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THE ANTIBIOTIC ERYTHROMYCIN DID NOT AFFECT MICRONUCLEUS FREQUENCY IN HUMAN PHA-STIMULATED LYMPHOCYTES

D. GRUJIČIĆ[1], IVANA STOŠIĆ[1], and OLIVERA MILOŠEVIĆ-DJORDJEVIĆ[1,2]

[1]*Faculty of Science, University of Kragujevac,* 34000 Kragujevac, Serbia
[2]*Faculty of Medicine, University of Kragujevac,* 34000 Kragujevac, Serbia

Abstract — The aim of the present study was to investigate the ability of the antibiotic erythromycin to induce micronucleus (MN) frequency and the cytokinesis block proliferation index (CBPI) in human peripheral blood lymphocytes in vitro using the cytokinesis-block micronucleus test (CBMN). Cell cultures were treated with five different concentrations of erythromycin ranging from 0.68 x 10-4 to 5.45 x 10-4 M. The positive control cells were treated with a known mutagen, mitomycin C (MMC), in a concentration of 1.6 x 10-6 M. None of the tested concentrations of erythromycin significantly changed the average MN frequency or CBPI in PHA-stimulated lymphocytes ($p > 0.05$).

Key words: Erythromycin, human peripheral blood lymphocytes, *in vitro* effect, micronucleus test

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INTRODUCTION

Erythromycin is a macrolide antibiotic that is used to treat various infections, particularly in patients allergic to penicillin (Czeizel et al., 1999). This medicament is essentially bacteriostatic, that is it inhibits protein synthesis by binding reversibly to the 50 S subunits of sensitive microorganisms, but in high concentrations erythromycin is bactericidal against very susceptible organisms (Kapusnik-Uner et al., 1996). The antibiotic is concentrated in the liver and is excreted as the active form in the bile. It is most often administered orally or intravenously. The usual daily dose for adults ranges from 1 to 2 g, while for children the usual dose is 30-50 mg/kg per day (Sweetman, 2002).

Because erythromycin has a broad spectrum of activity, it is used in the treatment of a wide variety of infections. This antibiotic is most effective in vitro against anaerobic gram-positive cocci and bacilli. It is ineffective against most aerobic enteric gram-negative bacilli, but shows activity against some gram-negative organisms in vitro, including Haemophilus influenzae, *Neisseria meningitidis,* and *N. gonorrhoeae* (Kapusnik-Uner et al., 1996). Erythromycin is currently recommended for the treatment of syphilis, diphtheria, and Legionnaire's disease (Klein, 1997). Also, this is the drug of choice for treating pregnant women with *Chlamydia infections* (Miller, 2006). On the other hand, fungi, yeasts, and viruses are resistant to erythromycin (Sweetman, 2002).

Side effects caused by erythromycin are rare. Derby et al. (1993) reported a case of choleastatic hepatitis that developed after treatment with erythromycin. Erythromycin has in rare cases been reported to cause arrhythmia – most often in combination with other drugs, for example verapamil (Goldschmidt et al., 2001) – liver dysfunction, and pancreatitis (Sweetman, 2002). Results obtained by Lanbeck and Paulsen (1995) suggest dose-dependent cytotoxicity of erythromycin for endothel cells after 24 h of exposure. According to the National Toxicology Program (1988), treatment with erythromycin did not cause any neoplastic lesions.

When determining genotoxic agents, the formation of micronuclei (MN) is a common cytogenetic end point. Micronuclei are small extranuclear bodies which occur as a result of chromosome damage (Mateuca et al., 2006; Zalacain et al., 2006) in divid-

ing cells from an acentric chromosome, chromatic fragments, or whole chromosomes or chromatids that are not included in daughter nuclei in telophase (Fenech and Morley, 1985). Numerous studies showed that different factors can cause increase of spontaneous micronucleus (MN) frequency (Neri et al., 2005), as can chemical (Pedersen et al., 2006), physical (An and Kim, 2002), and biological (Suarez et al., 2007) agents. Moreover, the presence of MN in circulating lymphocytes is a relevant biomarker reflecting the biological effect of exposure to genotoxic agents (Milošević-Djordjević et al., 2007). Since erythromycin is widely used in humans as a broad-spectrum antibiotic, the objective of the present study was to investigate the effect of different concentrations of this antibiotic on MN frequency in human peripheral blood lymphocytes in vitro.

MATERIAL AND METHODS

Peripheral blood of three healthy donors (two 28-year-old females and a 40-year-old man), non-smokers who had not been exposed to known environmental mutagen agents, was used for determination of spontaneous and induced MN frequency. The cytokinesis block micronucleus test (CBMN) was applied (Fenech, 2000).

