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Inhibition of methotrexate-induced chromosomal damage by folinic acid in V79 cells

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Abstract

Methotrexate (MTX), an anticancer compound, is widely used in the treatment of leukemia. It induces cytogenetic damage as well as cytostatic effects on a variety of cell systems. Folinic acid (Leucovorin) is generally administered along with MTX as a rescue agent to decrease MTX-induced toxicity. However, information regarding the inhibitory effect of folinic acid against cytogenetic damage caused by MTX is limited. This study was conducted to assess the cytogenetic effect of MTX and its inhibition by folinic acid (FA) using the micronucleus and chromosomal aberration assays concurrently. Exponentially growing V79 cells were treated with MTX at five different concentrations (5–100 μg ml^{-1}) with S9 microsomal fraction for 6 h and post-treated with two concentrations of FA (5 or 50 μg) for 40 h. Results indicate that MTX alone induced a concentration-related increase in % micronucleated binucleated cells (MNBN) and % aberrant cells (Abs). There was a decrease in nuclear division index (NDI) with increase in MTX concentration. Similarly, the mitotic index (MI) also decreased in all concentrations of MTX tested. The addition of FA at 50 μg ml^{-1} significantly reduced % MNBN (40–68%) and % Abs (36–77%). Inhibition was also seen at 5 μg FA (12 to 54% for MNBN and 20 to 61% for Abs). These results indicate that FA is capable of reducing the cytogenetic damage induced by MTX and appears to be an anticlastogenic agent. © 1998 Elsevier Science B.V.

Keywords: Methotrexate; Anticancer drug; Folinic acid (leucovorin); Dihydrofolate reductase; Micronucleus; Chromosomal aberration; V79 cell

1. Introduction

Methotrexate (MTX) is an antineoplastic agent used in the treatment of acute lymphoblastic leukemia, non-Hodgkin's lymphoma, breast cancer and several other cancers. It also induces cytogenetic damage as well as cytostatic activity in a variety of test systems. MTX competitively binds to the enzyme dihydrofolate reductase (DHFR) thereby preventing its normal function of reducing dihydrofolate

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to tetrahydrofolate, an essential cofactor for nucleic acid synthesis. MTX is also a mitotic inhibitor that arrests the cell cycle in interphase and leads to prolongation of metaphase. Dalen et al. [1] have shown that the mitotic index was decreased drastically in Chang cells when MTX was administered at a concentration of 5×10^{-5} mg ml^{-1} or higher. However, when MTX was added simultaneously with leucovorin (folinic acid, clinically known as leucovorin), no reduction in mitotic index was seen. In vivo studies indicate that micronucleus (MN) induction was enhanced after repeated treatments of MTX compared to a single treatment in male mice [2,3]. Similar results were found in studies of bone marrow and peripheral blood cells [4–6].

Folinic acid (FA), is an ubiquitous member of biological folate pools, usually comprising 10–20% of total cellular folates [7,8]. It is widely administered as a rescue agent for methotrexate toxicity [9]. Studies have shown that leucovorin protects against repeated daily dose toxicity of trimetrexate (TMX), a drug structurally similar to MTX, in rats. TMX was administered orally at doses of 25, 35 and 45 mg kg^{-1} body weight and leucovorin was administered at doses 1.5, 20 or 50 mg kg^{-1} twice daily both as co-treatment along with TMX and post-treatment at 6 h after TMX administration [10]. Also, a study by Theiss et al. [11] showed an increase in TMX genotoxicity as evidenced by chromosomal aberrations and was reversed by administration of leucovorin. The above studies show the cytotoxic effect of MTX and can be reversed by the administration of FA which bypasses the major site of MTX action, the binding of DHFR and subsequent interference with the formation of tetrahydrofolic acid. FA has also been used as an antigenotoxic agent to prevent chromosomal damage in bone marrow cells of mice receiving MTX [12].

Although there are several studies indicating that MTX is genotoxic, knowledge of its cytogenicity in V79 cells is limited. Also, knowledge of the cytogenetic effects of FA as an antigenotoxic agent on MTX-induced DNA damage is limited. Therefore, this study was undertaken to investigate the extent of MN and structural chromosomal aberrations (SCA) formation by MTX treatment in V79 cells and the protective effect of FA on MTX-induced cytogenetic damage.

