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Discrimination between micronuclei induced by spindle poisons and clastogens by using toads bone marrow polychromatic erythrocytes

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ABSTRACT

A proposal for discrimination between aneugens and clastogens by using bone marrow polychromatic erythrocytes (PCEs) of *Bufo regularis* was submitted. Two common aneugens (colchicine and vinblastine sulphate) and two common clastogens (cyclophosphamide and mitomycin C) were used. The micronuclei (MN) appeared as small spherical or oval bodies present inside MN-PCEs and obviously separated from the nucleus. The used chemicals induced high percentages of micronuclei with variable sizes, which clarify the sensitivity of bone marrow cells of *Bufo regularis* to genotoxic action of chemicals. Twenty-five MN-PCEs induced by each chemical were randomly selected, photomicrographed and measured by the ruler for subsequent analysis. The micronucleus relative length was calculated by dividing the nucleus length over micronucleus length of the same MN-PCE. According to calculations, the proposal stated that, the aneugens induced large micronuclei with relatively large micronuclei (> 20 % of nucleus length) and conversely, the clastogens induced relatively much smaller micronuclei (< 20 % of nucleus length). The results of this work pointed out: (1) the benefit of using of bone marrow micronucleus test in *Bufo regularis* for detecting genotoxic action of chemicals, (2) the availability of discrimination between micronuclei produced by spindle poisoning agents and those produced by chromosome breaking agents.

KEYWORDS: bone marrow polychromatic erythrocytes, , clastogens, micronuclei.

INTRODUCTION

The micronucleus test on the mouse bone marrow polychromatic erythrocytes - originally developed by Schmid (1975) - is probably the most frequently used *in vivo* short-term genotoxicity test. This test can be used as an alternative for a chromosomal aberration assay. Mammalian bone marrow micronucleated erythrocytes provide a simple and rapid method for detection of some types of chromosomal damage caused by chemical and physical agents (Krishna & Hayashi 2000). Bone marrow cells of the maculated toad, *Bufo regularis*, were proven to be sensitive for genotoxic agents using both micronucleus test and chromosomal aberrations assay (Mosallam 2000). The micronuclei in circulating blood cells of newts have also been used in an attempt to evaluate amphibian haematopoeic tissues for sensitivity to some genotoxicants (Djomo *et al.* 2000). Other trials have been carried out to test systems other than amphibians and mammals using the micronucleus assay, e.g. chick (Bhunya & Jena 1996), fish (Ayllon & Garcia-Vazquez 2000) and plants (Cotelle *et al.* 1999).

Many trials have also been carried out to evaluate the use of developing micronuclei in different organs and tissues other than bone marrow as indicators of genotoxicity, from rat tracheal epithelial cells (Zhu *et al.* 1991) and human exfoliated bladder cells (Smith *et al.* 1993). The developing micronuclei indicate the occurrence of a chromosomal aberration(s), but does not specify the type of the aberration. However, micronuclei induced by spindle poisons are, in general, considerably larger than those induced by clastogens (Vanhauwaert *et al.* 2001). It is evident that micronuclei originating from chromosomal fragments are generally ought to be smaller than those resulting from whole chromosome(s).

The main objective of this work was to evaluate micronucleus induction in polychromatic erythrocytes from bone marrow cells of *Bufo regularis* caused by the genotoxicity of well-known clastogens and spindle poisoning agents, as well as to submit a

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proposal for discrimination between micronuclei developed by clastogens and spindle poisons agents.

MATERIALS AND METHODS

Animals: Animals were captured from public gardens to avoid exposure to contaminants in the cultivated areas. The animal weights ranged from 30-40 g. The animals were housed in glass containers (aquaria 90x50x50 cm). The aquarium was filled with water to about half its height. A large piece of cork inside the aquarium was used to enable animals to settle on it. The water inside the aquarium was changed with new tap water every 2 days. A stainless steel net with slots (1x1 cm) was used to cover the aquarium. The animals were acclimatized for about two weeks before the start of experimental procedure. During this period the animals were fed once every two days with small pieces of minced fresh beef liver. For chemical treatments, the animals were assorted into 26 groups (6 animals each) and were settled in a small aquarium (30x15x15 cm).

