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Evaluation of the genotoxicity and fetal toxicity of the herbicide Paraquat

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ABSTRACT. The genotoxicity and fetal toxicity of the herbicide Paraquat was assessed in female mice treated with acute subcutaneous doses of the herbicide at different stages of pregnancy. The micronucleus test (MN) in peripheral blood erythrocytes and an evaluation of embryonic losses were applied. The results of the present work demonstrate that a single acute treatment in the period after-implantation did not produce a significant reduction in the size of the offspring at the lower dosage tested, 10 mg/kg bw (body weight). Genotoxicity was observed at 24 and 30 hours after treatment in animals receiving this dosage on the ninth and third day of pregnancy respectively. Our data suggest that the *in vivo* treatment with the lower herbicide dose induces embryonic losses in females exposed to the agent during the pre-implantation period. The dose of 20 mg/kg bw did not show genotoxic effects 24 hours after treatment, probably having induced cellular defenses due to high toxicity of the herbicide. This dose subsequently caused the death of the animals, hindering the analyses of MN at 30 hours post-treatment and of fetal toxicity determinations.

Key words: genotoxicity, fetal toxicity and Paraquat

RESUMO. Avaliação da genotoxicidade e da toxicidade fetal do herbicida Paraquat. A genotoxicidade e a toxicidade fetal do Paraquat foram avaliadas em camundongos fêmeas após tratamento agudo e subcutâneo do herbicida em diferentes períodos gestacionais. Foram utilizados o teste do micronúcleo (MN) em eritrócitos de sangue periférico e a avaliação de perdas embrionárias. Os resultados deste trabalho mostraram que o tratamento agudo com a menor dose testada, 10mg/kg pc (peso corpóreo), no período de pós-implantação, não produziu diminuição significativa do tamanho da prole. Observou-se genotoxicidade 24 e 30 horas após o tratamento de animais que receberam esta dosagem no 9º e 3º dia de prenhez, respectivamente. Nossos dados sugerem, ainda, que o tratamento *in vivo,* com a menor dose, induz perdas embrionárias em fêmeas expostas ao agente no período de pré-implantação. A dose de 20mg/kg pc não mostrou efeito genotóxico após 24 horas do tratamento, provavelmente por ter induzido seleção celular devido à alta toxicidade do herbicida. Esta dose causou a morte dos animais, impedindo as análises de MN após 30 horas do tratamento e da toxicidade fetal.

Palavras-chave: genotoxicidade, toxicidade fetal e Paraquat.

Agricultural chemical actions upon genetic material can occur in several ways. *In vivo* cytogenetic tests in order to analyze the metaphasic chromosomes or the micronucleus can be used to identify clastogenic compounds. Both tests are widely used and regarded with special importance by many regulating agencies because the whole animal is used, avoiding the artificial deficiencies in metabolic activation when *in vitro* tests are used (IPCS, 1985). Moreover, precocious or delayed mortality of embryos or fetuses can be evaluated, as well as reductions in fetal weight or the presence of gross malformations.

Paraquat is one of many herbicides that have been used to substitute mechanical weed control in Brazilian agriculture (Alves, 1986). It is a contact herbicide and therefore non-selective, killing weeds along with any other plant it comes into contact with

(Zambrone, 1986). Genotoxic effects of this herbicide were investigated by Parry (1975) and Salam *et al* (1993) in yeast; Alekperov *et al.* (1967), Bell *et al*. (1976), Benigni *et al.* (1979), Njagi and Gopalan (1981), Badr *et al*. (1983), Seehy *et al.* (1989) and Salam *et al*. (1993) in higher plants; Yossef *et al*. (1985) and Salam *et al*. (1993) in Drosophila; Nicotera *et al.* (1985), Sofuni and Ishidate (1988), Sawada *et al*. (1988) and Tanaka and Amano (1989) in Chinese hamster cells; Salam *et al.* (1993) in human lymphocytes and by Rios *et al*. (1995) in mouse somatic and germ cells.

