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EVALUATION OF GENOTOXIC EFFECTS OF IPRONIDAZOL (GASTROGAL 10®) IN CULTURES OF HUMAN PERIPHERAL BLOOD LYMPHOCYTES

MARKOVIĆ BILJANA*, STANIMIROVIĆ Z*, DJELIĆ N* and ANĐELKOVIĆ M**.

*Department of Biology, Faculty of Veterinary Medicine, University of Belgrade, **Department of Genetics, Faculty of Biology, University of Belgrade, Yugoslavia

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In this work, we examined the genotoxic effect of the antibiotic preparation ipronidazol (Gastrogal 10). An experiment was performed in vitro on human peripheral blood lymphocytes using the chromosome aberration and sister chromatid exchange tests. Clastogenic effects of ipronidazol were examined at three experimental concentrations (25 μg/ml, 50 μg/ml i 100 μg/ml) in eight experimental cycles. The results demonstrate that Gastrogal 10 has the ability to change the human lymphocyte kariotype, i.e. it induces numerical aberrations (aneuploidies and polyploidies), as well as chromosome gaps and breaks. Moreover, Gastrogal 10 induces a significant increase of sister chromatid exchange in human lymphocytes. The obtained results demonstrate that the examined preparation exhibits a certain genotoxic potential.

Key words: chromosome aberrations, Gastrogal 10, ipronidazol, SCE

INTRODUCTION

The application of genotoxic agents and their possible accumulation in living systems cause ireversible consequences such as lethal mutations, frequent gamete loss, embryo mortality, congenital malformations, changes of genetic variability in populations and increased incidence of carcinoma (Anderson *et al.*, 1994). Therefore genotoxicological studies should be supplemented by research on the pharmacokinetics of toxic compounds, in order to broaden our knowledge about the role of genotoxic agents in such complex processes as mutagenesis and carcinogenesis.

Environmental mutagenesis has special features, because various compounds can increase gene mutation frequency, despite the possibility of a high level of survival in exposed populations. (Legator, 1994; Muller and Kasper, 2000). Namely, mutations can be induced even at concentrations that exhibit very low cytotoxicity (Kirkland, 1998; Kovalkovicova et al. 2001). Since the genotoxic agents comprise a large number of pharmaceutical preparations that can express the above mentioned properties, it is important to evaluate their potential genotoxicity (FAO/WHO, 1995). Literatury data on the genotoxic potential of ipronidazol are scarce. Voogd *et al.* (1977) demonstrated that ipronidazol

(fluctuation test) increases mutation frequency during the following three to four generations in a prokaryotic system. In addition, ipronidazol influences redox processes in heart mitochondria. Low concentrations of ipronidazol decrease redox processes by 20%, whereas higher concentrations cause a more profound effect – 60% a decrease. Metabolic changes under the influence of ipronidazol comprise a decrease of FMN protein level and lowered intensity of ADP phosphorylation in the presence of gluto-malate substrate (Aicardi and Solani, 1982).

According to the World Health Organisation (WHO, 1998), ipronidazol exhibits carcinogenic properties. Therefore restricted administration of ipronidazol is recommended. Since there is a general tendency to use pharmacological preparations with lower toxicity and genotoxicity, the aim of the present investigation was to test Gastrogal 10, a widely used preparation in veterinary medicine, for genotoxicity under *in vitro* conditions.

MATERIALS AND METHODS

Test substance. Gastrogal 10 (ICN Galenika, Beograd) contains 100 mg of ipronidazol per ml of solution.

Treatment. Freshly prepared dilutions were added to cultivation vials to obtain final concentrations of 25 μg/ml, 50 μg/ml and 100 μg/ml at the begining of incubation. Control (untreated) cultures were set up in each experimental cycle. There were eight experimental cycles.

In vitro chromosome aberration test. Metaphase spreads were obtained according to the method of Evans and O'Riordan (1976) as modified by Zimonjić *et al.* (1990). Briefly, human lymphocyte cultures were prepared heparinised whole blood of healthy men under 35 years of age, added to Parker 199 media (Torlak, Belgrade), containing 30% heat inactivated calf sera (VZ, Subotica) and 0.04 mg/ml of phytohaemagglutinin (Murex, Dartford, England). The cultivation vials were incubated for 72 h at 37°C. Two hours before harvesting colchicine (Sigma Chemical Co., St. Louis, MO) was added to a final concentration of 0.5 μg/ml. After the hypotonic treatment (0.075 M KCl) followed by three repetitive cycles of fixation in methanol/acetic acid solution (3: 1, v/v), centrifugation, and resuspension, the cell suspension was dropped on chilled grease-free microscopic slides, air dried and stained in 10% Giemsa (Kemika, Zagreb, Croatia) solution.

