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COMPARISON OF MICRONUCLEI FREQUENCY IN BONE MARROW CELLS OF THREE RAT LINES



The aim of this paper is to compare the spontaneous and induced with cyclophosphamide micronucleus indexes in bone marrow cells of the Sprague Dawley, Lewis and Wistar rat lines. Five experimental groups were formed (10 animals of each sex and of each line, in every group). The first group was used as the negative control (intact animals), the second one was exposed to oral administration of drugs; other conditions were the same as for the other groups. The third group was treated with 2 % Tween 65 and the fourth group was treated with 0.9 % NaCl. Both substances were administered by oral way to 2 ml/kg during 14 days. The fifth group was treated intraperitoneally with strong mutagen cyclophosphamide in the dose of 50 mg/kg (10 ml/kg in solution), on 48th and 24th hours before euthanasia. The Sprague Dawley line (both sexes) was significantly different from the other lines. Rats of this line had lower index of spontaneous formation of micronuclei, higher index of cyclophosphamide-induced micronucle formation, percent of micronucleated erythrocytes in bone marrow and the index of cytotoxicity. The results obtained make it possible to identify the most appropriate line of rats as model animals for studies of genotoxicity. It will allow also to obtain more accurate estimates of genotoxicity of various substances.

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Introduction. Usually for testing genotoxicity of different substances researchers use different lines of rats. Due to genetic differences between lines their results cannot be compared. That is why the correct choosing of model organisms remains the very important problem [1]. Comparative studies of spontaneous and induced genome damages in different lines of rats could provide researchers with efficient way of choosing the most appropriate genetic lines of rats for their tests.

One of popular test of genotoxicity is the micronucleus assay in bone marrow cells. This test is easy to perform and it provides researchers with clear information on the rate of genome damages in bone marrow cells. This approach allows to register in vivo an ability of chemical substances to induce chromosome breaks [2]. The increasing of frequency of polychromatic erythrocytes with micronuclei indicates the respective increasing of chromosome damages [2, 3]. The detection of micronucleated erythrocytes can also be performed in species, whose spleen cannot eliminate the micronucleated erythrocytes or species sufficiently sensitive to detect agents that cause numeric or structural chromosome aberrations [2, 3]. The micronucleus assay must include the positive control with mutagenic substance – usually cyclophosphamide [2]. This mutagen is an alkylating agent that forms monoadducts and cross-connections between chains [3] and does not possess the specificity dependent on the cell cycle [4]. The aim of this article is to compare the spontaneous and cyclophosphamide induced micronucleus indexes of Sprague Dawley, Lewis and Wistar rat lines.

Materials and methods. *Animals and environmental conditions.* Sprague Dawley, Lewis and Wistar rat lines, young adults (6–8 weeks of age) of both sexes with body mass about 180–210 g at the end of the quarantine were used in our work. They were held under temperature 23 ± 2 °C, relative humidity 60 ± 10 % and 12 h cycles of light-darkness. The access to the water and the food (CENPALAB) was *ad libitum*. These conditions were common for all groups. We adhered to the ethical principles for the investigation with laboratory animals according to the Canadian Advice of Care Animal [5].

Doses and treatment procedures. All the substances were administered in the «two days» schedule and the concentrations were adjusted weekly according to the body mass increasing. The ani-

mals were randomly distributed among 5 groups with 10 rats of each sex of each line per group. The first group was used as the negative control (intact animals), the second one was exposed to oral administration of drugs (technique control group). The third experimental group was treated with 2 % Tween 65 (carrying substance 1) which is used as carrier of many nonpolar hydrophobic substances, useful as tensoactive agent [6]; the fourth group was treated with 0.9 % NaCl (carrying substance 2), useful as solvent of most hydrophilic substances [7]. Both substances were prepared for 2 hours before using and were applied per os to 2 ml/kg during a period of 14 days. The fifth group was treated intraperitoneally with cyclophosphamide (Ledoxina®. Lemery. CORP) previously dissolved in 0,9 % NaCl, in the dose of 50 mg/kg [8]. The solution of cyclophosphamide was used immediately after being prepared and applied to animals (in dose 10 ml/kg), on the 48th and 24th hours before euthanasia [8].

