostraron un incremento significativo en las frecuencias de MN (5.08 - 9.80 MN/1000 células) en los grupos tratados durante los meses de octubre a enero del año siguiente, la estación seca de esta área de México. Las frecuencias de MN de los ratones tratados disminuyeron a niveles testigos (1.29 - 3.20 MN/1000 células) después de 6 a 7 meses de exposición continua. Los datos obtenidos indican que el ensayo de MN en los eritrocitos de ratón es adecuado para las pruebas de clastogenicidad crónica de las aguas contaminadas con tiempo límite máximo de alrededor de 6 meses, que corresponde al 20% de la juventud del ratón.



## INTRODUCTION

The Mouse-Erythrocyte-Micronucleus (Mus-EMN) assay is a modified clastogenicity testing system (MacGregor *et al.* 1980, Salamone *et al.* 1980) from the mouse bone marrow erythrocyte micronucleus test (Heddle 1973, Schmid 1975). In the Mus-EMN test, the blood samples can be collected from the eye socket or the tail of the animal repeatedly for an extended period. Thus the test can be applied to the same animal under the chronic or subchronic exposure to chemical agents (Salamone *et al.* 1980, Barale *et al.* 1985, Choy *et al.* 1985, Ma *et al.* 1985, Rithidech *et al.* 1988) or X-rays (Harris *et al.* 1984, 1985). Schlegel and MacGregor (1982) demonstrated that micronuclei induced by chronic treatment with triethylenemelamine could persist in normachromatic erythrocyte (NCE) for 30-40 days in the circulating blood of mice. The Mus-EMN test was conducted to test the clastogenicity of drinking water from a shallow well of a rural community (Ma *et al.* 1987) at the monthly intervals for 6 months. Increased MN frequencies in the treated group over the control were observed 3 months after feeding the mice with the polluted shallow well water. This demonstrated that the Mus-EMN assay was efficient for detecting clastogenicity of water pollutants at very low concentration under the chronic exposure. The current study is one year long project to monitor the clastogenicity of wastewater samples collected from the Arena Canal containing effluents from the Benito Juarez industrial district of the Queretaro City in Mexico (Fig. 1). We also intended to detect the possible seasonal variation of the clastogenicity, and the efficacy of this test for long range monitoring of clastogenicity of water pollutants.

## MATERIALS AND METHODS

The Mus-EMN assay with a double staining procedure (Harris hematoxylin and Giemsa) was used throughout this study. The double stained permanent slides enhanced the

clarity of the micronuclei and allowed more time for careful, repeated scoring. Twenty four young (about 2 month old) CD-1 white mice were divided into three groups of 8, with 4 males and 4 females in each group for this *in vivo* study. Each one of these mice was caged individually for repeated blood sampling and data collection. Two treated groups were fed either with 1:5 (wastewater: tapwater) ratio (treated group 1) or 1:2 (wastewater: tapwater) ratio (treated group 2) of wastewater collected from the Arena Canal diluted with tapwater, and the control group was fed with clean water which was obtained from the tapwater supply of the City of Queretaro. 100 ml of the diluted wastewater or tapwater were supplied to each cage every week, and the blood samples were collected from a small puncture on the tail of the mouse at monthly intervals beginning on September 19, 1986 and ending on August 18, 1987 with the exception of the month of March. About 3  $\mu$ L of peripheral blood collected from each mouse was immediately mixed with 3% ethylene diamine tetraacetic acid (EDTA) solution in a cell-well and smeared onto a clean slide. The erythrocytes were fixed in 100% methanol for 1 minute and allowed to dry in an high temperature (40°C) incubator overnight. The micronuclei (Fig. 2) in the fixed erythrocytes were double stained with Harris hematoxylin (10 minutes) and 0.1% Giemsa (15 minutes). Usually 3 slides were made and repeatedly double scored by two different observers. The scoring procedure involves counting of the number of evenly distributed erythrocytes under a 1000X magnification field of view with the help of a cross-hair reticle in the ocular lens. Generally, the counts were made from 10 typical slides with even-spread erythrocytes in order to establish an average number of erythrocytes per field for a given series of experiments. The number of MNs was actually counted from a number of fields (generally in the neighborhood of 350-500 cells) totalling around 10000 erythrocytes (NCE) from each slide. The means and standard deviations and standard errors of the micronuclei frequencies were derived from 8 slides of each of the experimental groups. The data were statistically analyzed with F-test and Dunnett-t statistics (Dunnett 1955) for the difference (0.05 significance) among two treated against a negative control group.