Chemicals and treatment: The following chemicals were used: Colchicine (COL; 0.2, 0.4 & 0.8 mg/kg b.w.) in 1-g vials (Sigma chemicals, USA); Vinblastine sulphate (VLB; 0.01, 0.1 & 0.4 mg/kg b.w.) in 10-mg vials (under the trade name, Velbe, Lilly, USA); Cyclophosphamide (CPA; 5, 20 & 40 mg/kg b.w.) in 100-mg vials (under the trade name Endoxan, Asta Verke, Germany); Mitomycin C (MMC; 0.05, 0.1 & 0.4 mg/kg b.w.) in 5-mg vials (under the trade name Mutamycin, Bristol Myers Squibb, USA).The doses of all chemicals were chosen on the basis of their effectiveness in developing micronuclei in bone marrow polychromatic erythrocytes of mammalian test systems (Grawe *et al.* 1994).

All the chemicals were dissolved in amphibian saline (7g/L NaCl in bidistilled water). All the solutions were freshly prepared directly before treatment of the animals. The injected volume was 0.1 mL/10 g body weight. All the doses were applied as a single intraperitoneal injection.

Micronucleus test: The animals were anaesthetized by ether and bone marrow collected from the femur bone of one leg in a centrifuge tube containing an amphibian saline (7g/L sodium chloride in bidistilled water) and mixed gently. After centrifugation for 5 min at 500 r.p.m., the supernatant was discarded and the cell pellet resuspended in about 1 mL saline. One drop of the cell suspension was smeared on clean glass slides and air-dried. The bone marrow cells were fixed in methyl alcohol for 10 min and air-dried. The slides were stained with Giemsa stain (2 % in phosphate buffer solution, pH 6.8 for 5 min) and examined using a phase-contrast binocular microscope (Hund, Germany) with x10 eyepieces and x100 plane achromatic oil immersion objective lens. Micronuclei were scored among 1000 PCEs from one or two slides from each animal.The percentage of PCEs was counted according to the following equation:

$$\% \text{ PCEs} = \text{PCEs}/100 \ (\text{PCEs} + \text{NCEs}).$$

Scoring criteria of the work were based on the following criteria:-

1- Polychromatic and normochromatic erythrocytes were differentiated by the colour of their cytoplasm.The cytoplasm of PCEs appears reddish blue (due to the presence of remnants of RNA in the cytoplasm) while that of NCEs is red.
2- Micronuclei were scored only when spherical or oval in shape and with a blue colour, the same intensity of the nucleus of the same cell to avoid artifacts.
3- Micronucleus must be obviously separate from the nucleus of the PCE.
4- MN-NCEs were not included in the counts.

The 1000 scored PCEs were observed in randomly selected locations on one or two slides. About 35 MN-PCEs were randomly selected for each chemical -regardless of dose- to be photographed for subsequent measurements.

Photomicrography: All conditions for microphotography were kept constant to obtain photographs with the same quality. These constant conditions included the magnification, illumination, temperature and chemical composition of the developer and fixer for developing the film and the sensitive photographic papers as well as the enlarging power of the magnifier. In order to control the microscope illumination an electric voltage stabilizer was used. The microphotography was carried out with x100 oil immersion plane achromatic objective lens and x7 subjective lens with exposure time 2 sec, using black and white Kodak T-Max 100 film printed at 60 x magnification.
Measurements: Measurements were made by ruler from the photomicrographs.The long axes of the micronuclei and nuclei were measured and tabulated for further analysis.
Statistical analysis: The significance of difference between the control value versus the results of the preliminary experiment was assayed by Student's *t* test. Nested analysis of variance (anova) was carried out to compare between the data of MN-PCEs or PCEs resulted from aneugens (COL and VLB) or those resulted from clastogens (CPA and MMC) and a *P*-value less than 0.05 was considered statistically significant.