Clinical observations. We performed two daily clinical observations, during 8:30–10:30 a.m. and during 3:00–4:30 p.m. During each observation we had investigated the general clinical state of the animal, including the palpation for the detection of lesions, possible breathing affectations, of the nervous, cardiovascular, gastrointestinal system, state of the skin, hair, mucous and eyes coloration.

Euthanasia method. All animals were sacrificed with ether. We provided this process in a way that the animals did not show pain signs or suffering.

Bone marrow cells micronucleus assay. The micronucleus assay was performed according to the modified protocol [9, 10]. One femur per animal was extracted and the medullar cavity was washed out with 3 mL of fetal bovine serum. The obtained cells were centrifuged at 200 g for 10 min; and the pellet was spreaded onto the slides [9]. The slides (minimum 2 slides per animal) were dried 24 h under ambient temperature, fixed in absolute methanol (5 min) and stained with 5 % Giemsa for 12–15 min [10]. The analysis was performed by three independent observers, using a microscope Olympus BH-2 (100× lens with immersion).

The polychromatic erythrocytes (PE) and normochromatic erythrocytes (NE) were counted among 2000 cells/animal. The young eryth-

rocytes are the PE, microscopically they are observed as blue cells and the normochromatic erythrocytes (adults) are observed as pink [2, 9]. The percent of PE with micronucleus (MN-EP) was calculated in 2000 PE/animal. Later the cytotoxicity index (PE/NE) was calculated for the total population of erythrocytes per group and total number of micronucleus (MN) [10]. In this work we presented the average results of three independent observers.

Statistical analysis. We calculated the average (X) and standard deviation (S.D) of studied variables. The comparisons between controls and cyclophosphamide-treated (CF) groups for the same line, among different lines, sex and observers were performed with ANOVA with significance level $\alpha = 0.05$. The categorical variables (total number of micronuclei) were analysed by Chi-Square, with significance level of $\alpha = 0.01$. All the analyses were performed with STATISTICA (version 6) (StatSoft, Inc, 2003).

Permission of ethics committee. The authors declare that this work was made on the base of good practices of preclinical laboratory that are present in the national regulation of protocols of research, approved in the Cuban republic. When this research began it was obtained the protocol approval in writing.

Results and discussion. We did not observe any systemic damages of the whole organism of animals. We did not observe also animals with symptoms of intoxication. The damages were noted at the level of somatic cells. It let us conclude that the doses and the schedule of treatments were appropriate.

The groups of cyclophosphamide-treated animals differed from other groups of rats, while groups of negative controle, groups of control of the treatment way and two solvent substances did not differe each from other in the same sex groups and lines [7, 11]. The results obtained for Sprague Dawley rats are in good accordance with our previous works [12, 13].

The low (but significant) intoxication (PE/NE) was noted for Sprague Dawley rats of both sexes. No differences were found among sexes of the same group and rat line (Table 1) [7, 11]. In other studies differences among sexes were not observed also [14–16].

In Sprague Dawley line the PE/NE is equal 1.18 ± 0.06 ; the genotoxicity index was about

Table 1

The cytotoxicity indexes (PE/NE) and percentage of PE with micronucleus in bone marrow of Sprague Dawley, Lewis and Wistar rats