## RESULTS AND DISCUSSION

The monthly micronucleus frequencies of two treated and one control groups through the year are given in Table I.

The MN frequencies of the treated groups of mice which drank two different dilutions of wastewater in the months of October of 1986 through January of the following year were significantly higher than that of the control. This indicates the clastogenicity of these diluted wastewaters (Table I). Preliminary tests on undiluted wastewater were fatal to the animals. During this 4 month period, the MN frequencies of two different treated groups showed similar patterns of

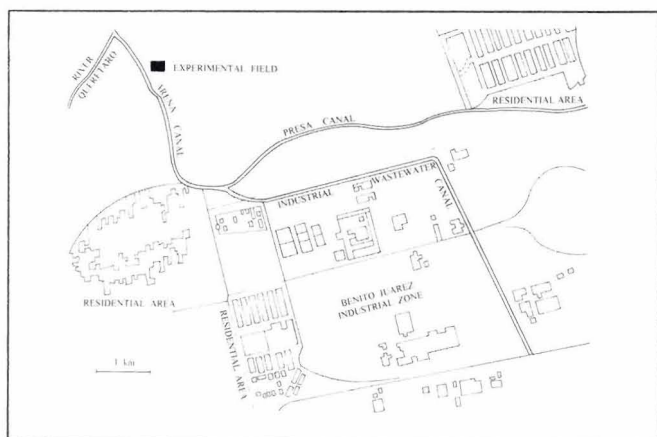


Fig. 1. The site of water sample collection at the Arena Canal which receives the wastewater of the Benito Juarez industrial zone of the Queretaro City.



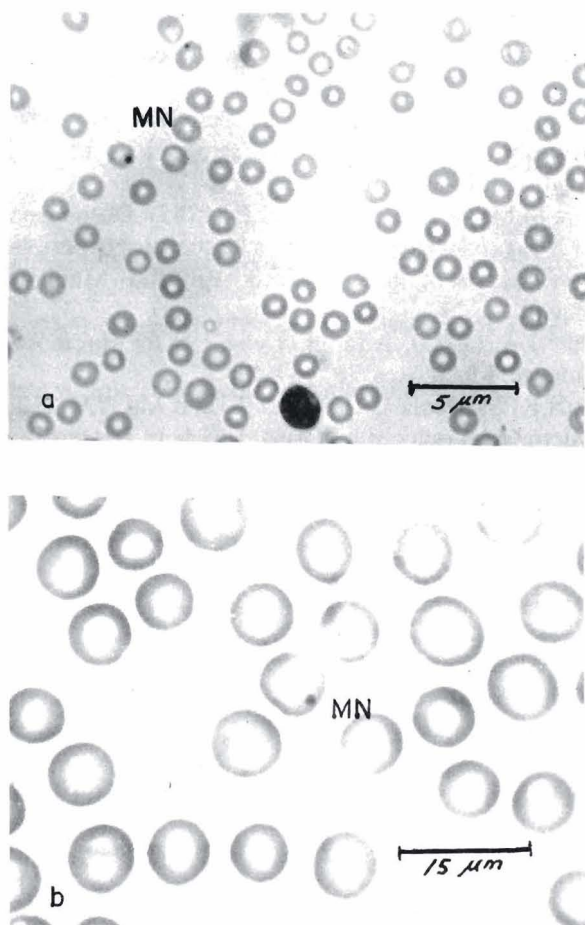


Fig. 2. Photomicrographs of the normochromatic erythrocytes with micronuclei, a) erythrocytes under high dry magnification, and b) under oil immersion.