This study evaluated the possible genotoxic effects of the herbicide Paraquat using the micronucleus test and its fetal toxicity through examination of the embryos of mice submitted to acute treatment with the product at various stages of their pregnancy.

Materials and methods

1. Chemical agent. Paraquat, 1,1'- dimethyl - 4,4'- bipyridylium dichloride (CAS No. 1910-42-5), 99% purity, whose technical name is Gramoxone, is formulated by Nortox and indicated for various crops, for example: soybean, wheat, maize, coffee and citrus fruits. The commercial product was dissolved in distilled water at sub-lethal concentrations for the treatments, based on LD_{50}, previously calculated to approximately 35 mg/kg bw.

2. Treatment of the animals. The animals used in this study were virgin female mice (*Mus musculus*) Swiss line, weighing around 30g, from the *Biotério Central, Universidade Estadual de Londrina, Londrina*, PR. The animals were kept in polyethylene boxes with a wire lid normally used in laboratories, with food and water *ad libitum*.

Two samples of female mice were taken at distinct times after mating and identification of the "plug" (solidification of sperm in the vagina) (Batelman and Epstein, 1971) and treated with subcutaneous injections with an aqueous solution of Paraquat at doses of 10 and 20 mg/kg bw.

There were four groups of eight animals tested, segregated according to their stage of pregnancy: Group I (3rd day), Group II (6th day), Group III (9th day) and Group IV (12th day).

For the evaluation of fetal toxicity, we utilized four control groups of eight animals each, whose females had received an injection of 10 mg/kg bw of NaCl 0.9%, on the following periods of gestation: CI (3rd day), CII (6th day), CIII (9th day) and CIV (12th day).

3. Micronucleus test on peripheral blood erythrocytes. Blood samples were collected from the tails of the animals and the blood smears were performed following Barale *et al*. (1985) methodology. The chosen sampling times were 24 hours before and 24 hours after the treatment, with each animal being its own control. A 30 hours post-treatment sample was added only for the subjects treated with 10mg/kg bw because the subjects treated with 20mg/kg bw died before this sampling time.

The slides were fixed with absolute methanol for 10 minute and 24 hours later they were stained with Giemsa diluted in phosphate buffer (pH = 6.8) at a 1:10 ratio for 20 minutes. The slides were then coded.

Eight thousand (4,000 control, 4,000 experimental) and 6,000 (3,000 control, 3,000 experimental) polychromatic erythrocytes (PCE) were analyzed per animal from subjects submitted to treatments with respectively 20mg/kg bw and 10mg/kg bw.

The Kruskal-Wallis statistical test was applied to the data and $p \leq 0.05$ values were considered significant.

4. Effects on the offspring from different gestational ages. The procurement of fetus from animals of the experimental and control groups was made by laparotomy in the 18th day of gestation and the presence of a delayed reabsorption was recorded when it was possible to define the embryo and placenta (abortive body); numbers of living embryos and the weight of the litter was also recorded. Student's T-test ($P \leq 0.05$) was applied for comparison of the control and experimental groups.

Results

Table 1 shows the mean frequencies of micronucleated PCEs (PCEMNs) obtained after treating female mice at different stages of pregnancy. In the groups treated with 20 mg/kg bw, 24 hours after exposure to Paraquat, the PCEMNs frequencies were not statistically different from the controls, as confirmed by the Kruskal-Wallis test.

Twenty-four hours after treatment with 10 mg/kg, Group III had PCEMNs frequencies statistically different from its control ($H=4.30$; $P \leq 0.05$). At the 30-hour interval, a statistically significant increase in micronucleated polychromatic erythrocytes in treated animals of pregnancy Group I was observed ($H=4.34$; $p \leq 0.05$) when compared with the control data.