RESULTS

The cell cycle in amphibians is longer relative to mammals, so the appropriate interval between animal treatment and bone marrow sampling must be determined beforehand. Since we had no prior data about the profile of the genotoxic action of these chemicals on toad bone marrow cells, a preliminary experiment was designed to determine the optimal time for bone marrow sampling. An expected intermediate dose was chosen which induced PCEs with micronuclei.These micronuclei appeared in the form of spherical bodies inside the cytoplasm of the nucleated PCEs. The MN-PCEs had small (Fig. 1), large (Fig. 2) and in a few instances multiple micronuclei (Fig. 3). This preliminary experiment (Fig. 4) shows that the optimal time for elevated MN-PCEs production –for all chemicals- was two days after treatment ($p < 0.001$). Treatment also resulted in a remarkable cytotoxicity in the form of decreased percentages of PCEs ($p < 0.01$).

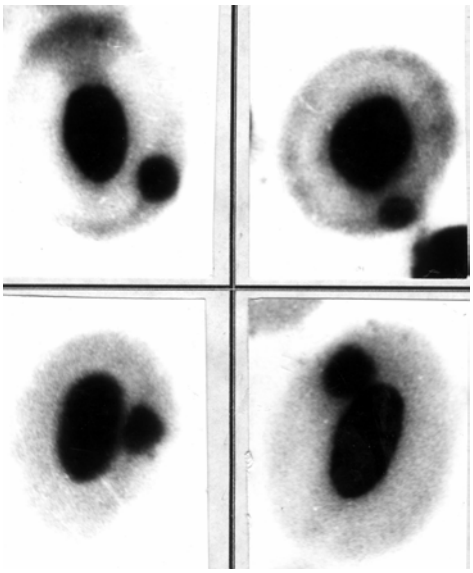


Figure 1: Photomicrographs of bone marrow micronuleated polychromatic erythrocytes (MN-PCEs) showing large-type micronuclei from colchicine treated animals. The micronucleus length is larger than 1/5 that of the nucleus.

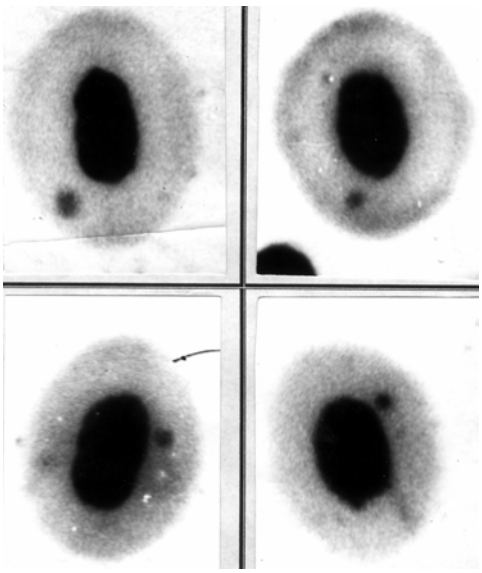


Figure 2: Photomicrographs of bone marrow micronuleated polychromatic erythrocytes (MN-PCEs) showing samll-type micronuclei from cyclophosphamide treated animals. The micronucleus length is less than 1/5 that of the nucleus.