Whole heparinized blood (0.5 ml) was cultured in 5 ml of PBMax Karyotyping (Invitrogen, California, USA), the complete medium for lymphocyte cultivation. Double cell cultures were incubated at 37°C for 72 h. The tested substances were dissolved in the cultivation medium without serum and added to cell cultures 24 h after the beginning of incubation. The effect of erythromycin was tested in five different concentrations, 0.68×10^{-4}, 1.36×10^{-4}, 2.73×10^{-4}, 4.10×10^{-4}, and 5.45×10^{-4} M. Cells treated with a known mutagen, mitomycin C (MMC, Sigma, St. Louis, MO, USA), in a concentration of 1.60×10^{-6} M were used as the positive control.

Forty-four hours after the beginning of incubation, cytochalasin B (Sigma, St. Louis, MO, USA) was added to the culture in a final concentration of 4 μg/ml. The cultures were incubated for the next 28 h and then subjected to preparation. The preparation was standard. A hypotonic 0.56% KCl solution was used for this purpose, after which the cell suspension was fixed in methanol: glacial acetic acid (3: 1) three times. Slides were stained with 2% Giemsa solution (Alfapanon, Novi Sad, Serbia).

Micronucleus frequency was determined by analyzing 1000 binucleated (BN) cells per culture according to the criteria for MN analysis described by Fenech (2000) and Kirsch-Volders et al. (2000). In order to determine the cytotoxic effect of the investigated concentrations of the medicament, the cytokinesis block proliferation index (CBPI) was calculated using the formula: $CBPI = [M_I + 2M_{II} + 3(M_{III} + M_{IV})/500]$, where M_I-M_{IV} denotes the number of cells with 1-4 nuclei (Surralles et al., 1995).

Statistical significance of the difference between average spontaneous and induced MN frequencies was determined using Student's t-test. The correlation between the investigated medicament concentrations and the percentage of BN cells in cultures treated with different doses of the medicament was determined by applying Pearson correlation coefficient. The variability of MN frequency was determined by analysis of variance (ANOVA).

RESULTS

Tables 1-4 present the results of MN analyses in peripheral blood lymphocytes before and after in vitro treatment with different concentrations of the antibiotic erythromycin.

Table 1 shows individual values of MN frequencies and CBPI in control and treated groups of cells obtained from three donors in good health. In control untreated cells, MN frequencies varied from 7 to 8 MN per 1000 analyzed BN cells. Treatment with MMC in positive control cells caused a statistically significant increase of MN frequency (102 MN per 1000 BN cells) in comparison to control untreated cells and all groups of cells treated with different concentrations of erythromycin ($p < 0.001$). The values of CBPI ranged from 1.81 to 2.01 in untreated cultures, but dropped to 1.65 in the positive control. The values of CBPI in cultures treated with erythromycin were in the range of 1.80-1.84.

Table 2 shows average MN frequencies and

Table 1. Frequency of MN in peripheral blood lymphocytes before and after in vitro treatment with different concentrations of erythromycin (E). Abbreviations: BNMN - binucleated cells with MN, CBPI - cytokinesis-block proliferation index, C - control untreated cells, MMC - Mitomycin C.

	Concentrations (M)	Frequency MN/1000	Analyzed BN	BNMN	Distribution of MN 1MN	2MN	3MN	4MN	5MN	CPBI
Donor A										
C	-	8	1000	7	6	1				2.01
E_1	0.68×10^{-4}	7	1000	7	7					1.83
E_2	1.36×10^{-4}	9	1000	7	5	2				1.84
E_3	2.73×10^{-4}	7	1000	7	7					1.82
E_4	4.10×10^{-4}	8	1000	7	6	1				1.84
E_5	5.45×10^{-4}	8	1000	7	6	1				1.82
Donor B										
C	-	7	1000	7	7					1.90
E_1	0.68×10^{-4}	8	1000	8	8					1.83
E_2	1.36×10^{-4}	7	1000	6	5	1				1.82
E_3	2.73×10^{-4}	10	1000	10	10					1.84
E_4	4.10×10^{-4}	7	1000	7	7					1.81
E_5	5.45×10^{-4}	7	1000	7	7					1.80
Donor C										
C	-	7	1000	7	7					1.81
E_1	0.68×10^{-4}	7	1000	6	5	1				1.80
E_2	1.36×10^{-4}	8	1000	7	6	1				1.81
E_3	2.73×10^{-4}	10	1000	8	6	2				1.81
E_4	4.10×10^{-4}	10	1000	8	6	2				1.84
E_5	5.45×10^{-4}	10	1000	9	8	1				1.84
Positive control (MMC)	1.60×10^{-6}	102	1000	84	76	2	5		1	1.65