fluctuation and then fell to the background level after the month of April. The MN frequencies induced by the higher concentration of wastewater (group 2) were relatively higher than that induced by the lower concentration (group 1). This pattern of fluctuation and relative difference in MN frequencies of these two treated groups could serve as the signs of seasonal variation of the clastogenicity of the wastewater and the clastogenicity may be preferentially elevated during the dry season of this area of Mexico. We regret that no chemical analysis data of the water samples were available to substantiate this postulation. The MN frequencies of the control group stayed at the steady rate except the month of January. Whether this was due to elevated clastogenicity of tapwater in this month requires further confirmation with bioassay and chemical analysis. The elevated MN frequencies of the treated groups in the current study began at the end of the first month after treatment as compared with the earlier tests on the shallow well water (Ma *et al.* 1987) which began at the end of the third or fourth month after continuous exposure. The MN frequencies of the current study which reached the significantly higher level in a relatively short time is the sign of higher clastogenicity of wastewater than the shallow well water.

The decline of the MN frequencies after 6-7 months of drinking the diluted wastewater followed a general trend which was demonstrated in earlier tests on shallow well water (Ma *et al.* 1987). A study on low concentrations of formaldehyde (5-15 mg/kg, i.p. injections) treated CD-1 mice (Loarca *et al.* 1989) reached the peak MN frequencies two weeks after treatment and the MN frequencies started to decline 60 days after treatment. A similar decline of

TABLE I. RESULTS OF A YEAR-LONG MONITORING OF THE CLASTOGENICITY OF INDUSTRIAL WASTEWATER WITH THE Mus-EMN ASSAY

Months	Control Tapwater: water MN/1000 NCE Mean $\pm$ S.E.		T-1 (1:5 dilution) Wastewater: Tapwater MN/1000 NCE Mean $\pm$ S.E.		T-2 (1:2 dilution) Wastewater: Tapwater MN/1000 NCE Mean $\pm$ S.E.	
Sep. 1986	3.3	0.53	3.0	0.25	4.5	0.25
Oct.	1.5	0.04	5.4*	0.81	8.2*	0.50
Nov.	3.0	2.47	7.0*	0.25	7.0*	0.32
Dec.	3.2	0.18	8.1*	0.42	9.6*	0.46
Jan. 1987	4.4	0.50	9.0*	0.53	10.0*	0.56
Feb.	3.4	0.25	7.5	1.60	7.6	1.10
Mar.			poor slides			
Apr.	2.8	0.43	3.8	0.42	3.3	0.35
May	2.4	0.21	3.8	0.53	5.0	0.84
Jun.	2.0	0.25	2.2	0.11	2.9	0.21
Jul.	2.6	0.18	2.5	0.18	3.6	0.11
Aug.	2.0	0.15	3.0	0.28	2.0	0.18

The mean MN frequencies of the months of October, November, December and January are C = 3.0, T-1 = 7.4, and T-2 = 8.7, which are relatively higher than the other months of the year and are significantly ( $p < 0.05$ ) higher than control values

\* Significant,  $p < 0.05$

MN frequencies was observed after 42 or 60 days of continuous treatment with benzene (Barale *et al.* 1985, Rithidech *et al.* 1988). The two-year continuous treatment of B6C3F<sub>1</sub> mice with benzene (Choy *et al.* 1985) showed a linear increase of MN frequencies under the dose range of 25-600 mg/kg in blood samples collected on the 120th day. Progressive decline of MN frequencies was observed at the end of one and two years. By comparing the Mus-PEMN test results of these chronic or subchronic continually treated mice, the onset of the peak MN frequency and the time of decline of MN frequency depend upon the magnitude of clastogenicity of the agents. There is a trend that the higher the potency of the treating agent, the quicker it reaches the peak, and also the sooner it starts to decline. This general trend of non-linear increment and decline of MN frequencies after a relatively long duration of chronic or subchronic exposure to the clastogens could be the result of overdose and toxic effect in this test system. For this reason, the Mus-EMN assay has the limited effective duration for chronic or subchronic clastogenicity testing of different chemical agents. The efficient duration falls between 1 and 6 months after the continuous exposures to various agents. Considering the two and one half years of the average life span of the laboratory mice, this 6-month youthful duration in chronic tests of water pollutants covers as much as 20% of their useful life. The efficacy of the Mus-EMN test on chronic effects of water pollutants is well justified. For chemical tests, the Mus-EMN is suitable for subchronic exposure within two-month duration. The current study did not show sex dependent sensitivity as demonstrated in benzene studies (Barale *et al.* 1985, Choy *et al.* 1985).

