



Effect of intratumoral application of methotrexate *in vivo* on frequency of micronuclei in peripheral blood lymphocytes

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BACKGROUND: Methotrexate is a cytostatic drug belonging to the group of antimetabolites; it is an antagonist of folic acid with an intensive application in gynecology practice.

METHODS: The frequency of micronuclei (MN) in peripheral blood lymphocytes of 30 patients with myoma uteri was analyzed before and after intratumoral application of methotrexate (MTX) in total dose from 50 to 115 mg. Analysis of micronuclei was performed by the application of cytokinesis-block technique (CB).

RESULTS: Average frequency of MN in lymphocytes of patients before the therapy was 4.6 ± 0.4 MN/1000 analyzed cells. After the completion of therapy with six separate doses during six consecutive menstrual average frequency of MN increased 1.5 times (7.0 ± 0.6) in comparison to control frequency before the therapy. Statistically significant difference ($p < 0.001$) was established by the Student *t* test.

CONCLUSION: Methotrexate intratumoral treatment of the myoma uteri significantly increased micronuclei in peripheral blood lymphocytes.

KEY WORDS: Methotrexate; Injections, Intralesional; Myoma; Uterine Neoplasms; Lymphocytes; Micronuclei

INTRODUCTION

Methotrexate (MTX) is a cytostatic drug belonging to the group of antimetabolites; it is an antagonist of folic acid, which has an intensive application in the treatment of malignant diseases, psoriasis, and rheumatoid arthritis; lately, it has been used as a form of medicamentous treatment of myoma uteri by subendometrial injection (1).

Effects of MTX are used to antagonize the index of mitotic activities in endometrium and myomas increase by estrogens; they also increase the binding of thymidine in target cells. Action of MTX completely fits into mechanism of acting of folate antagonists and it is the most effective in S phase of cell cycle. Like most metabolites, MTX is partially selective for tumorous cells and toxic for all normal cells that divide fast. Therefore, we decided to investigate the effect of the mentioned drug on the frequency of micronuclei

(MN) in peripheral blood lymphocytes of patients as a consequence of intratumoral application *in vivo*. Since 1985, cytokinesis-block micronucleus test (CBMN) in human peripheral blood lymphocytes has been accepted by many laboratories as an optimal method for evaluation of genotoxic effect (2,3). It is known that micronuclei (MN) increase consequence of chromosomal damages in dividing cells (2), and that there is a positive correlation between these two biological endpoints (4).

The aim of our study was to determine MN frequency in peripheral blood lymphocytes of patients diagnosed with myoma uteri, before and after intratumoral application of MTX.

PATIENTS AND METHODS

In the investigation of mutagenic effect of MTX on the MN frequency in peripheral blood lymphocytes, we applied CBMN test (2) before and after the completed therapy on the sample of 30 patients with the diagnosis myoma uteri. The median age of surveyed patients was 45 ± 0.8 years, (range, 36-52 years). MTX was applied intratumorally in single doses on day 8 of follicular phase of menstrual cycle, and the procedure was repeated during six consecutive cycles.

We used standard method for cultivation of peripheral blood lymphocytes in duration of 72 hours (5,6) together with some modi-

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fications necessary for application of the CBMN test (2) in our cytogenetic studies.

For the cultures of peripheral blood lymphocytes 77% Parker 199 (Torlak, Belgrade), 20% fetal calf serum (Institute of Veterinary Medicine, Novi Sad) and 3% phytohemagglutinin (PHA-INEP) were used. Under sterile conditions, 20 drops of whole heparinized blood were put into bottles containing 5ml of medium. Cultures were incubated at 37°C for 72 hours. Cytochalasin B (Fluka) was added after 48 hours of incubation into the final concentration of 4µg/ml.

The preparation was standard. Hypotonic solution was 0.56% KCl, and fixation was carried out during 2x15 minutes by fixative (glacial acetic acid: methanol = 1:3). The slides were stained by 2% alkaline Giemsa (Alfapanon, Novi Sad) and dried under a lamp. The analyses of MN were carried out on 1000 binucleated lymphoblasts per patient. We applied Student *t* test and analysis of variance (ANOVA) when comparing the obtained results.