Groups	n	Sex	MN ^R	PE/NE ^T	MN-PE (%) ^T
Sprague Dawley Rats					
Negative Control (Intact)	10	F	28a	1,21 ± 0,05	0,20 ± 0,03a
	10	M	26a	1,17 ± 0,04	0,18 ± 0,04a
Technique Control	10	F	32a	1,19 ± 0,06a	0,22 ± 0,01a
	10	M	27ac	1,20 ± 0,03a	0,19 ± 0,05a
Vehicle Substance 1	10	F	29a	1,21 ± 0,02a	0,20 ± 0,02a
	10	M	30a	1,19 ± 0,04a	0,21 ± 0,02a
Vehicle Substance 2	10	F	25a	1,18 ± 0,04a	0,17 ± 0,05a
	10	M	22a	1,18 ± 0,06a	0,15 ± 0,06a
Cyclophosphamide ¹	10	F	250**a	0,89 ± 0,04*a	1,74 ± 1,03*a
	10	M	241**ac	0,90 ± 0,03*a	1,68 ± 0,92*ac
Lewis Rats					
Negative Control (Intact)	10	F	46b	1,10 ± 0,03b	0,31 ± 0,04b
	10	M	48b	1,11 ± 0,03b	0,33 ± 0,03b
Technique Control	10	F	48b	1,11 ± 0,02b	0,33 ± 0,05b
	10	M	47b	1,09 ± 0,03b	0,32 ± 0,06b
Vehicle Substance 1	10	F	49b	1,12 ± 0,02b	0,34 ± 0,06b
	10	M	50b	1,13 ± 0,02 b	0,34 ± 0,05b
Vehicle Substance 2	10	F	42 b	1,10 ± 0,03 b	0,29 ± 0,07b
	10	M	44 b	1,13 ± 0,02 b	0,30 ± 0,07b
Cyclophosphamide ¹	10	F	295**b	0,82 ± 0,02*b	2,03 ± 1,48*b
	10	M	291**b	0,81 ± 0,02*b	2,00 ± 1,08*b
Wistar Rats					
Negative Control (Intact)	10	F	53b	1,11 ± 0,02b	0,36 ± 0,03b
	10	M	47bc	1,10 ± 0,04b	0,33 ± 0,02b
Technique Control	10	F	54b	1,12 ± 0,03b	0,37 ± 0,02b
	10	M	49bc	1,14 ± 0,02b	0,34 ± 0,04b
Vehicle Substance 1	10	F	50b	1,10 ± 0,03b	0,34 ± 0,05b
	10	M	55bc	1,11 ± 0,03b	0,38 ± 0,03b
Vehicle Substance 2	10	F	51b	1,10 ± 0,03b	0,35 ± 0,03b
	10	M	46bc	1,12 ± 0,04b	0,32 ± 0,05b
Cyclophosphamide ¹	10	F	292**bc	0,79 ± 0,03*b	2,01 ± 1,56*b
	10	M	298**b	0,80 ± 0,02*b	2,05 ± 1,16*b

Inducation. PE – Polychromatic Erythrocytes. NE – Normochromatic Erythrocytes. MN – Micronucleus. Count in 2000 cells/animal of 10 animal/group/sex/line. ^T Analyzed variable using the ANOVA test (X: average; SD: Standard Deviation). ^R Analysed variable, χ^2 non parametric test. ¹ Intraperitoneal treatment. *p < 0,05 (comparison with the negative control group in the same genetic line and sex, ANOVA test). **p < 0,01 (comparison with the negative control group in the same genetic line and sex, Chi Square (χ^2) non parametric test). a, b – Different letters it differs when comparing among lines keeping in mind the same variable in the same experimental group and sex. c – Comparison between sexes in the same experimental group and rat lines.