2. Materials and methods

2.1. Cell line

V79 Chinese hamster lung fibroblast cell line was obtained from Dr. C.C. Chang (Michigan State University, East Lansing, MI). The cell line was tested for mycoplasma contamination. The cells were grown in 75 cm^2 Falcon tissue culture flasks containing 15 ml Eagle's minimum essential medium (MEM; Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Gibco), 100 U penicillin/ml medium and 100 μg streptomycin/ml medium (Gibco). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2 and were subcultured using trypsin-EDTA solution (Gibco) in phosphate-buffered saline (PBS).

2.2. Chemicals

Methotrexate (Sigma Chemical Company, St. Louis, MO), was dissolved in 0.05 M NaOH and a 2.5 mg ml^{-1} stock solution was prepared. The stock solution was diluted immediately prior to use. Concentrations of 5, 10, 25, 50 or 100 μg ml^{-1} of medium were used in this study. Concentration selection was based on our exploratory studies and those conducted by Pienkowska and Koziorowska [12]. The cell cultures, in duplicate, were treated with MTX for 6 h. The exposure time was determined based on our preliminary studies. MEM and mitomycin C (MMC) were used as negative and positive controls, respectively. NaOH was used as a solvent control for MTX.

Folinic acid (Sigma, MO) was dissolved in distilled water at 2 mg ml^{-1} and diluted immediately prior to use. The concentration selection for FA (5 or 50 μg ml^{-1}) was based on studies conducted by Burres and Cass [13]. The cultures were exposed to MTX and were post-treated with FA for 40 h. Based on our preliminary studies, a reasonable number of binucleated (BN) cells could be obtained only at 40 h.

Cytochalasin B (CYB, Sigma) was dissolved in dimethyl sulfoxide (DMSO) at 2 mg ml^{-1}, stored at −20°C and diluted with PBS immediately prior to use. A final concentration of 3 μg CYB/ml culture medium [14,15] was used. CYB was added simulta-

neously along with post-treatment of FA. To arrest cells at metaphase for chromosomal aberration studies, Colcemid (Gibco), at a concentration of 0.025 μg ml^{-1} culture medium, prepared in Hanks Balanced Salt Solution with phenol red at 10 μg ml^{-1}, was added 2 h prior to harvesting cells.

2.2.1. S9 Mixture

The S9 microsomal fraction, prepared according to previous reports [16,17], was from the liver tissue of adult male rats (Sprague–Dawley) pretreated with Aroclor 1254. All experiments were carried out with the same batch of S9 fraction which was stored in −80°C. The S9 mix was prepared fresh by combining 30% S9 fraction with appropriate cofactors, and 0.5 ml of S9 mix was added to V79 cell cultures containing 5 ml culture medium. Treatments with S9 were carried out for 6 h along with or without MTX, the cells were washed twice, and post-treated with FA at different concentrations.

2.3. Micronucleus assay

The MN assay was performed according to the procedure described by Krishna et al. [18]. Briefly, 1–2 million cells were seeded in each 25 cm^2 tissue culture flask with 5 ml culture media. The cells were allowed to attach and grow for approximately 24 h. Duplicate cultures were included for each treatment group. MTX at varying concentrations along with S9 mix was added to the cultures and incubated for 6 h. Following treatment, the medium with S9 mix and MTX was aspirated and flasks were rinsed twice with PBS. FA and CYB were added simultaneously to each culture and incubated for an additional 40 h. After post-treatment incubation, the medium was removed, flasks were rinsed with PBS, and cells dislodged with trypsin-EDTA solution at 37°C were collected and centrifuged at 1000 rpm for 5 min. The supernatant was removed and the pellet resuspended in the remaining solution. The cells were treated with 5 ml hypotonic solution (75 mM KCl, dropwise) at 37°C for 3 to 5 min and recentrifuged. The supernatant was removed, pellet resuspended, and two drops of cell suspension per slide were carefully dropped onto prelabeled clean dry slides angled at 45° and air dried for 5 min. The slides were then dipped in absolute methanol and air dried to fix the cells. The time between hypotonic treatment and fixing is very critical, as cells continue to swell and may burst if not fixed in a timely fashion. The slides were stained with Diff Quik (Baxter). For scoring, a Carl Zeiss (Oberkochen, Germany) microscope was used. For cell cycle analysis, 400 cells per treatment group were scored for the presence of one, two, or more than two nuclei per cell and the nuclear division index (NDI) was calculated. For MN analysis, 1000 binucleated cells per treatment group were analyzed. The morphological criteria for MN scoring in binucleated cells were similar to those reported by Countryman and Heddle [19] and Roberts et al. [20].