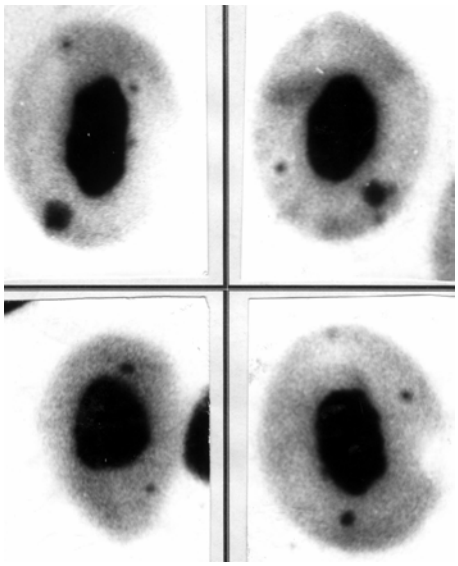
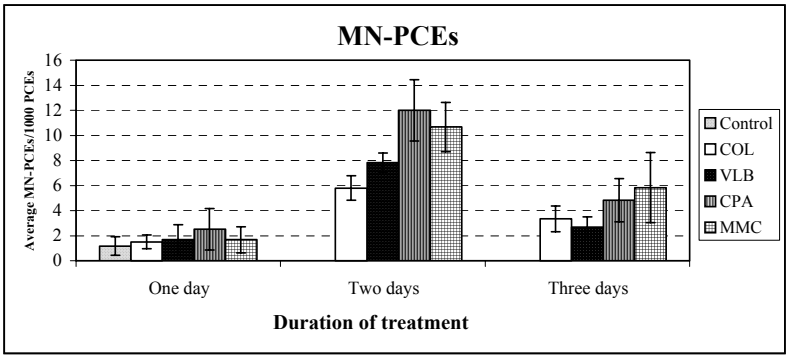


Figure 3: Photomicrographs of bone marrow micronuleated polychromatic erythrocytes (MN-PCEs) showing multiple micronuclei from mitomycin C treated animals. The same MN-PCEs have many micronuclei with variable



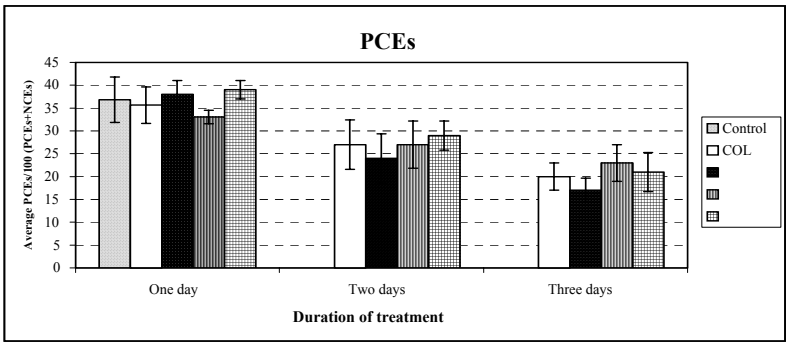


Figure 4 : The results of the preliminary experiment on the averages of MN-PCEs and PCEs in bone marrow of toads treated with aneugens (COL 0.4 mg/kg and VLB 0.1 mg/kg) and clastogens (CPA 20 mg/kg and MMC 0.1 mg/kg) for one two and three days.

The Effect of the four chemicals: Toads treated with aneugens (colchicine and vinblastine sulphate) and clastogens (cyclophosphamide and mitomycin C) are significantly increased the MN-PCEs over the control (Fig. 5). Moreover, the clastogens induced statistically significant increases in the frequencies of MN-PCEs than aneugens ($p < 0.05$). It is clear that, the effect of MMC is dose dependent and linear. All the chemicals induced a significant linear reduction in the percentages of PCEs (Fig. 6). For aneugens, the reductions in the percentages of PCEs are very similar, but for clastogens, MMC induced a much greater reduction in the percentages of PCEs than CPA ($p < 0.05$).

Table 1 show measurements and calculations of 25 MN-PCEs -randomly selected for each chemical -regardless the dose level. The two aneugens (COL and VLB) on average induced relatively large micronuclei (> 20 % of nucleus length), but conversely, the two clastogens (CPA and MMC) induced much smaller micronuclei (Fig, 7), < 20 % of nucleus size on average. There were differences in the frequency of large and small micronuclei, since the aneugens induced only 16 % (COL) and 4 % (VLB) of the small type, where as clastogens induced 88 % (CPA) and 72 % (MMC).

