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EXAMINATION OF CARBADOX GENOTOXICITY *IN VITRO* AND *IN VIVO*

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Due to its bactericidal activity Carbadox is used for treatment of swine dysentery and salmonellosis. Also, it can be used as a factor for growth stimulation. Carbadox is the only drug for certain health problems in swine production. At the same time Carbadox is slowly metabolised.

The aim of this work was an examination of Carbadox genotoxicity in vivo and in vitro. In vitro genotoxicity of different doses of Carbadox was examined on cultured human peripheral blood lymphocytes. Damage to chromosomal DNA was observed under the microscope as sister chromatid exchanges (SCEs).

In vivo genotoxicity of Carbadox was examined on metaphase chromosomes from bone marrow cells of BALB/c strain male mice. Experimental animals were intragastrically treated with three different doses of Carbadox. In addition there were negative and a positive control groups of mice. Animals in the positive control were treated with a known clastogenic agent, cyclophosphamide, in a dose of 0,4 mg/kg b.w. In vivo genotoxicity of Carbadox was examined through eight experimental cycles.

The results showed that this preparation with an antimicrobial mode of action induced damage to chromosomal DNA in human lymphocytes as well as structural and numerical chromosomal changes in bone marrow cells of BALB/c mice. All the examined concentrations of Carbadox significantly increased SCEs and consistently manifested a dose-dependent pattern. Also, all the examined doses caused karyotypic changes in vivo inducing numerical and structural chromosomal aberrations in mouse bone marrow cells. There was a positive correlation between the number of bone marrow cells with cytogenetic changes and the dose of Carbadox.

It could be concluded that Carbadox expressed potential genotoxicity, especially at the highest investigated doses.

Key words: Carbadox, genotoxicity, peripheral blood lymphocytes, DNA damage, sister chromatid exchanges, bone marrow cells, chromosomal changes.

INTRODUCTION

Drugs and their metabolites can alter living matter at all levels of organisation. Genotoxic and mutagenic actions of some drugs are of special importance. In human and veterinary medicine antimicrobials are widely used and any genotoxicity or mutagenicity is very undesirable. Residues of antibiotics in human and animal tissues might aid their genotoxic mode of action. Carbadox, widely used in veterinary medicine, may induce negative biological effects in treated animals, such as toxic, cytotoxic and cancerogenic effects. Therefore effects, in some countries the use of Carbadox is prohibited. Its toxicity for the endocrine system of mammals has been demonstrated. Thus Carbadox interferes with adrenal functions (Vander Molen et al., 1986). From 2,5 to 5 hours after treatment, Carbadox reduced the level of NH_3 in portal plasma (Yen and Pond, 1990, 1993) and Carbadox mixed with food has been used for prevention of swine dysentery. The toxicity of Carbadox residues was studied by Yen et al. (1992), who concluded that Carbadox would be toxic for animals if used for long a time at doses higher than a prescribed amount. They pointed out the potential cancerogenic activity of Carbadox and recommended a dose of 25 ppm Carbadox for use in food. On the contrary Ohta et al. (1984) showed negative results in the SOS induction test but positive results for Carbadox mutagenicity in the Ames test. That was the reason for the classification of Carbadox in the third class of substances with a cancerogenic mode of action. Experimental rats were orally treated with Carbadox, but its cancerogenic activity could not be confirmed (Truhaut et al. 1981). According to a FAO/WHO report (1990) Carbadox should be further investigated. Also, there is a risk for human and animal populations from the ingestion and inhalation of Carbadox. Considering all these facts, the aim of this study was to examine the possible genotoxicity of different doses of Carbadox in vitro (human peripheral blood lymphocytes and SCEs) and in vivo (chromosomes of bone marrow cells of BALB/c mice).

MATERIALS AND METHODS

Carbadox genotoxicity to human peripheral blood lymphocytes *in vitro* was investigated by observing DNA damage as sister chromatid exchanges. Cultures of lymphocytes were treated with a therapeutic dose of Carbadox (40 μg/ml), as well as with doses 25% (10 μg/ml) and 50% (20 μg/ml) below the therapeutic dose. Carbadox was dissolved in ethanol. In addition to the experimental groups, there were control cultures of lymphocytes treated with ethanol only. Also, there were non-treated and positive controls (treatment with cyclophosphamide in a dose of 40 μg/ml). After 72 hours all cultures were examined for sister chromatid exchanges. The treatment of cultures with chemicals, preparation of lymphocytes and differential staining of sister chromatids were done by the method of Perry and Evans (1975) as modified by Zimonjić et al. (1990).