Sister-chromatid exchange test. Cultures were set up in the same way except that 5-bromo-2'-deoxyuridine (BrdUrd, Sigma Chemical Co., St. Louis, MO) was added at the beginning of incubation at a final concentration of 25 μM. The visualisation of differentially stained chromatids was achieved by the method of Perry and Evans (1975), slightly modified by Zimonjić *at al.* (1990).

Statistical analysis. Comparison of the significance between treated and untreated cultures was performed by Student's t-test.

RESULTS

The results obtained in this experiment demonstrate that ipronidazol (Gastrogal 10) induced numerical aberrations (aneuploidies and polyploidies) and structural lesions (chromatid gaps and breaks) at all experimental concentrations. (Table 1. Figure 1).

Table 1. Cytogenetic parameters in control and experimental cultures of human peripheral blood lymphocytes treated with increasing doses of Gastrogal 10

	control		Gastrogal 10 25 µg/ml		Gastrogal 10 50µg/ml		Gastrogal 10 100µg/ml	
	X±SD	%	X±SD	%	X±SD	%	X±SD	%
Aneuploidy	1.63	0.82	14.75	7.38	21.88	10.94	31.75	15.88
Polyploidy	0	0	2.13	1.07	5.5	2.75	7.87	3.94
Lesions	2	1	11.63	5.82	19.13	10.17	24.25	12.13
Breaks	0.63	0.32	6	3	13.62	6.81	18.25	9.13
All cytogenetic changes	4.26	2.14	34.51	17.27	60.13	30.67	82.12	41.08

There was positive dose-dependence, in that the lowest concentration (25µg/ml) caused aberrations in 17.27% cells, whereas the concentration of 50 µg/ml induced cytogenetically detectable changes in 30.67% of the examined mitoses. Finally, the highest concentration (100 µg/ml) caused cytogenetic changes in 41.08% cells. Statistical analysis by Student's *t*-test revealed significant differences between control and treated cultures. Moreover, the same level of statistical significance existed between cultures treated with different concentrations of Gastrogal 10 (Table 2).

In addition to the chromosome aberration assay, we investigated possible effects on DNA using a sister chromatid exchange (SCE) test *in vitro*. The results are shown in Table 3 and Figure 2. The mean value of SCE per cell frequency in the control group amounted to 1.07±0.04. Treatment with Gastrogal 10 caused a dose-dependent increase of SCE frequency per cell. Thus, in cultures treated with 25 g/ml the mean value was 4.40 ± 0.15. The intermediate concentration (50 g/ml) and the highest concentration applied (100 µg/ml) caused more profound elevation of SCE frequency per cell with mean values of 7.47 ± 0.30 and 9.97± 0.17, respectively. The level of statistical significance in relation to the control group was relatively high ($p<0.01$) even after treatment with the lowest concentration of Gastrogal 10, whereas at higher concentrations statistical significance was more profound ($p<0.001$).