Table 2

Variation of data obtained by three observers

Groups	Sex	MN ^R			PE/NE ^T			MN-PE (%) ^T		
		Ob1	Ob2	Ob3	Ob1	Ob2	Ob3	Ob1	Ob2	Ob3
Sprague Dawley Rats										
NC	F	25a	30a	29a	1,26b	1,18a	1,19a	0,15a	0,19a	0,26a
	M	24a	24a	30a	1,14a	1,19a	1,18a	0,22a	0,15b	0,17a
TC	F	27a	34a	35b	1,18a	1,23a	1,16a	0,25a	0,22a	0,19a
	M	22a	29a	30b	1,22a	1,20a	1,19a	0,17a	0,19a	0,21a
VS1	F	26a	27a	34b	1,26b	1,19a	1,18a	0,16a	0,20a	0,24a
	M	33a	30a	27a	1,16a	1,20a	1,21a	0,22a	0,24a	0,17a
VS2	F	20a	29b	26ab	1,17a	1,18a	1,19a	0,16a	0,15a	0,20a
	M	20a	19a	27b	1,15a	1,20a	1,19a	0,15a	0,13a	0,17a
CF	F	241a	249ab	260b	0,79a	0,98a	0,90a	1,82b	1,56a	1,84a
	M	235a	248a	240a	0,94a	0,77a	0,99a	1,55a	1,62a	1,87a
Lewis Rats										
NC	F	50a	43a	45a	1,10a	1,13a	1,07a	0,29ab	0,27a	0,37b
	M	53a	46a	45a	1,17b	1,10ab	1,06a	0,36a	0,34a	0,29a
TC	F	43a	46a	55a	1,08a	1,11a	1,14a	0,38a	0,28a	0,34a
	M	43a	45a	53a	1,15a	1,07a	1,06a	0,29a	0,30a	0,37a
VS1	F	43a	55b	49ab	1,09a	1,10a	1,17a	0,34a	0,32a	0,36a
	M	42a	52ab	56b	1,08a	1,16a	1,15a	0,25a	0,38ab	0,39b
VS2	F	40a	46a	40a	1,12a	1,11a	1,07a	0,34a	0,26a	0,27a
	M	47a	45a	40a	1,15a	1,14a	1,10a	0,30a	0,33a	0,27a
CF	F	286a	289ab	310b	0,66a	0,92b	0,88ab	2,09ab	1,76a	2,25b
	M	280a	288ab	305b	0,90b	0,83ab	0,70a	2,50b	1,84a	1,67a
Wistar Rats										
NC	F	42ab	50b	37a	1,10a	1,13a	1,10a	0,35a	0,35a	0,39a
	M	42a	49a	50a	1,07a	1,14a	1,09a	0,29a	0,37a	0,33a
TC	F	55a	48a	59a	1,13a	1,09a	1,14a	0,39a	0,37a	0,35a
	M	46a	49a	52a	1,14a	1,17a	1,11a	0,34a	0,37a	0,32a
VS1	F	44a	56b	50ab	1,11a	1,12a	1,07a	0,33a	0,35a	0,34a
	M	51a	58a	56a	1,10a	1,15a	1,08a	0,37a	0,36a	0,42a
VS2	F	49a	51a	53a	1,07a	1,12a	1,11a	0,32a	0,36a	0,37a
	M	44a	45a	49a	1,13a	1,16a	1,07a	0,33a	0,32a	0,31a
CF	F	285a	296a	295a	0,87b	0,90b	0,60a	1,94a	2,04a	2,05a
	M	299a	300a	295a	0,83a	0,78a	0,80a	2,09a	2,00a	2,06a

Inducation. NC – Negative Control. TC – Technique Control. VS1 – Vehicle Substance 1. VS2 – Vehicle Substance 2. CF – Cyclophosphamide. O – number of observers. PE – Polychromatic Erythrocytes. NE – Normochromatic Erythrocytes. MN – Micronucleus. Count in 2000 cells/animal of 10 animal/group/sex/line. ^T Analyzed variable using the ANOVA test (X: average; SD: Standard Deviation, p < 0,05). ^R Analyzed variable using the χ^2 non parametric test (p < 0,01). a, b – Different letters it differs when comparing the results of each observer in the same variable, rat line, sex and experimental group.

0.15 ± 0.06 % of polychromatic erythrocytes with micronucleus. The response of Sprague Dawley line to the cyclophosphamide was high, but smaller than the clastogenicity results obtained in both sexes of Lewis and Wistar lines. The total MN level in Sprague Dawley rats was lower than in animals of other lines. On the other hand, the cyclophosphamide revealed its biggest clastogenic effect in the males of Wistar rats [11, 16]. Significant differences between sexes were not observed. The different response of rat lines on cyclophosphamide is the result of their differences in drug metabolism [7, 17, 18]. Hepatic enzymes are fundamental in the first phase of xenobiotic metabolism that participates in the cyclophosphamide metabolism in the liver [7, 11, 17–21]. The differences in expressions of enzymes lead to differences in their metabolisms [20, 21]; different concentrations of the metabolites, active mutagen levels and so on.