Genotoxicity of Carbadox was in vivo examined on bone marrow cells of BALB/c mice. Experimental animals were treated intragastrically with Carbadox in doses of 0.2 mg/kg b.w., 0.4 mg/kg b.w. and 0.8 mg/kg b.w. and the cells harvested after 72 hours. Also, there were three more groups of mice. The first group was treated with ethanol and the second with Endoxan in a dose of 0,4 mg/kg b.w. (positive control). The third group was intact (negative control).

Chromosomes were prepared from bone marrow cells and examined by the method of Hsu and Patton (1969), as modified by Stanimirović (1995).

RESULTS AND DISCUSSION

The results for frequency of sister chromatid exchanges in human peripheral blood lymphocytes and karyotypic analysis of bone marrow cells of BALB/c mice are shown in Tables 1 and 2. According to the other authors (Crosen et al., 1977; Kato, 1980; Carrano et al., 1978, 1980,1981; Okinava et al., 1981) the spontaneous level of SCEs per cell varies from 1.4 to 4.5 SCEs. A characteristic low average value of SCEs/cell was found in our negative control (1.07). In cultures treated with ethanol the frequency of DNA damage was 1.8 SCEs/cell. There were 14.32 SCEs/cell in cultures treated with cyclophosphamide (positive control group). In experimental cultures treated with different doses of Carbadox there were 7.60 SCEs/cell (10 μg/ml), 12.87 SCEs/cell (20 μg/ml) and 15.57 SCEs/cell (40 μg/ml). Thus, the highest Carbadox dose induced the highest frequency of SCEs. Carbadox has been clearly shown to expressed clastogenic activity on exposed chromosomes. It is possible that Carbadox in higher doses interacted with DNA structures or interfered with the function of the DNA molecule (Bil and Kren, 1992).

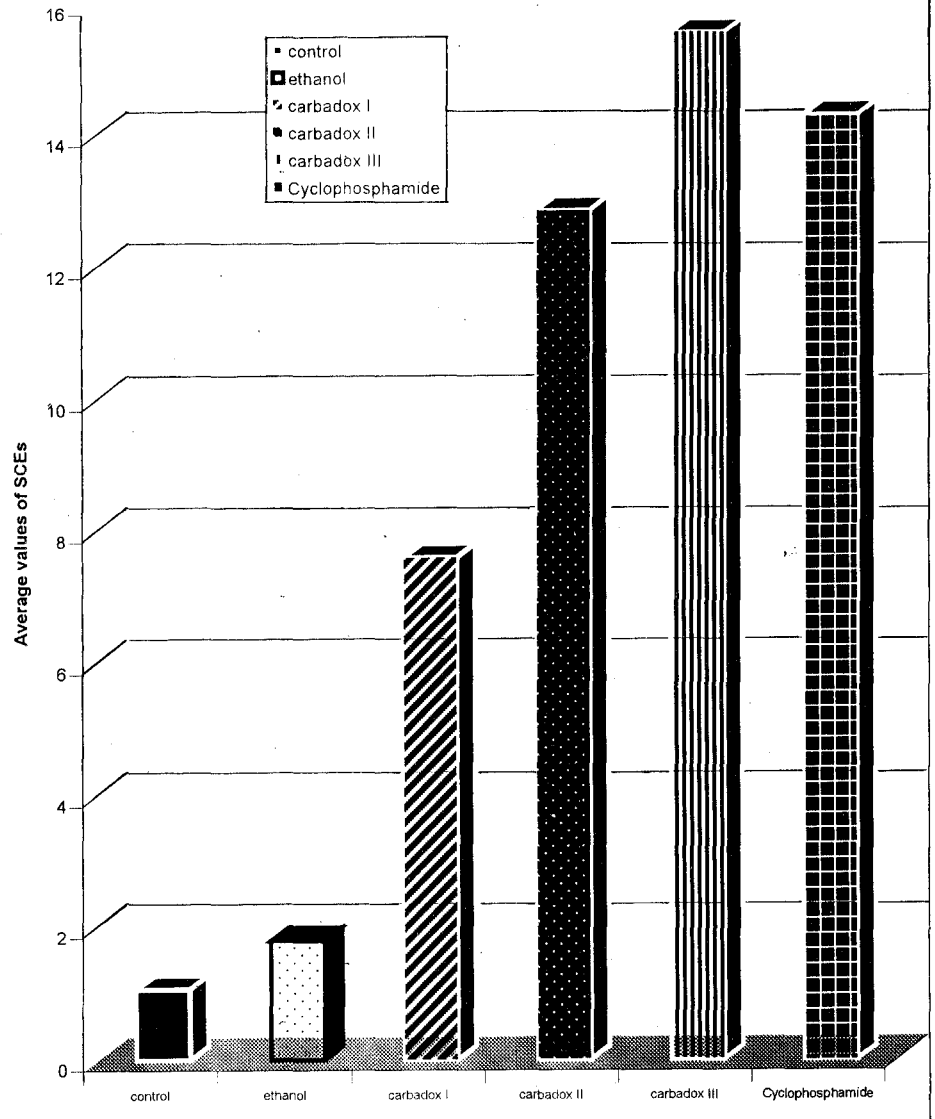
Table 1. Frequency of sister chomatid exchanges (SCEs) in control and experimental cultures of human peripheral blood lymphocytes treated with increasing doses of Carbadox