CBPI in control and cell cultures treated with the tested concentrations of erythromycin. Comparison of control untreated and treated cells revealed that the tested concentrations of erythromycin did not influence the MN rate significantly ($p > 0.05$). The average frequency of MN per 1000 BN cells in control untreated cells was 7.33 ± 0.58. Analysis of the distribution of MN revealed that the investigated concentrations of erythromycin did not significantly change the number of BN cells containing MN. Almost all tested concentrations of erythromycin, except the lowest dose (0.68×10^{-4} M), slightly

Table 2. Average MN frequencies, MN distribution, and CBPI in peripheral blood lymphocytes after in vitro treatment with different concentrations of erythromycin (E) and in control cells (C). Abbreviations as in Table 1.

Treatment	Concentrations (M)	Average MN ± S.D./1000 BN cells	Total of analyzed BN cells	BNMN	Distribution of MN 1MN	2MN	3 MN	mean CBPI ± S.D.
C	-	7.33 ± 0.58	3000	21	20	1		1.91 ± 0.10
E_1	0.68 x 10^{-4}	7.33 ± 0.58	3000	21	20	1		1.82 ± 0.02
E_2	1.36 x 10^{-4}	8.00 ± 1.00	3000	20	16	4		1.82 ± 0.02
E_3	2.73 x 10^{-4}	9.00 ± 1.73	3000	25	23	2		1.82 ± 0.02
E_4	4.10 x 10^{-4}	8.33 ± 1.53	3000	22	19	3		1.83 ± 0.02
E_5	5.45 x 10^{-4}	8.33 ± 1.53	3000	23	21	2		1.82 ± 0.02

Table 3. Presence of mononucleated (mono), binucleated (BN), and polynucleated (poly) lymphocytes after in vitro treatment with different concentrations of erythromycin (E) and in control cells (C). Abbreviations: % BN - percentage of BN cells, *Student's t-test $p < 0.05$.

Treatment	Concentrations (M)	mono (%)	BN (%)	poly (%)
E_1	0.68 x 10^{-4}	20.96	76.40	2.64
E_2	1.36 x 10^{-4}	18.66	80.41	0.93*
E_3	2.73x 10^{-4}	19.66	78.48	1.86
E_4	4.10 x 10^{-4}	18.02	80.81	1.17
E_5	5.45 x 10^{-4}	20.49	77.03	2.48
C		17.02	75.00	7.98

Table 4. Analysis of variance for MN before and after in vitro treatment with different concentrations of erythromycin.

Variance	Sum of squares	df	Mean square	F	Sig.
Between groups	4.400	4	1.100	0.611	0.664
Within groups	18.000	10	1.800		
Total	22.400	14			

increased the number of MN in BN cells. The same table shows average CBPI values as well. All tested concentrations of erythromycin lowered the CBPI, but without statistical significance ($p > 0.05$).

Table 3 presents the percentage of mononucleated, binucleated, and polynucleated cells in treated cultures in relation to control untreated cells. In all treated cell cultures, erythromycin decreased the percentage of polynucleated cells. A statistically significant decrease was observed only in cultures treated with a 1.36 x 10^{-4} M concentration of erythromycin ($p < 0.05$). The percentage of mononucleated and BN cells was slightly higher than in control cells, but without statistical significance. The Pearson correlation coefficient showed no correlation between different concentrations of erythromycin and the percentage of BN cells ($R = 0.39$, $p > 0.05$).

Application of ANOVA to MN (Table 4) did not reveal any statistically significant difference in comparison of between group (bg) vs. within group (wg) variance ($F = 0.611$, $p > 0.05$). The inter-individual variance of MN was greater than the intra-individual.

DISCUSSION

Because of its rapidness, simplicity, and potential for automation, the measurement of micronucleated cells is often preferred in routine genotoxicity testing (Miller et al., 1997; Bonassi et al., 2007). The CBMN test became one of the most promising methodologies for assessing DNA damage because it affords the possibility detecting both chromosome and genome mutations in BN cells (Kirsch-Volders and Fenech, 2001). Apart from this characteristic, the relationship between MN frequency and genetic damage, as well as that between increase of MN frequency and cancer risk, were the main arguments for using this test for evaluating the genotoxic effect of erythromycin in our study.

We found published sources which treat the effect of erythromycin on genetic material. However, in none of these studies was the MN test used as the biomarker of chromosomal damage. According to the National Toxicology Program (1988), erythromycin did not produce an increase in the frequency of biomarkers such as sister chromatid exchanges (SCE) or chromosomal aberrations in either the presence or absence of metabolic activation (S9) after treatment of cultured Chinese hamster ovary cells.