2.4. Structural chromosomal aberration (SCA) analysis

The SCA assay was performed using the procedure described in previous studies [21]. Initial chemical treatments were similar to that for the MN assay. Forty hours after treatment with FA, 0.025 μg of Colcemid/ml culture medium was added to each flask to arrest cells at metaphase. Cells were harvested 2 h later by trypsinization and collected into 15 ml conical tubes. Five ml of hypotonic solution (75 mM KCl) was added dropwise to each tube and the cells were incubated for 15 min at 37°C. Approximately five drops of freshly prepared fixative (3:1 methanol:acetic acid) was added to each tube. The cells were then centrifuged for 5 min at 1000 rpm. The supernatant was decanted and the pellet was resuspended with the remaining solution. Five ml of fixative was added to each tube for 20 min at 4°C, and the cells were spun for 5 min at 1000 rpm. This step was repeated twice. Two to four drops of cell suspension were dropped onto a cold, wet slide. Following air drying at room temperature for approximately 24 h (or overnight), cells were stained with 10% Gurr's Giemsa in Gurr's phosphate buffer (pH 6.8) and 100 metaphase cells in each treatment group were analyzed for SCAs. The types of SCAs were classified according to standard cytogenetic procedures. The following types of SCAs were recorded: chromatid gaps, chromatid breaks, chromatid deletions, fragments, minutes, triradials, quadriradials,

complex rearrangements, chromosome gaps, chromosome breaks, acentric fragments, double minutes, dicentrics and rings. For cytotoxicity, mitotic index (MI) was calculated based on the number of metaphase cells present in a total of 1000 cells per treatment group. All slides were blind coded before being scored.

2.5. Statistical analysis

The MN and SCA data were analyzed by χ^2 test. A sequential linear concentration-trend test was performed to compare the test groups with their respective controls. Correlation coefficients (r) were calculated for MN, SCA, NDI and MI.

3. Results

3.1. Methotrexate-induced chromosomal damage

Five concentrations (5, 10, 25, 50 or 100 μg ml^{-1}) of MTX were used in this study. MEM served as the negative control, NaOH the solvent control and MMC as the positive control. S9 was used in all concentrations of MTX and the control treatment received S9 alone. Two concentrations of FA, 5 or 50 μg ml^{-1}, were used.

Results of the MN assay indicated a decrease in NDI values with increasing MTX concentrations when MTX was added alone (Table 1). No significant difference ($p \leq 0.05$) in NDI was found among

Table 1
Inhibition of methotrexate-induced micronuclei by folinic acid in V79 cells

Concentration		Cell cycle kinetics/400 cells			NDI[a]	MNBN cells/1000 BN cells				MNBN cells (%)	MNBN inhibition (%)
MTX (μg ml^{-1})	FA (μg ml^{-1})	1 N	2 N	$>2N$		1MN	2MN	$>$2MN	Total		
0	0 (MEM)	88	113	199	2.78	11	4	0	15	1.5	–
0	0 (NaOH)	75	113	212	2.87	14	2	5	21	2.1	–
0	0 (MMC)	49	130	221	2.98	87	19	6	112**	11.2*	–
0	0	21	104	275	3.32	14	0	0	14	1.4	–
0	5	22	80	298	3.44	12	1	0	13	1.3	–
0	50	14	78	308	3.51	10	0	0	10	1.0	–
5	0	86	50	264	3.11	25	7	2	34	3.4	–
5	5	71	45	284	3.24	22	3	0	25	2.5	26.5
5	50	56	45	299	3.36	16	2	0	18*	1.8*	47.1
10	0	80	58	262	3.11	38	19	6	63	6.3	–
10	5	61	80	259	3.14	28	4	7	39*	3.9*	38.1
10	50	53	84	263	3.18	19	1	0	20**	2.0**	68.3
25	0	94	66	240	2.97	45	11	9	65	6.5	–
25	5	67	73	260	3.13	23	4	3	30**	3.0**	53.9
25	50	62	90	248	3.09	19	3	2	24**	2.4**	63.1
50	0	94	124	182	2.68	50	11	11	72	7.2	–
50	5	80	105	215	2.88	23	9	4	6**	3.6**	50.0
50	50	78	79	243	3.02	28	8	7	43**	4.3**	40.3
100	0	155	123	122	2.22	49	10	17	76	7.6	–
100	5	144	55	201	2.65	46	14	7	67	6.7	11.8
100	50	104	68	228	2.88	17	8	7	32**	3.2**	57.9