Finally, a trial was carried out to investigate 100 MN-PCEs induced by each chemical, regardless of dose, classifying them by direct observation under the microscope into large (> 20 % of nucleus length) and small (< 20 % of nucleus length) types. This showed that the vast majority of micronuclei were singlets, and confirms that aneugens induced mainly the large type and clastogens the small type of micronuclei (Fig. 8).

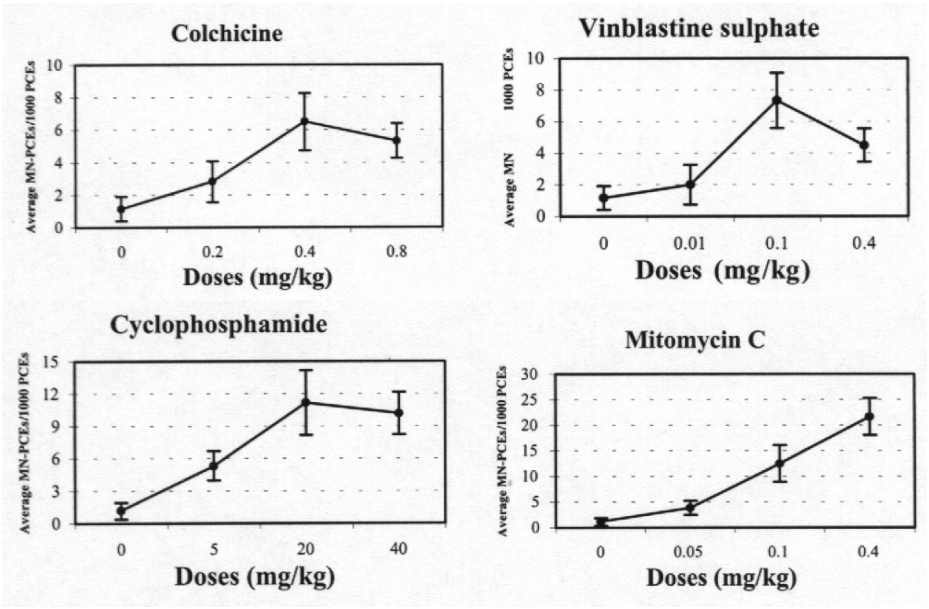


Figure 5: The effect of treatment with aneugens (colchicine and vinblastine sulphate) and clastogens (cyclphosphamide and mitomycin C) on MN-PCEs counts in bone marrow of the toads.