The results obtained on the average MN frequencies in human peripheral blood lymphocytes after the end of treatment with different concentrations of erythromycin in vitro did not show a statistically significant increase ($p > 0.05$) in relation to MN values in control untreated cultures. As expected, only the positive control caused a significant increase of MN frequency ($p < 0.001$).

The CBMN test is also used to detect effects on cell proliferation (Pastor et al., 2003) and cytotoxicity (Fenech, 2008) by following changes in CBPI values (Laffon et al., 2001). This index shows the number of cell cycles through which treated cells have passed (Surralles et al., 1995). A significant decrease of CBPI is associated with severe genomic damage which can stop further division of cells or activate the apoptosis mechanism (Kirsch-Volders and Fenech, 2001). Our results showed that all tested concentrations of erythromycin resulted in a decrease of CBPI, but without statistical significance ($p > 0.05$).

Analysis of the percent of binucleated cells in treated cultures showed that the examined concentrations of erythromycin induced a nonsignificant increase in the percentage of binucleated cells in relation to control untreated cells ($p > 0.05$). Moreover, at all tested erythromycin concentrations, the percentage of mononucleated cells slightly increased. Only in cultures treated with a 1.36×10^{-4} M erythromycin concentration did we obtain a statistically significant decrease in the percentage of polynucleated cells ($p < 0.05$).

Previous investigations showed that MN distribution in BN cells can indicate the level of genomic damage. Thus, an increase of MN is related to chromosomal damage (Fenech and Morley, 1985), and there is positive correlation between these two biological endpoints (Joksić, 1999). Minozzo et al. (2004) demonstrated that cells with a greater number of MN suffered more genome damage. Analysis of MN distribution showed that the tested concentrations of erythromycin, except for the lowest concentration (0.68×10^{-4} M), slightly increase the number of MN in BN cells. In addition, the number of BN cells with MN slightly increases in cultures treated with the three highest concentrations of erythromycin (2.73×10^{-4}, 4.10×10^{-4}, and 5.45×10^{-4} M).

Many authors point out that different life conditions (such as work, living environment, and diet) can influence the baseline frequency of MN (Xue et al., 1992; Rekhadevi et al., 2009). The lymphocytes of patients before therapy showed an individual variation in MN/1000 BN cells of from 7 to 8, whereas after therapy it ranged from 7 to 10. By applying ANOVA to MN, we noticed that the intra-individual variance was lower than the inter-individual. Variability in the induced MN frequency may be attributable to individual reactivity to the applied therapy. Numerous studies showed that in different persons subjected to treatment with the same dose of agents, the level of chromosomal damage was different, which was explained in terms of specificity

of their reaction (Saritha and Ahuja, 1990; Grujičić et al., 2007).

Our results obtained using the CBMN test indicate that the tested concentrations of erythromycin (0.68×10^{-4}, 1.36×10^{-4}, 2.73×10^{-4}, 4.10×10^{-4}, and 5.45×10^{-4} M) did not affect micronucleus frequency in PHA-stimulated human lymphocytes. Finally, on the basis of these results and assuming that MNs can be used as biomarkers for substance genotoxicity, we conclude that this antibiotic in the tested concentrations in vitro did not show any genotoxic effect in human peripheral blood lymphocytes.

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АНТИБИОТИК ЕРИТРОМИЦИН НЕ ПОВЕЋАВА ФРЕКВЕНЦУ МИКРОНУКЛЕУСА У ХУМАНИМ РНА СТИМУЛИСАНИМ ЛИМФОЦИТИМА

Д. ГРУЈИЧИЋ[1], ИВАНА СТОШИЋ[2] И ОЛИВЕРА МИЛОШЕВИЋ-ЂОРЂЕВИЋ[1, 2]

[1]*Природно-математички факултет, Универзитет у Крагујевцу,* 34000 Крагујевац, Србија
[2]*Медицински факултет, Универзитет у Крагујевцу,* 34000 Крагујевац, Србија

У овој студији је испитивана способност антибиотика еритромицина да индукује микронуклеусну (MN) фреквенцу и цитокинезис блок пролиферациони индекс (CBPI) у хуманим лимфоцитима периферне крви ин витро, помоћу цитокинезис-блок микронуклеусног теста (CBMH). Ћелијске културе су третиране са пет различитих концентрација еритромицина од 0,68 x 10^{-4} do 5,45 x 10^{-4} M. Позитивне контролне ћелије су третиране познатим мутагеном, митомицином C (MMC) у концентрацији 1,6 x 10^{-6} M. Све тестиране концентрације еритромицина нису статистички значајно мењале просечну MN фреквенцу и CBPI у РНА стимулисаним лимфоцитима ($p > 0,05$).