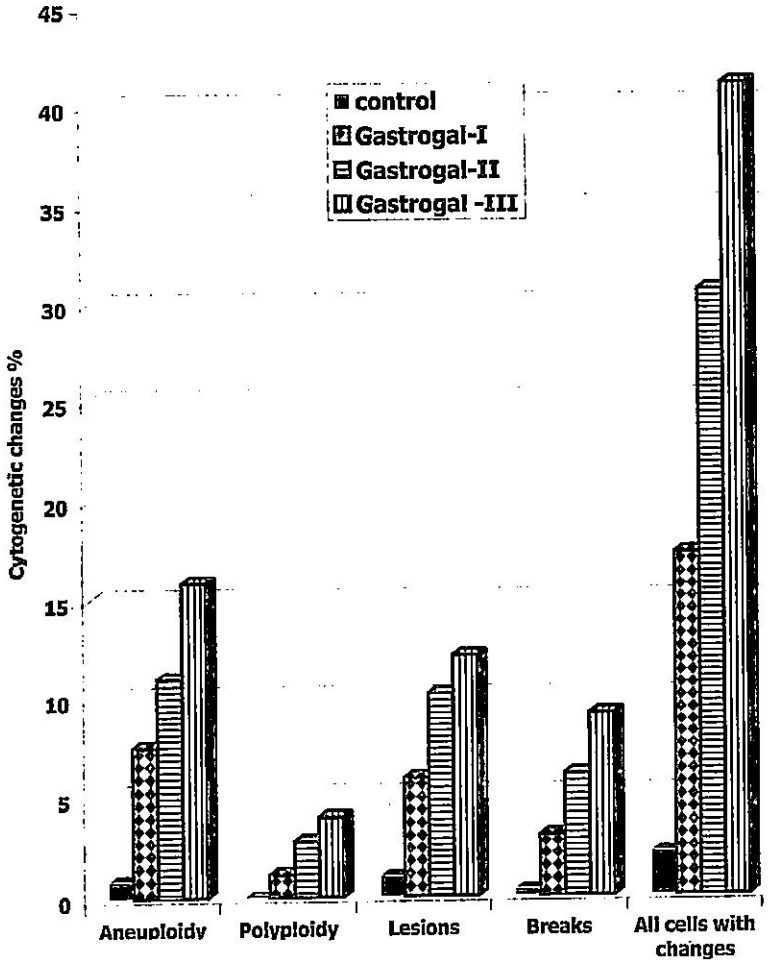

Figure 1. Percentage values of cytogenetic parameters in control and experimental cultures of human periphoral blood lymphocytes treated with increasing doses of Gastrogal 10

Table. 2. Level of statistical significance between controls and Gastrogal 10 - treated groups for total cytogenetic parameters monitored.

	X ± SD	Gastrogal 10 25 µg/ml SD	t	Gastrogal 10 50 µg/ml SD	t	Gastrogal 10 100µg/ml SD	t
Contol	4.26 ± 1.83	0.94	*** 32.18	0.85	*** 49.88	0.99	*** 90.44
Gastrogal10 25 µg/ml	34.51 ± 1.92			1.14	*** 22.47	0.88	*** 52.98
Gastrogal10 50 µg/ml	60.13 ± 2.58					1.07	*** 19.63
Gastrogal10 100 µg/ml	82.12 ± 1.55						

Table 3. Frequency of sister chromatid exchanges (SCEs) in control and experimental cultures of human peripheral blood lymphocytes treated with increasing doses of Gastrogal 10

Treatments	SCE range min	max	Mean value of SCE/cell	SD	t-test
Control	0	3	1.07	0.04	
Gastrogal10 25 µg/ml	1	8	4.40	0.15	4.28**
Gastrogal10 50 µg/ml	3	12	7.47	0.30	42.49***
Gastrogal10 100 µg/ml	4	15	9.77	0.17	93.45***

**p<0.01
***p<0.001

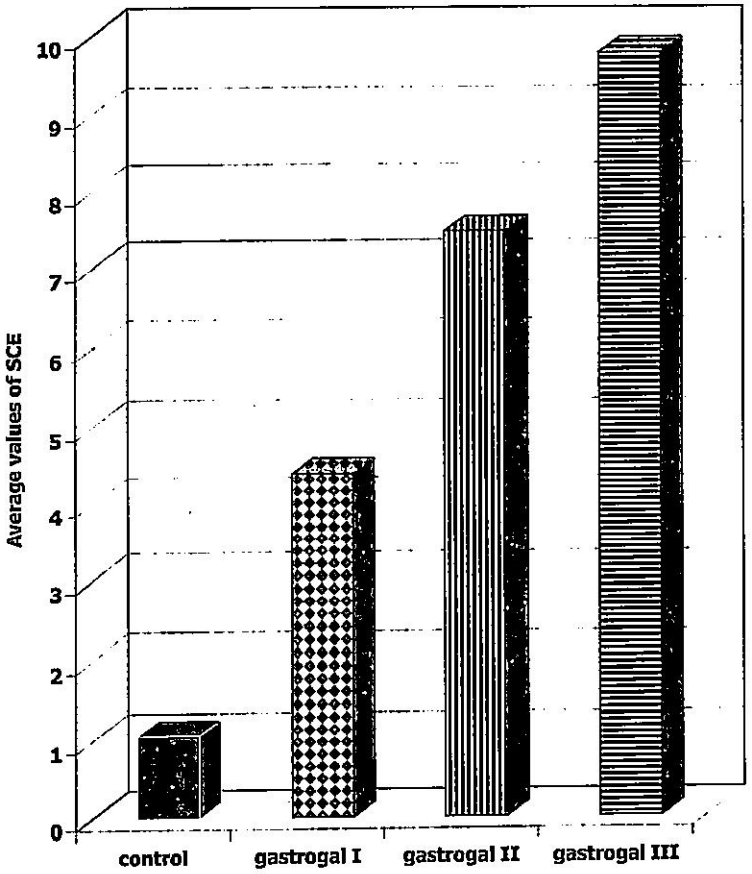

Figure 2. Average values of SCE in control and experimental cultures of human peripheral blood lymphocytes treated with increasing doses of Gastrogal 10