RESULTS

Results of the investigation are presented in Tables 1 and 2, and in Figure 1.

Table 1. Frequencies of micronuclei (MN) in peripheral blood lymphocytes of patients with myoma uteri before and after methotrexate intra-tumoral treatment of the myoma

Patients	Age (years)	Before therapy		Doses (mg)	After therapy	
		Analyzed CB cells	Frequency MN/1000 CB cells		Analyzed CB cells	Frequency MN/1000 CB cells
1.	40	214	5	50	1000	1
2.	43	1000	4	50	1000	4
3.	52	1000	5	50	1000	9
4.	47	1000	8	50	1000	10
5.	40	1000	8	50	1000	8
6.	51	1000	4	55	1000	5
7.	50	1000	5	55	1000	5
8.	49	1000	8	60	1000	10
9.	41	1000	6	60	1000	9
10.	42	1000	3	60	1000	8
11.	50	1000	8	60	1000	8
12.	40	1000	7	60	1000	16
13.	45	1000	4	60	1000	8
14.	48	1000	9	60	1000	7
15.	47	1000	4	60	930	4
16.	40	1000	3	60	1000	10
17.	46	1000	4	60	1000	7
18.	53	1000	1	60	1000	3
19.	44	1000	1	60	1000	3
20.	36	1000	6	60	1000	5
21.	49	1000	3	60	1000	4
22.	43	1000	5	60	1000	8
23.	42	1000	4	60	1000	10
24.	48	1000	1	60	1000	6
25.	52	1000	2	60	1000	7
26.	45	1000	5	60	1000	8
27.	45	1000	5	60	1000	9
28.	44	1000	7	60	1000	9
29.	48	1000	4	70	1000	7
30.	36	1000	0	115	1000	3
<i>X</i> ± <i>SE</i>	45.2±0.84	943.86±56.14	4.63±0.43	60.17±2.06	997±2.06	7.03±0.55

t=4.85 df= 29 p<0.001

Table 2. ANOVA for frequency of micronuclei in lymphocytes before and after methotrexate therapy

Variability	Dispersion (D)	df	Variance S ²	F	p
Between group	86.40	1	86.40	11.88	0.001
Within group	421.93	58	7.27		
General	508.33	59			

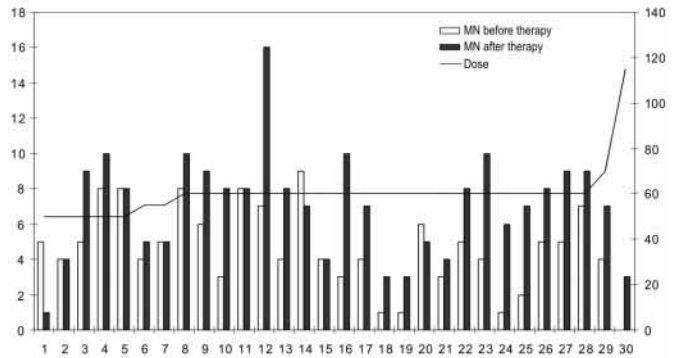


Figure 1. Average frequencies of MN/1000 in peripheral blood lymphocytes of patients before and after metotrexate therapy

After the application of MTX therapy (7.03±0.55), average MN frequency (Table 1) was increased 1.5 times in comparison to average MN frequency in the same patients before the therapy (4.63.±0.43), with the statistical significance of p<0.001.

From 29 930 analyzed binucleated cells after the treatment, 272 (0.91%) cells with MN were found, which was double in comparison with control cells before the therapy (0.46%). Binucleated lymphoblasts with 1 MN were exclusively present in the analyzed sample.

Frequencies of MN were increased in most patients in relation to their values before the therapy. This increase was individual - from 1.25 (25%) to 6 times (600%). The change of MN frequency did not occur in the analyzed cells of patients numbered 2 and 5 who received total dose of 50 mg, patient number 7 who received total dose of 55 mg, and patients 11 and 15 who received total dose of 60 mg. In patient number 1 who received total dose of 50 mg, and patients 14 and 20 who received total dose of 60 mg, the frequencies of MN after the therapy decreased in relation to their values before the therapy.