The comparison of data of three independent observers is shown in the Table 2. In general the results demonstrated little variation of the variables and studied experimental groups. These data are in good correspondence with results of other investigators [23, 24] where high repeatability and consistency in their micronucleus assay in bone marrow cells were demonstrated. Thus, it may be recommended to explore genotoxic effects of drugs at the chromosome level [9].

Being based on the data obtained, we suppose that the Sprague Dawley rat line is more genetically stable than the other lines. This line revealed the lower spontaneous indexes and acceptable cyclophosphamide-induced indexes [25, 26]. These results are important also due to heterogeneity of response of the Sprague Dawley rats, similar to those in human populations [25, 26]. Further, we suggest to use Wistar rats in experiments with cyclophosphamide intraperitoneal treatment as positive control. In our experiments Wistar line was the most sensitive to the mutagenic action of cyclophosphamide and this line may be good biomodel to detection of mutagenic effects of similar substances.

Conclusions. The Sprague Dawley rats of both sexes differ significantly from the other lines in lower spontaneous indexes and acceptable induced with cyclophosphamide indexes in the bone marrow cells micronucleus assay.

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СРАВНЕНИЕ ЧАСТОТЫ МИКРОЯДЕР В КЛЕТКАХ КОСТНОГО МОЗГА ТРЕХ ЛИНИЙ КРЫС

Сравнивали спонтанные и индуцированные циклофосфамидом микроядерные индексы в клетках костного мозга трех линий крыс – Sprague Dawley, Льюис и Вистар. Сформировали пять экспериментальных групп (по 10 животных каждого пола и линии в каждой группе). Первую группу использовали в качестве негативного контроля (интактные животные), вторая служила контролем влияния перорального приема препаратов. Все условия содержания были одинаковыми для всех групп животных. Крысы третьей группы обрабатывали 2 % Tween 65, а четвертой группы – 0,9 % NaCl. Оба вещества вводили перорально в дозе 2 мл/кг в течение 14 дней. Животных пятой группы обрабатывали внутрибрюшинно циклофосфамидом в дозе 50 мг/кг (10 мл/кг раствора), за 48 и 24 ч до эвтаназии. Линия Sprague Dawley (оба пола) существенно отличается от других. Крысы этой линии имели низкие показатели спонтанного образования микроядер и высокий индекс при индукции циклофосфамидом, а также процент эритроцитов с микроядрами в костном мозге и индекс цитотоксичности. Полученные результаты позволяют определить наиболее подходящие линии крыс в качестве модельных животных для изучения генотоксичности. Это также позволит получать более точные оценки генотоксичности различных веществ.

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ПОРІВНЯННЯ ЧАСТОТИ МІКРОЯДЕР В КЛІТИНАХ КІСТКОВОГО МОЗКУ ТРЕХ ЛІНІЙ ЩУРІВ

Порівнювали спонтанні та індуковані циклофосфамідом микроядерні індекси в клітинах кісткового мозку трьох ліній щурів – Sprague Dawley, Льюіс і Вистар. Сформуливали п'ять експериментальних груп (по 10 тварин кожної статі і лінії в кожній групі). Першу групу використали як негативний контроль (інтактні тварини), друга слугувала контролем впливу перорального прийому препаратів. Всі умови утримання були однаковими для всіх груп тварин. Щурів третьої групи

обробляли 2 % Tween 65, а четвертої групи – 0,9 % NaCl. Обидві речовини вводили перорально в дозі 2 мл/кг впродовж 14 днів. Тварин п'ятої групи обробляли внутрішньочеревно циклофосфамідом в дозі 50 мг/кг (10 мл/кг розчину), за 48 і 24 год до евтаназії. Лінія Sprague Dawley (обидві статі) істотно відрізнялась від інших. Щури цієї лінії мали низькі показники спонтанного утворення мікроядер і високий індекс при індукції циклофосфамідом, а також відсоток еритроцитів з мікроядрами в кістковому мозку та індекс цитотоксичності. Отримані результати дозволяють визначити найбільш відповідні лінії щурів як модельних тварин для вивчення генотоксичності. Це також дозволить отримувати точніші оцінки генотоксичності ризикних речовин.

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