DISCUSSION

Analysis of metaphase spreads obtained from cultures treated with various concentrations of Gastrogal 10, revealed that all concentrations applied in this investigation induced numerical aberrations (aneuploidies and polyploidies) and structural alterations visible as chromatid gaps and breaks. The appearance of aneuploid cells can be explained by changes or dysfunction of microtubules (Clements and Tood 1981). Therefore, we assume that Gastrogal 10 may interfere with the formation or normal function of microtubules, thereby inducing aneuploidies. Moreover, the tested antibiotic preparation was able to induce polyploidies, thereby reflecting the genotoxic potential of ipronidazol (Marković, 1999). In addition to numerical aberrations, all applied concentrations induced structural changes (gaps and breaks) on human lymphocyte chromosomes. These changes result either from primary DNA lesions or damage to the chromosomal protein substrate (Đelić 1997; Stanimirović *et al.* 1998; Marković, 1999). It is evident that the percentage of overall cytogenetic changes increased with the elevation of Gastrogal 10 concentration in the culture media. Although Gastrogal 10 had the ability to induce chromosome aberrations at all concentrations applied, the close dose-dependence clearly demonstrates its genotoxic potential.

Abundant literature data indicate that the level of spontaneous SCE frequency per cell varies between 1.4 and 4.5, depending on the cell type, culture conditions, age of donors and other circumstances (Marković *et al., 2000).* Thus, the mean value of spontaneous SCE frequency per cell is relatively low and according to some authors is approximately 1.1. The analysis of SCE frequency per cell on cultured human lymphocytes treated with Gastrogal 10, revealed that mean value of SCE increased steadily from 4.40 0.15 at the concentration of 25g/ml to 9.97±0.17 at the highest concentration (100 g/ml). While the SCE frequency per cell induced by the lowest concentration of Gastrogal 10 is comparable to the spontaneous level observed in some laboratories, the higher concentrations induced a highly significant increase of SCE frequency compared to the control values of spontaneous SCE occurrence. The ability of Gastrogal 10 to increase SCE frequency per cell possibly results primary damage at the level of DNA (Oikawa *et al.,* 1983). It is conceivable that ipronidazol directly or indirectly interacts with nucleic acids and/or facilitates the appearance of DNA damage (Bila and Kren, 1992).

On the basis of the obtained results it can be concluded that the antibiotic preparation Gastrogal 10 has the ability to change the kariotype of human lymphocytes. Gastrogal 10 has genotoxic potential that can be detected via induction of structural and numerical chromosomal aberrations. The SCE per cell demonstrates that tested concentrations exhibit various level of genotoxicity. Namely, the lowest concentration of Gastrogal 10 induces SCE per cell frequency comparable to spontaneous changes, whereas higher concentrations induce a strong genotoxic effect exhibited by the very high level of SCE.

Address for correspondence:
Dr Biljana Marković
Department of Biology,
Faculty of Veterinary Medicine,
Bul. JNA 18, Belgrade, Yugoslavia
e-mail: biolog@vet.bg.ac.yu