Table 1. Relative frequencies of erythrocyte polichromatic micronuclei (PCEMNs) obtained from female mice before and after 24-hour and 30-hour of acute treatments with 20 and 10 mg/kg bw of Paraquat on the 3rd, 6th, 9th or 12th day of gestation. 4,000 and 3,000 cells for two subjects (20 and 10 mg/kg bw) in a total of 8 animals for each gestational group were analyzed

Treatment			Relative Frequency of PCEMNs		
mg/kg bw	Exposure Time (hours)	Days of gestation (Group)	Control	Experimental	H Value calculated
20	24	3 (I)	0,12	0,16	1,63
		6 (II)	0,05	0,09	2,98
		9 (III)	0,11	0,14	1,37
		12 (IV)	0,13	0,17	1,79
10	24	3 (I)	0,04	0,08	3,63
		6 (II)	0,03	0,03	0,01
		9 (III)	0,03	0,07	4,30*
		12 (IV)	0,01	0,02	0,94
10	30	3 (I)	0,04	0,10	4,34*
		6 (II)	0,03	0,05	1,48
		9 (III)	0,03	0,07	2,68
		12 (IV)	0,01	0,04	2,97

Statistical analysis with Kruskal-Wallis test: * = P≤ 0,05

Table 2 shows the mean number of living embryos, delayed reabsorptions (abortive bodies) and weight of the litter after acute treatment with 10mg/kg bw aqueous solution of Paraquat.

Table 2. Effects of Paraquat on live fetuses, abortive bodies and fetal body weight after acute treatment (10mg/kg bw) of female mice, on 3rd, 6th, 9th or 12th day of gestation and their respective controls

Days of gestation (Group)	Mean ± Standard Deviation					
	Live Fetuses		Abortive bodies		Fetal body weight	
	Control	Experimental	Control	Experimental	Control	Experimental
3 (I)	10,25±1,28	8,87±1,55	0,62±0,91	3,00±2,67*	1,40±0,09	1,36±0,14
6 (II)	10,12±2,23	8,87±1,72	0,50±0,92	0,87±0,99	1,37±0,11	1,34±0,09
9 (III)	10,37±1,40	9,62±2,44	0,50±0,75	1,25±1,48	1,29±0,11	1,30±0,04
12 (IV)	10,13±1,35	8,37±2,77	0,50±0,75	0,75±0,70	1,56±0,23	1,23±0,11*

Statistical analysis with Student "t"test: * = P ≤ 0,05

The mean number of embryos surviving the treatment of the females from different gestational periods was not significantly different in relation to the control groups.

Group I, treated on the 3rd day of gestation, presented a significant difference in the average of delayed reabsorption (abortive bodies) compared to its control group. The average weight of the litters was significantly different after treatment with aqueous Paraquat in females at 12th day of gestation (Group IV) relative to its control group.

Discussion

The micronucleus test results after the acute Paraquat treatment on female mice indicate that the 20 mg/kg bw dose was not genotoxic for any of the pregnancy groups 24 hours after acute treatment with the product. The herbicide showed genotoxic effects in animals treated with 10 mg/kg bw on the 9th day of pregnancy (Table 1) for the same sampling time.

In rodents, the adequate sample time for bone marrow cells (24 hours) is not suitable for PCE studies of peripheral blood, as these take more time to reach the blood flow (Slesisnski and Guzzie, 1988). Due to these findings, the sampling time of 30 hours was added to the 10mg/kg bw treatment, observing the genotoxic effect only in the specimens which were treated on the 3rd day of pregnancy. Table 1 shows that the use of a more adequate time interval for sampling the blood cells leads to a genotoxic response in precocious gestations when compared with the 24-hour sampling. An increase in statistically calculated values (Table 1) for Groups I, II and IV relative to the 24-hour sampling time point was also observed. This fact confirms that the best sampling interval required to attain maximum numbers of micronuclei by the peripheral blood method is 30 hours. However the decrease of this value observed in gestational group IV requires further investigation.

The correlation between the frequency of chromossomal damage and the assessment of embryonic mortality demonstrated here is in accordance with the results of Becker and Schöneich (1982). Paraquat influenced the continuity of the embryonic development and led to an increase in the frequency of PCEMNs, 30 hours after treatment, in females subjected to 10mg/kg bw of the product on the 3rd day of pregnancy. The losses in these females were significant (Table 2) and demonstrated that, despite the zygote not being implanted at the time of the treatment, the toxic effect of the Paraquat hindered the development of many embryos and consequently led to embryonic death.