A report by Parton *et al.* (1989) indicated that the *in vivo* induced MN could be expelled from polychromatic erythrocytes in the bone marrow of the mouse. Whether or not this could indirectly contribute to the decline of the MN frequency in the peripheral blood erythrocytes requires further investigation. At least, so far, there is no report available for such expulsion process in peripheral blood erythrocytes.

## REFERENCES

- Barale R., Giorgelli F., Ciranni R., Casini D., Succoni D. and Loprieno N. (1985). Benzene induced micronuclei in circulating erythrocytes of chronically treated mice. *Mutat. Res.* 144, 193-196.
- Choy W.N., MacGregor J.T., Shelby M.D. and Maronport R.R. (1985). Induction of micronuclei by benzene in B6C3F<sub>1</sub> mice: retrospective analysis of peripheral blood smear from the NTP carcinogenesis bioassay. *Mutat. Res.* 143, 55-59.
- Dunnett C.W. (1955). A multiple comparison procedure comparing several treatments with a control. *J. Amer. Stat. Assoc.* 50, 1096-1121.
- Harris M.M. and Ma T.H. (1984). Peripheral-Erythrocyte-Micronucleus assay on the clastogenicity of X-rays. *Environ. Mutagen.* 6, 403.
- Harris M.M., Ma T.H. and Giles P. (1985). Dose response of X-ray induced micronucleus frequencies in peripheral erythrocytes of mice and rats. *Environ. Mutagen.* 7 (Suppl. 3), 29.
- Heddle J.A. (1973). A rapid *in vivo* test for chromosomal damage. *Mutat. Res.* 18, 187-190.
- Loarca F.P., Arreola G.G., Perez A. and Ma T.H. (1989). Micronuclei induced by formaldehyde in erythrocytes of mice. *Environ. Mol. Mutagen.* 14 (Suppl. 15), 117.
- Ma T.H., Anderson V.A., Harris M.M., Neas R.E. and Lee T.S. (1985). Mutagenicity of drinking water detected by *Tradescantia*-Micronucleus test. *Can. J. Genet. Cytol.* 27, 143-150.
- Ma T.H., Miller T.L. and Xu Z. (1985). Mouse peripheral erythrocytes micronucleus (Mus-MCN) test on genotoxicity of acetaldehyde. *Environ. Mutagen.* 7, 28-29.
- Ma T.H., Neas R.E., Harris M.M., Xu Z., Cook C. and Swofford D. (1987). *In vivo* tests (*Tradescantia*-and Mouse-Micronucleus) and chemical analysis on drinking water of rural communities. In: Short-term bioassays in the analysis of complex mixtures V. (S.S. Sandhu, D. DeMarini, M.J. Mass, M.M. Moore and J.L. Mumford, Eds.) Plenum Press, New York, pp. 189-205.
- MacGregor J.T., Wehr C.M. and Gould D.H. (1980). Clastogen-induced micronuclei in peripheral erythrocytes: the basis of an improved micronucleus test. *Environ. Mutagen.* 2, 509-514.
- Parton J., Beyers J. and Garriott M. (1989). The expulsion of micronuclei from polychromatic erythrocytes of mouse bone marrow *in vivo*. *Environ. Mol. Mutagen.* 14 (Suppl. 15), 152.
- Rithidech K., Au W.W., Sadogopa B., Whorton E.B. and Legator M.S. (1988). Persistence of micronuclei in peripheral blood normochromatic erythrocytes of subchronically treated male mice. *Environ. Mol. Mutagen.* 12, 319-329.
- Salamone M., Heddle J.A., Stuart E. and Katz M. (1980). Towards an improved micronucleus test: studies on 3 model agents: mitomycin C, cyclophosphamide and dimethylbenzanthracene. *Mutat. Res.* 74, 347-356.
- Schlegel R. and MacGregor G.T. (1982). The persistence of micronuclei in peripheral blood erythrocytes: detection of chronic chromosome breakage in mice. *Mutat. Res.* 104, 367-369.
- Schmid W. (1975). The micronucleus test. *Mutat. Res.* 31, 9-15.