Treatments	SCE/cell interval of variation (IV) min	max	Acrage X SCE/cell	SD	t-test
Control group	0	3	1,07	0,04	
Ethanol	0	4	1,8o	0,13	10,43***
Carbadox 10 μg/ml	2	14	7,60	0,36	36,28***
Carbadox 20 μg/ml	5	17	12,87	0,10	196,67***
Carbadox 40 μg/ml	8	21	15,57	0,14	207,14***
Cyclophosphamide 40 μg/ml	8	21	14,32	0,12	201,09***

***p<0,001

All the investigated doses of Carbadox induced numerical and structural chromosomal aberrations *in vivo*. Szabova (1988) also observed clastogenic activity of Carbadox on chromosomes of ICR mice during an experiment lasting seven days. Similar results for Carbadox clastogenic activity were obtained by

Figure 1. Average values of SCEs in control and experimental cultures of human peripheral blood lymphocytes treated with increasing doses of Carbadox



Cihak and Vontorkova (1983) who treated ICR mice with Carbadox in an experiment lasting 24 hours. All those results suggest that Carbadox has very strong and aggressive activity on the genetic structure of experimental mammals (mice of different strains) exposed to its action for different durations. Moreover, these results confirm that the highest level of Carbadox is genotoxic and mutagenic.

In our experiments all the investigated doses of Carbadox induced numerical chromosomal changes (aneuploidies and polyploidies), as well as structural chromosomal changes, such as lesions, gaps, breaks, rings, acentrics and Robertsonian translocations. Namely, 16,08 % cells were of the aneuploidy and 2,32% polyploidic in mice treated with Carbadox at the dose of 0,8 mg/kg b.w. Lower values for numerical chromosomal changes occured in animals treated with Carbadox at 0,4 mg/kg b.w. (13.25% - aneuploidies and 1.16% - polyploidies) and 0.2 mg/kg b.w. (9.94 % of aneuploidic cells and 0.93% of polyploidic cells). Cyclophosphamide induced 14.61% aneuploidic cells and 1.9% polyploidic cells. The great number of aneuploidic and polyploidic cells from mice treated with Carbadox points to its genotoxicity, or to the possibility of reaction with fibres of the mitotic spindle causing irregular separation of chromosomes during anaphase. Also, it is not possible to exclude inactivation of cinetochores or centrioles by Carbadox (Vaughan-Dellarso et al., 1985; Vučinić, 1994; Stanimirović, 1995; Marković et al., 1996; Marković,1996; Stanimirović et al., 1997; Stanimirović et al., 1998; Marković,1999).