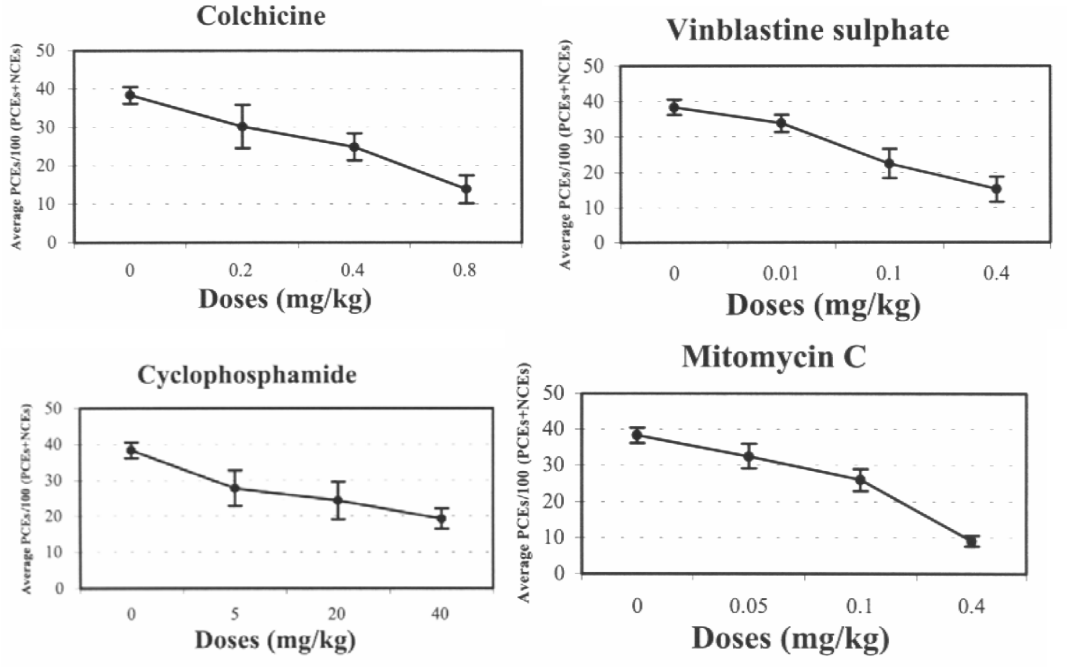


Figure 6: The effect of treatment with aneugens (colchicine and vinblastine sulphate) and clastogens (cyclphosphamide and mitomycin C) on MN-PCEs counts in bone marrow of the toads.

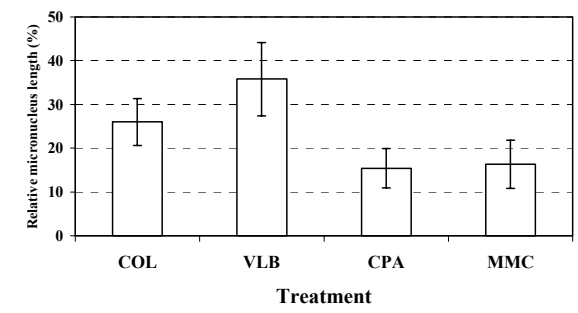


Figure 7 : Relative micronucleus length in relation to the nucleus length of the same MN-PCEs due to treatment with aneugens (COL and VLB) and clastogens (CPA and MMC).

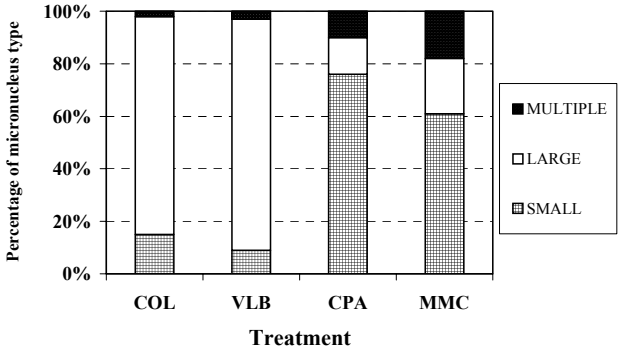


Figure 8: The distribution of micronucleus type among 100 MN-PCEs induced by each chemical and examined directly under the microscope.

DISCUSSION

There is a general agreement that the cell cycle in amphibians tissues is extended for more a longer time than in mammalian tissues (Wallace & Maden 1976; Masui & Wang 1998), and our pilot experiment showed that the optimum sampling time is two days after treatment. In the present study, bone marrow PCEs of the toad were shown to be sensitive to micronuclei induction by both aneugens and clastogens. Generally, the clastogens induced many more MN-PCEs than aneugens, mostly of the small type, and more frequently induced multiple micronuclei. These results are similar to those of mammalian bone marrow PCEs (Vanderkerken *et al.* 1989; Grawe *et al.* 1994). We could discriminate between the effects of aneugens (mostly large) and clastogens (mostly small) using a criterion of 20 %. The possible explanation for developing micronuclei is that COL and VLB are spindle poisons affecting microtubule assembly during the formation of spindle fibres, which leads to chromosome lagging. CPA and MMC are chromosome-breaking agents which lead to the formation of acentric chromosomal fragments. Such lagged chromosomes and acentric chromosomal fragments are then encapsulated with a membrane to form large and small micronuclei respectively, (Vanparys *et al*. 1990; Elhajouji *et al.* 1997). Various simple or sophisticated methods on micronuclei to discriminate between the effects of clastogens and aneugens have been used from micronucleus morphology, the analysis of DNA content by flow cytometric analysis, fluorescent in situ hybridization with a DNA centromeric probe, presence of kinetochore and C-banding techniques. Vanderkerken *et al.* (1989) classified micronuclei into two major types from morphology, classifying small and large micronuclei relative to cell size.They postulated that aneugens induced micronuclei of the large type (> 0.25 cell diameter) while clastogens induced small micronuclei (< 0.25 cell diameter). Verschaeve *et al.* (1988) and Vanderkerken *et al.* (1989) used the classical C-banding technique to discriminate between clastogens and aneugens, finding that C-band positive micronuclei were much more frequent with aneugens (> 45 %) than clastogens (< 25 %). Detecting centromeres in aneugen-induced micronuclei by antikinetochore antibodies (Surralles *et al.* 1995; Stopper *et al.* 1994) found more than 85% stained positive for the presence of kinetochores, implying that these micronuclei contained whole chromosomes. Another method depends on the DNA staining properties of micronuclei, the flow cytometric analysis. The mean DNA content of micronuclei induced by aneugens was greater than clastogen-induced micronuclei (Van Hummelen *et al.* 1995; Vanderkerken *et al.*1989; Grawe *et al.* 1994; Torous *et al.* 1998). Another method depends on the presence of a fluorescent signal in the MN following fluorescent in situ hybridization (FISH) with a DNA centromeric probe (Darroudi *et al.* 1996; Migliore *et al.* 1996).