[a] Nuclear division index (NDI) = $\{1N + (2 \times 2N) + (4 \times >2N)\}/400$ cells scored, N = number of nuclei; BN = binucleated cells; MN = micronucleus; MNBN = micronucleated binucleated cell.
MEM = minimum essential medium; MMC = mitomycin C.
* $P \leq 0.05$, ** $P \leq 0.01$, significant values compared with their respective methotrexate concentrations using χ^2 test.

negative, solvent and positive controls but addition of S9 increased the NDI from 2.8 to 3.3. The proportion of mononucleated cells increased with increasing MTX concentration; however, the proportion of binucleated cells decreased at lower concentrations (5, 10 or 25 μg MTX/ml). The % MNBN cells increased significantly ($p \leq 0.01$) from 1.5% to 11.2%, when treated with the positive control, MMC. The % MNBN cells increased significantly with increasing concentrations of MTX alone ($r = 0.72$, $p \leq 0.01$) (Table 1). In the SCA assay, the MI slightly decreased with increasing concentrations of MTX tested. The values of MI ranged from 4.7 in the control to 3.9 at the highest concentration tested (Table 2). The frequency of aberrant cells increased significantly with increasing concentrations of MTX alone ($p \leq 0.01$) in V79 cells.

3.2. Inhibition of methotrexate-induced chromosomal damage by folinic acid

Results of inhibition studies showed that there was no significant increase in NDI compared to their controls when 5 μg FA/ml was added to cultures with different concentrations of MTX. NDI values ranged from 3.4 in the control to 2.7 at the highest concentration of MTX. The NDI values were 3.3 and 2.2 when MTX was treated alone. Similar results were obtained with the addition of 50 μg FA/ml (NDI at 0 μg < 5 μg < 50 μg FA/ml; Table 1). The percent MNBN decreased significantly at higher concentrations of MTX when 5 μg FA/ml was post-treated, with the exception of the 100 μg MTX/ml treatment group (Fig. 1A). Statistically significant inhibition was observed at all concentra-

Table 2
Inhibition of methotrexate-induced chromosomal aberrations by folinic acid in V79 cells

Concentration		Mitotic index[a] (MI)	Aberrations per 100 metaphase cells[b]													Aberrations/100 metaphase cells (without gaps)	Abs[c] (%)	Abs[c] inhibition (%)
MTX (μg ml^{-1})	FA (μg ml^{-1})		Chromatid type							Chromosome type								
			tg	tb	td	f	m	tr	qr	sg	sb	af	dm	d	r			
0	0 (MEM)	4.6	2	0	1	0	5	0	0	0	0	0	1	1	0	8	7	–
0	0 (NaOH)	4.2	1	1	0	0	1	0	0	1	0	0	1	3	0	6	6	–
0	0 (MMC)	4.3	2	2	2	0	18	1	1	2	1	0	11	6	1	43	27**	–
0	0	4.7	3	1	0	0	3	1	0	0	0	0	1	2	0	8	7	–
0	5	4.7	2	0	0	0	7	1	0	0	0	0	1	1	0	10	6	–
0	50	4.9	1	0	0	0	4	0	0	0	0	0	1	1	0	6	4	–
5	0	4.8	2	3	0	0	5	1	0	2	0	0	4	2	0	15	9	–
5	5	5.4	0	0	0	0	3	0	0	0	0	0	1	2	0	6	4	55.6
5	50	5.0	1	0	1	0	2	0	0	0	0	0	0	2	0	5	3	66.7
10	0	4.3	3	2	0	3	7	0	0	0	0	0	4	3	0	19	13	–
10	5	4.6	0	0	2	0	1	0	0	0	0	0	1	1	0	5	5*	61.5
10	50	5.4	3	0	0	0	2	0	0	0	0	0	0	2	0	4	3**	76.9
25	0	4.1	5	3	1	0	26	0	0	0	0	2	7	5	0	44	25	–
25	5	4.7	3	2	0	0	17	0	0	0	0	0	3	4	1	27	20	20.0
25	50	6.1	1	3	0	0	10	0	0	0	0	0	4	2	0	19	16	36.0
50	0	4.0	2	5	1	2	19	2	0	2	2	1	7	4	0	43	32	–
50	5	5.8	3	4	0	0	16	0	0	0	1	0	9	1	1	32	18*	43.8
50	50	6.5	1	1	0	0	5	0	0	1	0	0	4	2	0	12	9**	71.9
100	0	3.9	1	8	0	0	22	1	1	1	1	0	12	2	3	50	41	–
100	5	5.6	4	4	0	1	12	0	0	0	0	1	4	0	4	26	16**	61.0
100	50	5.5	0	1	0	0	6	0	0	0	2	0	1	3	0	13	12**	70.7