Single MN frequencies before and after the therapy due to received doses are shown in Figure 1. Correlation between the received dose and MN frequency was negative (r=-0.17 p=0.36).

Summarizing the presented results, the analysis of variance-ANOVA (Table 2) shows a significant difference in average frequencies of MN between the observed groups, with a probability p<0.001. Influence of genetic factors on variability in the presence of MN is considerably greater (78.4%) than that of environmental factors (21.6%).

DISCUSSION

Micronuclei in mitotically active cells arise from structural chromosomal aberrations or disturbed function of mitotic spindle. That is why some authors consider that the follow up of MN frequencies in peripheral blood lymphocytes in the samples of human individuals could be a very effective test to estimate the effects of biological, physical and chemical agents (7).

At the of a six months long therapeutical MTX intratumoral treatment of the myoma uteri, the results of average MN frequencies in peripheral blood lymphocytes of 30 patients aging from 36 to 52 years showed statistically significant increase in relation to control MN values of the same patients before the therapy ($p < 0.001$).

The obtained results can be compared with data from literature. MTX produced a significant genetic damage, which was proved by the increased incidence of chromosomal aberration and MN formation in human as well as in animal model (8). Acar et al. (9) reported significantly higher MN frequencies in patients with acute lymphoblastic leukemia (ALL) after the treatment with antileukemic agent (vincristine, methotrexate, daunomycine, prednisone and asparaginase). Kasahara et al. (10) indicated that level of MN and chromosomal aberrations in mouse cells of bone marrow was far higher after multiple treatments than in single MTX treatments, and they concluded that effect of multiple doses of MTX on induction of MN and chromosomal aberrations could be explained by intracellular inhibition of dihydrofolate reductase (DHFR).

The primary site of action of MTX is the enzyme dihydrofolate reductase (DHFR). Inhibition of DHFR leads to toxic effects through partial consumption of the tetrahydrofolate cofactors, which are necessary for the synthesis of purines and thymidylate (precursors of DNA) (11-13), and through direct inhibition of folate-dependent enzymes of purine and thymidylate metabolism by the polyglutamates of MTX and dihydrofolate polyglutamates which accumulate with the inhibition of DHFR (14). The final result is the inhibition of DNA replication and cell death (13).

Among the lymphocytes of patients before therapy, individual variation in MN/1000 binucleated cells was significant, varying from 0 to 8; after the therapy MN ranged from 1 to 16. By the application of ANOVA for MN, we noted statistically significant difference in comparison between groups vs. within group variance ($F = 11.88$, $p < 0.001$). The present variability in the MN frequency could be attributed to an individual reactivity on MTX action. Interindividual variation in MN was almost five times greater than intraindividual variation. It is our impression that interindividual cell variation in MN frequency is mainly based on epigenetic, i.e. a regulatory-genetic variation whereas the differences among individuals are prevalently structurally genetic.

Previous investigations showed that in different persons treated by the same dose of mutagen, the level of chromosomal aberrations was different, which was explained as an individual reactivity (15,16). Individual sensitivity increased due to the difference in metabolic activation of mutagen and efficiency of DNA repair (17). Obtained results showed a negative correlation between received doses of MTX and MN frequencies. In 5 patients (No. 2, 5, 7, 11, 15 in Table 1) the change of MN frequency did not occur after the therapy, and in 3 patients (No. 1, 14 and 20 in Table 1) frequencies of MN after the therapy decreased in relation to their values before therapy.

Many authors demonstrated mechanisms of resistance to MTX action (18-21). DHFR levels in leukemic cells increase within 24 hours after the treatment with methotrexate. This probably shows the induction of new enzyme synthesis. Recent investigations demonstrate that the intracellular level of DHFR protein is controlled at the level of mRNA translational efficiency through an autoregulatory mechanism, whereby the DHFR protein may bind to and control the translational efficiency of its own mRNA (22).

CONCLUSION

Our results suggest that intratumoral application of MTX therapy by using sub-endometrial injection into the myoma in patients with myoma uteri significantly increased the frequency of micronuclei in human peripheral blood lymphocytes.

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