In conclusion, the bone marrow polychromatic erythrocytes of *Bufo regularis* not only could be used to detect genotoxic chemicals, but also can discriminate between clastogens and aneugens using micronucleus length relative to nucleus length. The ease with which the assay is performed and reproducibility of the results with clastogenic and aneugenic compounds indicate that the amphibian *Bufo regularis* is a promising animal for routine screening of suspected genotoxicants.

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الملخص العربى

التمييز بين الأنوية الدقيقة الناتجة عن المعالجة بمسممات خيوط المغزل ومكسرات الصبغيات فى خلايا الدم الحمراء متعددة الصبغات فى نخاع العظم للضفدعة الرقطاء

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تمت دراسة حساسية خلايا الدم الحمراء متعددة الصبغيات ذوات الأنوية الدقيقة الناتجة عن المعالجة بمسممات خيوط المغزل ومكسرات الصبغيات وكذلك تم تقديم اقتراح لإمكانية التمييز بين أنواع الأنوية الدقيقة الناتجة عن المعالجة بمسممات خيوط المغزل ومكسرات الصبغيات فى نخاع العظم للضفدعة الرقطاء. تمت معالجة الحيوانات عن طريق التجويف البريتونى بمركبي الكولشسين وكبريتات الفنبلاستين كعوامل مسببة لتسمم خيوط المغزل, ومركبي السيكلوفوسفاميد وميتوميسين سى كعوامل مسببة لتكسير الصبغيات, وتمت المعالجة بجرعات مفردة من المركبات على حدة. وبدراسة تأثير المعالجة بالمركبات الكيميائية المختلفة وجد أنه قد حدث زيادة فى معدل ظهور خلايا الدم الحمراء متعددة الصبغيات ذوات الأنوية الدقيقة وبلغت هذه الزيادة أقصاها فى سحبات نخاع العظم بعد مرور 48 ساعة بعد المعالجة، وحدوث نقص حاد فى عدد خلايا الدم الحمراء متعددة الصبغيات عند المعالجة بالجرعات المرتفعة, ولقد ظهرت الأنوية الدقيقة عل هيئة أجسام كروية أو بيضاوية مختلفة فى الحجم0 وجد أيضا أن الأنوية الدقيقة الناتجة من المعالجة بمركبي الكولشسين وكبريتات الفنبلاستين كانت ذوات أحجام أكبر بـ 20% من حجم النواة لنفس الخلية، بينما الأنوية الدقيقة الناتجة من المعالجة بمركبي السيكلوفوسفاميد وميتوميسين سى كانت ذوات أحجام أصغر بـ20% من حجم النواة لنفس الخلية

Table 1: Measurements of nucleus length (NL), micronucleus length (MNL) and the relative micronucleus length MNL/NL(%) for randomly selected 25 MN-PCEs with single micronucleus induced by each chemical.