[a] Mitotic index (MI) = number of metaphase cells × 100/1000 cells scored.
[b] tg, chromatid gap; tb, chromatid break; td, chromatid deletion; f, fragment; m, minute; tr, triradial; qr, quadriradial; sg, chromosome gap; sb, chromosome break; af, acentric fragment; dm, double minute; d, dicentric; r, ring.
[c] Abs = aberrant cells; MEM = minimum essential medium; MMC = mitomycin C.
* $P \leq 0.05$, ** $P \leq 0.01$, significant values compared with their respective methotrexate concentrations using χ^2 test.

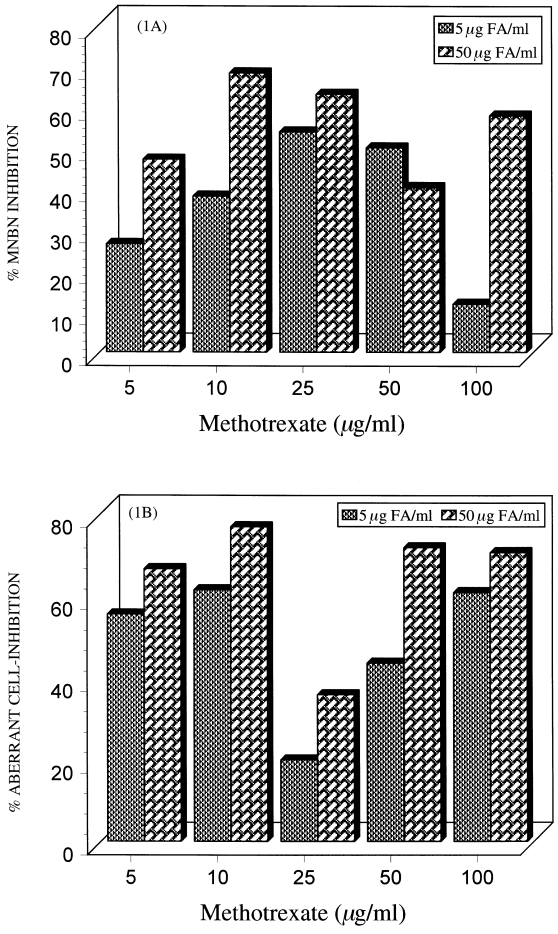

Fig. 1. Protective effect of folinic acid on MTX-induced chromosomal damage in V79 Chinese hamster lung cells: (A) percent inhibition of micronucleated binucleated cells (% MNBN); (B) percent inhibition of aberrant cells.

tions of MTX when cells were post-treated with 50 μg FA/ml. At 5 μg FA/ml, inhibition ranged from 12 to 54%. It was observed that the protection was greater when 50 μg FA/ml was added (40 to 68%).

The SCA results indicated that, with increasing concentrations of MTX alone, there was a numerical decrease in MI. The MI values ranged from 4.7 in the control to 3.9 in the highest MTX concentration tested ($r = -0.81$) (Table 2). Aberrations per 100 metaphase cells increased significantly with increasing MTX concentrations. The percent aberrant cells decreased significantly with the addition of 5 μg of FA at higher concentrations of MTX (10, 50 or 100 μg/ml). However, no significant effect was seen at the lowest concentration (5 μg MTX/ml). FA at 50 μg/ml significantly decreased percent aberrant cells at all concentrations except 5 and 25 μg MTX/ml. Inhibition studies showed that there was a significant inhibition of percent aberrant cells and that the percent inhibition ranged from 20 to 61.5 for 5 μg and from 36 to 77 for 50 μg FA/ml (Table 2, Fig. 1B).

4. Discussion

In our experiments, addition of rat liver S9 mix was necessary for a reliable detection of the clastogenicity of MTX. It is known that hepatic biotransformation of a chemical gives rise to a spectrum of metabolites. The exclusive formation of these metabolites are very specific to certain compounds. To date, no study has been conducted using S9 mix in mammalian cells to detect clastogenicity/cytotoxicity of MTX. However, an attempt was made to study the mutagenic effect of MTX in *Salmonella typhimurium* tester strains with and without addition of a mouse or rat liver microsomal fraction [22,23]. A study by Theiss et al. [11] clearly showed an increase in trimetrexate (structurally and functionally similar to MTX and works as an anticancer via dihydrofolate reductase pathway) genotoxicity was evidenced by chromosome aberrations in the presence of S9. Also, Bremnes et al. [24] indirectly showed that MTX undergoes biotransformation in the presence of rat-liver homogenate in vitro into 7-hydroxymethotrexate which enhances the cytotoxic effect of MTX. Based on our preliminary experiments, we conclude that MTX may be more clastogenic in the presence of S9 through conversion into its metabolites. However, the exact type of metabolite formed and its relative toxicity needs to be further investigated.