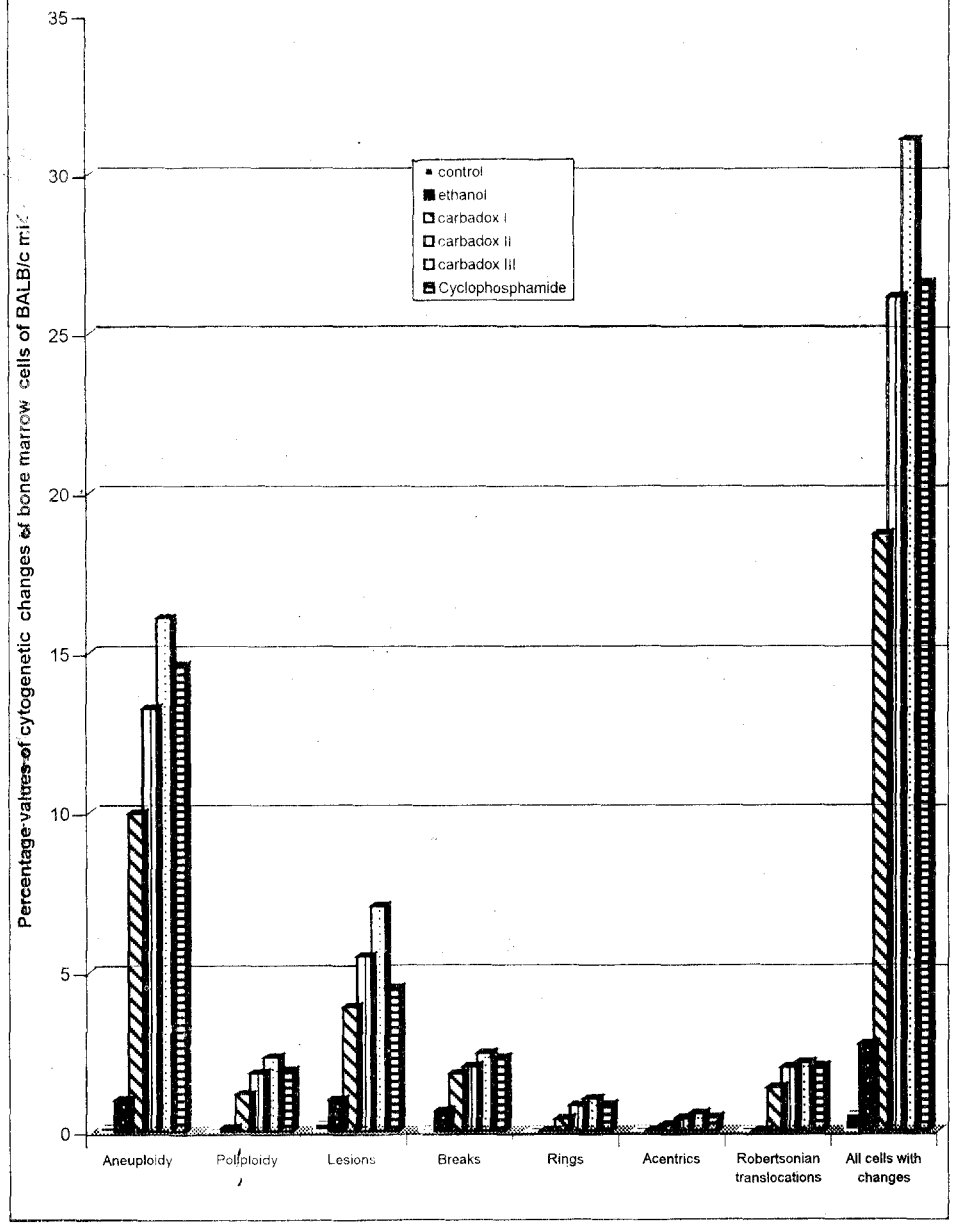
According to Lilp (1981) spontaneous gaps and breaks of *Mus musculus* chromosomes vary from 0 to 2,8% in somatic cells. Many authors later confirmed this level (Irans, 1985; WHO, 1985; Marković, 1999). Brogger (1982) suggested that the high frequency of gaps and breaks could be the consequence of chemical genotoxic effects. All experimental doses of Carbadox (0,2, 0,4 and 0,8 mg/kg b.w.) increased the frequency of cells with gaps and breaks (3,88%, 5,47% and 7,04%, respectively) compared to negative controls (0 - 2,8%). Also, all the experimental doses of Carbadox induced rings, acentrics Robertsonian translocations of chromosomes as types of structural chromosomal changes. In the cells of the positive controls treated with cyclophosphamide there were gaps and breaks, too (0.4% - rings and 0.81% - acentrics). From the results given in Table 2 it is possible to conclude that structural chromosomal changes of all types increased as the dose of Carbadox increased. The equivalent dose of Carbadox induced a similar total number of changes as cyclophosphamide. These results point to the strong genotoxic activity of Carbadox