Serial	Colchicine			Vinblastine sulphate			Cyclophosphamide			Mitomycin C		
	NL	MNL	MNL/NL	NL	MNL	MNL/NL	NL	MNL	MNL/NL	NL	MNL	MNL/NL
1	3.1	1.3	41.9	2.7	1.3	48.1	2.9	0.2	6.8	3.1	0.3	9.6
2	3.1	0.8	25.8	2.7	1	37	2.9	0.3	10.3	3.2	0.5	15.6
3	3.1	0.6	19.3	3.1	1.1	35.4	2.9	0.7	24.1	2.8	0.4	14.2
4	2.8	0.9	32.1	3.4	1.5	44.1	3	0.4	13.3	2.8	0.5	17.8
5	2.9	0.7	24.1	2.8	1.3	46.4	2.6	0.5	19.2	3.1	0.4	12.9
6	2.9	0.7	24.1	2.7	1.4	51.8	2.9	0.5	17.2	3	0.3	10.1
7	3	0.8	26.6	2.6	1.1	42.3	3.3	0.4	12.1	2.8	0.6	21.4
8	3	0.8	26.6	3.2	1.3	40.6	3	0.6	20.1	2.7	0.6	22.2
9	3	0.7	23.3	2.9	0.8	27.5	3	0.4	13.3	3	0.7	23.3
10	3	0.7	23.3	2.6	1	38.4	3.2	0.7	21.8	3.2	0.4	12.5
11	3	1	33.3	2.8	1	35.7	3	0.5	16.6	2.9	0.3	10.3
12	2.9	0.8	27.5	2.8	1.2	42.8	3	0.3	10.1	2.9	0.9	31.1
13	3	0.8	26.6	3.2	0.6	18.7	3	0.5	16.6	2.9	0.4	13.7
14	3	1	33.3	2.8	0.8	28.5	3.2	0.3	9.3	3	0.5	16.6
15	2.7	0.7	25.9	3	0.9	30	3.2	0.4	12.5	3	0.4	13.3
16	3.1	0.8	25.8	3.1	1.1	35.4	2.6	0.5	19.2	3.2	0.4	12.5
17	3	0.9	30	2.9	1.2	41.3	3.2	0.7	21.8	3	0.3	10.1
18	2.9	0.7	24.1	3.2	1.3	40.6	3.2	0.4	12.5	3.1	0.6	19.3
19	3.2	0.5	15.6	3	1.2	40	2.9	0.6	20.6	2.8	0.7	25.1
20	2.8	0.6	21.4	3	0.9	30	3.4	0.5	14.7	3	0.5	16.6
21	3	0.7	23.3	3.1	0.7	22.5	2.8	0.5	17.8	2.9	0.6	20.6
22	3.1	0.8	25.8	2.8	0.8	28.5	2.9	0.5	17.2	3.1	0.3	9.6
23	3.1	0.7	22.5	2.9	0.9	31	3.2	0.4	12.5	3.3	0.5	15.1
24	3.2	0.6	18.7	2.7	1	37	3.1	0.3	9.6	2.9	0.6	20.6
25	3	0.9	30	3.1	0.7	22.5	3	0.5	16.6	3	0.4	13.3
Total	74.91	19.5	650.9	73.1	26.11	896.1	75.42	11.61	385.8	74.81	12.11	407.4
Mean	3.1	0.78	26.0	2.9	1.04	35.8	3.02	0.46	15.4	2.99	0.48	16.3
S.D.	0.12	0.16	5.4	0.2	0.24	8.4	0.20	0.13	4.5	0.15	0.15	5.5
% small MN (< 20 %)			16			4			88			72
% large MN (> 20 %)			84			96			12			28

N.B.: The measurements were carried out by the ruler and the numbers in centimeters.
The relative micronucleus length (MNL/NL) is expressed in percentages.