A slight reduction in NDI was observed in MTX-treated cells. This reduction can be explained by the fact that MTX inhibits synthesis of thymidylate, purines, and glycine due to its inhibition of dihydrofolate reductase in vivo [25]. Induction of micronuclei by MTX has been reported previously. Kasahara et al. [5] have shown that the frequency of micronucleated reticulocytes increased in a concentration-de-

pendent manner. The present study showed similar results with V79 cells. Further studies by Kasahara et al. [4] have shown that a single treatment of MTX increased MN slightly and multiple injections of MTX not only induced more micronuclei but also severely reduced the percentage of polychromatic erythrocytes when compared to single injection. It is possible that the reduction of DHFR activity occurred due to intracellular accumulation of MTX. It has been suggested that MTX enters bone marrow cells, binds to DHFR and completely inhibits the activity of this enzyme. The continuous inhibition of DHFR activity might cause an imbalance in the deoxyribonucleotide triphosphate (dNTP) pool due to the storage of thymidylate and purine nucleotides and, as a consequence lead to DNA lesions [26]. Another possible mechanism, especially in the case of a single concentration of MTX, may be that a decrease in the dNTP pool is supplemented by a salvage pathway. However, because insufficient dNTPs remain, DNA lesions induced by MTX genotoxicity present themselves as micronuclei. Several in vivo studies have shown that multiple doses of MTX induced a dose related increase in MN [2–5]. The multiple dose effect on the induction of MN by MTX might be explained by the intracellular accumulation of the drug resulting in an imbalance or decrease in dNTP pool resulting from enzyme inhibition.

Studies have shown that FA deficiency results in chromosomal/chromatid breaks whereas, dihydrofolate reductase pathway was a result of altered nucleotide pool. In this study, the effect of FA on the number of double strand breaks (DSBs) was not directly shown. However, the extent of MTX-induced chromosomal damage decreased when cells were posttreated with FA. Similar rescue experiments were conducted by Pinedo et al. [27] who showed competitive reversal of MTX cytotoxicity by leucovorin on mouse bone marrow cells. The leucovorin concentration required for rescue was not strictly proportional to the concentration of MTX. Toxicity of 10^{-3} M MTX was completely reversed by equimolar concentration of leucovorin, but with higher MTX concentrations, relatively more leucovorin was required. While 10^{-5} M MTX-induced toxicity was rescued by 10^{-3} M leucovorin, rescue of the toxic effect of 10^{-4} M MTX by 10^{-3} M leucovorin was not observed. It is well established that MTX is a mitotic inhibitor that arrests cell cycle in interphase and leads to prolongation of metaphase in several cell types. Prolongation of, or complete arrest in metaphase was demonstrated after the addition of three different concentrations of MTX (0.05 μg, 3.2 μg and 204 μg/ml) to Chang cells in vitro [1].

Folinic acid is widely used as a rescue agent for MTX-induced toxicity by itself and in combination with 5-fluorouracil. Recent studies suggest that a therapeutic advantage with reduced toxicity can be achieved by the administration of intensive high consentrations of MTX in malignant disease. However, folate deficiency acts synergistically with mutagens/carcinogens to magnify genetic damage [28,29].

In our study, MTX-induced damage was reversed by the addition of FA which may directly or indirectly influence the synthesis and/or repair of DNA after damage. When there is damage, a certain amount of repair occurs within the cell using the nucleotide pool present in the cell. However, excessive breaks caused by MTX may deplete the nucleotide pool leading to a deficiency of repair process. It is known that DHFR catalyses the reduction of dihydrofolate to tetrahydrofolate, a cofactor required for synthesis of thymidylate used to synthesize DNA. Inhibition of DHFR ultimately leads to the depletion of folate cofactor involved in purine biosynthesis, thus decreasing DNA synthesis and/or repair subsequently leading to cell death. The study presented here provide evidence that FA inhibits the chromosomal damage induced by methotrexate.

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