On the basis of the results *in vitro* and *in vivo* it could be concluded that all experimental doses of Carbadox expressed genotoxicity and possible mutagenicity due to its clastogenic effects.

Table 2. Cytogenetic parameters of bone marrow cells from control and experimental groups of mice treated with increasing doses of Carbadox

Cytogenetic parameters	Control group		Cyclophophamide 0.4 mg/kg b.w.		Ethanol		Carbadox 0.2mg/kgb.w.		Carbadox 0.4 mg/kg b.w.		Carbadox 08mg/kg b.w.	
	X	%	X	%	X	%	X	%	X	%	X	%
Aneuploidy	1.21	0.17	102.56	14.6	6.94	0.99	69.96	9.94	92.73	13.25	112.5	16.08
Poliploidy	0	0	13.3	1.9	0.63	0.09	8.15	.16	12.71	1.82	16.27	2.32
Lesions	1.83	0.26	31.39	4.48	6.9	0.99	27.19	.88	38.31	5.47	49.29	7.04
Breaks	0.77	0.11	16.2	2.31	4.42	0.63	12.52	1.79	17.11	2.44	20.42	2.02
Accentrics	0	0	5.7	0.81	0	0	2.7	0.38	5.55	0.79	7.67	1.01
Rings	0	0	2.8	0.4	0	0	1.33	0.19	2.75	0.39	3.8	0.54
Robertson transloc.	0	0	14.17	2.02	0	0	9.52	.36	13.94	1.99	17.67	2.1
All cytog. changes	3.81	0.54	186.15	26.54	18.89	2.7	131.34	18.7	183.1	26.15	227.68	31.11

Figure 2. Percentage values of cytogenetic parameters in bone marrow cells from control and experimental groups of mice treated with increasing doses of Carbadox



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ISPITIVANJE GENOTOKSIČNOG EFEKTA CARBADOXA *IN VITRO* I *IN VIVO*

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S A D R Ž A J

Carbadox je sintetsko organsko jedinjenje (Metil-3(2quinoksal-metilen) karbozat-N1-N4 dioksid) sa baktericidnim dejstvom koje se koristi u suzbijanju svinjske dizenterije i salmoneloze i kao faktor koji stimuliše rast. Zbog spore metaboličke obrade i dugog zadržavanja u organizmu tretiranih životinja, a još više zbog njegovog toksičnog, citotoksičnog i kancerogenog dejstva, Carbadox u novije vreme predmet detaljnih proučavanja.

U ovom radu su izneti rezultati *in vitro* ispitivanja efekta Carbadoxa na limfocite periferne krvi čoveka praćenjem promena na nivou molekula DNK na osnovu učestalosti razmene sestrinskih hromatida. Citogenetička ispitivanja uticaja Carbadoxa, posle intragastrične aplikacije, na nastanak hromozomskih promena u ćelijama kostne srži obavljena su na miševima BALB/c soja. Hromozomi za kariotipsku analizu su dobijeni korišćenjem direktne metode ispiranja srži dugih cevastih kostiju.

Rezultati ispitivanja efekata različitih doza Carbadoxa *in vitro* na limfocitima čoveka i *in vivo* na ćelijama kostne srži miševa BALB/c ukazali su da ovaj antibiotik poseduje visok genotoksični potencijal i jasno manifestuje mutageni efekat.