However, the females treated in the periods of the 6th, 9th and 12th gestational days did not suffer significant embryonic losses and thus it was proved the toxic effect of Paraquat in the continuity of embryonic development did not occur in the post-implantation phase.

According to Hollander and Strong (1950), 72% of natural mortality occurs in the period after implantation, frequently in the first 3 days following implantation. Our results indicated a different picture of this natural mortality. We observed losses that were more significant when the treatment was implemented in the pre-implantation period. The parameter "abortive bodies" exhibited high variability in both control and experimental groups (Table 2). This variability refers to the number of

abortive bodies per female: 0 to 2 and 0 to 7, and to the number of females that aborted: 0 to 3 and 0 to 7 for control and experimental groups respectively.

Some researchers discuss abortive effects in mice and rats by some compounds, such as the DEHP (Tomita *et al.,* 1982); colchicine and derivatives (Tuchmann, 1977); and LSD (Alexander *et al.,* 1967) among others, not considering, however, the agrotoxins.

Severe mitostatic effects resulting from the Paraquat herbicide were described in *Vicia faba* (Njagi and Gopalan,1981), *Allium cepa* (Salam *et al.,* 1993) and in mice bone marrow cells (Rios *et al.,* 1995). Cassaletti *et al*. (1991) showed that this product reduced growth and sporulation in *Aspergillus nidulans,* while Benigni *et al.* (1979) observed induction of genetic mutations. Rios *et al.* (1995) found clastogenic effects of Paraquat in mice chromosomes under acute treatment but did not find them in multiple treatments.

According to Yonei *et al.* (1986), the capacity of the herbicide to produce damage in DNA is only possible when complexed with iron ions. It has also been suggested that the molecular mechanism of this herbicide is mediated by the formation of free radicals produced when it is reduced by redox enzymes, such as NADPH-cytochrome - reductase and glucose-6 phosphatase dehydrogenase (Gage *et al.,* 1968; Yeh *et al.,* 1987 and Kuo and Lin*.,* 1993). The genotoxic potential of the sub-products formed during Paraquat fabrication was assessed by Kuo and Lin (1993) in V79 cells. The authors showed that the production of free radicals by the pyridyl derivatives associated with metal ions, especially iron, had genotoxic effects on the biological system employed.

One explanation for our negative results for all the treatment groups with 20mg/kg bw and for the majority of the pregnancy groups treated with 10 mg/kg bw of Paraquat may be the cellular defense mechanism. According to Yonei *et al.* (1986) the toxicity of oxygen is dependent upon the balance between free radical production and their removal by the defense mechanism. Hassan and Fridovich (1977, 1979) suggested that the O_2^- generated by the Paraquat herbicide induces biosynthesis and consequently an intracellular increase in the superoxide dismutase enzyme levels which acts to protect against the toxicity of species with active oxygen, thus reducing chromosomal damage. This hypothesis also explains the results obtained in the present study with mice treated with 20 mg/kg bw.

Ribas *et al*. (1998) has not detected a genotoxicity of Paraquat in MN and CA (chromosomal aberration) tests with human lymphocytes. However, the herbicide induced a slight increase in the frequency of sister chromatid exchange (SCE) principally in the higher tested concentrations of the herbicide. Tanaka and Amano (1989), however, had shown that the induction of SCEs in mammalian cells has been produced even at low concentrations.

According to Matkovics *et al.* (1979), *in vivo* Paraquat effects depend on its concentration. The results in the present study showed that single acute treatments produced genotoxicity and fetal toxicity only at a dose of 10mg/kg bw.

Our results show that the greatest concentration tested was highly toxic and probably induced cellular defense mechanisms. Ribas *et al.* (1998) also had observed one high cytotoxicity of Paraquat in the cultures of human lymphocytes. Such results do not corroborate the data in the literature where clastogenic Paraquat effects have been observed only when high concentrations of the product were used (Tanaka and Amano, 1989; Rios *et al.,* 1995) or after long periods of exposure (Tanaka and Amano, 1989). In light of these considerations, studies should be continued.

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