REFERENCES

1. *Aicardi G, Solani G.* 1982, Effects of ipronidazole and 5-nitromidazoles on heart mitochondrial respiration, *Biochem Pharmacol,* 15: 3703 - 5.
2. *Anderson S, Sadinski W, Lee S, Ford T, Wirgin I, Wogan G.* 1994, Genetic and molecular ecotoxicology, a research framework. *Environmental Health Perspectives,* 102: 3 - 8
3. *Bila V, Kren V.*1992, Teratogenicity testing based on the interaction with a mutant allele, *Folia-Biol-Praha,.* 38: 40 - 7.
4. *Clements J, Tood NK.*1981, Halothane and non disjunction in Drosophila. *Mutat Res,* 91: 225 - 8.
5. *Đelić N.* 1997, Citogenetički efekti estradiola, tiroksina, insulina i adrenalina na humane limfocite in vitro. Doktorska disertacija, Univerzitet u Beogradu.
6. *Evans HJ, O'Riordan ML.* 1976, Human peripheral blood lymphocytes for the analysis of chromosome aberrations in mutagen tests. *Mutat Res.* 31: 135 - 48.
7. *FAO/WHO.* 1995, Forty-second Meeting of the Joint FAO/WHO Expert Committee on Food Additives. Evaluation of certain veterinary drug residues in food. WHO Techical Report Series No 851, 19 - 212. WHO, Geneva.
8. *Kirkland D.* 1998, Chromosome aberration testing in genetic toxicology past, present future. *Mutat, Res,* 404:173 - 85.
9. *Kovalkovicova N, Šutiakova I, Kacmar P, Šulik E, Legath H, Pistl J, Mlynarcihova H, Mikula I.* 2001, Chromosomal aberrations induced by the insecticide Endosulfan in sheep peripheral lymphocytes *in vitro, Acta Vet.* 51: 365 - 72.
10. *Legator S.* 1994, Application of integrated genetic monitoring: The optimal approach for detecting environmental carcinogens. Environmental Health Perspectives 102: 125 - 32.
11. *Marković B.* 1999, Genotoksični efekat antibiotskih preparata Carbadox, Tiamulin S i Gastrogal 10. Doktorska disertacija, Univerzitet u Beogradu.
12. *Marković B, Stanimirović Z, Vučinić M, Ćupić V.* 2000, Examination of Carbadox genotoxicity *in vitro* and *in vivo. Acta Vet, Beograd* 50: 387 - 96
13. *Muller L, Kasper P.* 2000, Human biological relevance and the use of threshold-arguments in regulatory genotoxicity assessment: experience with pharmaceuticals, *Mutat Res,* 242: 1 - 7.
14. *Oikawa A, Sakai S, Horaguchi K, Tohda H.* 1983, Sensitivities of peripheral lymphocytes from healthy humans to induction of sister chromatid exchanges by chemicals. *Cancer Res.,* 43: 439-42.
15. *Perry P, Evans HJ.* 1975, Cytological detection of mutagen carcinogen-exposure by sister chromatid exchange. *Nature,* 258: 121 - 5.
16. *Stanimirović Z, Vučinić M, Soldatović B.* 1998, Cytogenetic changes in bone marrow cells of Wistar rats induced by levamisole hydrochloride. *Acta Vet, Beograd* 48: 255 - 62.
17. *Voogd CE, Van der Stel JJ, Jacobs J.* 1977, The mutagenic action of nitorimidazoles. II. Tinidazole, ipronidazole, panidazole, and ornidazole. *Mutat Res,* 48: 155 - 61.
18. *World Health Organization (WHO).* 1998, Essential Drugs and Medicines Policy. WHO Pharmaceuticals Newsletter 9 &10. September & October 1998.
19. *Zimonjić D, Savković N, Anđelković M,* 1990, Genotoksični agensi. Efekti,principi i metodologija detekcije. Naučna knjiga, Beograd.

EVALUACIJA GENOTOKSIČNOG EFEKTA IPRONIDAZOLA (GASTROGALA 10®) U KULTURI HUMANIH LIMFOCITA PERIFERNE KRVI

MARKOVIĆ BILJANA, STANIMIROVIĆ Z, ĐELIĆ N i ANĐELKOVIĆ M

SADRŽAJ

U ovom radu su izneti rezultati ispitivanja genotoksičnih efekata antibiotskog preparata Gastrogala 10 čija je aktivna supstanca ipronidazol. Eksperiment in vitro je obavljen na humanim limfocitima periferne krvi, primenom testa indukcije hromozomskih aberacija u kulturi ćelija, kao i testa ispitivanja

oštećenja na nivou molekula DNK, praćenjem razmene sestrinskih hromatida (SCE). Ispitivanje klastogenog efekta odgovarajućih doza Gastrogala 10 (25μg/ml, 5oμg/ml i 100μg/ml) obavljeno je kroz 8 eksperimentalnih ciklusa. Rezultati istraživanja *in vitro* pokazuju da ispitivani antibiotiski preparat ima sposobnost promene kariotipa limfocita čoveka i indukcije numeričkih hromozomskih aberacija tipa aneuploidija i poliploidija i strukturnih hromozomskih promena tipa lezija i prekida. Gastrogal 10 indukuje značajno povećanje razmene sestrinskih hromatida u humanim limfocitima. Dobijeni eksperimentalni rezultati, ukazuju da ispitivani antibiotski preparat Gastrogal 10 ima određeni genotoksični potencijal.