Evaluation of the Genotoxic Effects of Methoprim in Whit Mice

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Abstract:

Genotoxicity of methoprim was evaluated in white mice using cytogenetics and molecular biology markers. Micronuclei in polychromatic erythrocytes (PCEs), chromosome aberrations (CAs) in bone-marrow, and types of damage in DNA with Comet assay were scored after treatment of mice with 0.96, 1.92, 3.07 and 15.35mg/kg.bw. for five successive days. The results showed significant increase in numbers of micronuclei, types of chromosome aberration in bone- marrow cells in treated groups compared with control groups. In addition, significant differences in levels of damage in DNA of both liver and bone-marrow cells in treated groups compared with control groups. The study reveals that high doses of methoprim induce features of genotoxicity in whit mice.

The researchers recommend that extreme supervision must be applied upon uncontrolled using of methoprim and the high doses must not be used except when highly needed.

تقدير التأثيرات السمية الوراثية للعقار ميثوبريم في الفئران البيض

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المستخلص:

تم تقدير السمية الوراثية للعقار ميثوبريم في الفئران البيض باستخدام مؤشرات الوراثة الخلوية والبيولوجي الجزيئي. وقد تم تسجيل النوى الصغيرة في كريات الدم الحمر غير الناضجة في نقي العظم، وكذلك الشذوذ الكروموسومي في خلايا نقي العظم فضلا عن أنواع

التلف في المادة الوراثية (الدنا) باستخدام تقنية تقدير الهالة بعد معاملة الفئران البيض بجرعات مختلفة من العقار ميثوبريم مساوية للجرعة العلاجية 0.96 ملغم\كغم وزن جسم وضعف الجرعة العلاجية 1.92ملغم\كغم وزن جسم، إضافة إلى جرعة تساوي عشر قيمة الجرعة المميتة النصفية 3.07 ملغم\كغم وزن جسم وأخرى تساوي نصف قيمة الجرعة المميتة النصفية 15.35 ملغم\كغم وزن جسم حسب الترتيب. أظهرت نتائج الدراسة وجود ارتفاع معنوي في أعداد النوى الصغيرة وأنواع الشذوذ الكروموسومي في خلايا نقي العظم في المجاميع المعاملة مقارنة مع مجموعات السيطرة. فضلا عن وجود فروق معنوية في مستويات التلف في الدنا لكل من خلايا الكبد وخلايا نقي العظم للمجاميع المعاملة مقارنة مع مجاميع السيطرة. تكشف الدراسة عن أن الجرعات العالية من العقار ميثوبريم تؤدي الى حث مظاهر السمية الوراثية في الفئران البيض.

ويوصي الباحثون بفرض رقابة مشددة على الاستخدام غير المنظم للعقار وعدم استخدام الجرعات العالية إلا عند الضرورات القصوى.

Introduction

Trimethoprim belongs to a family of synthetic 2,4 diaminopyrimidines, whichhave a potent microbial activity against wide variety of bacterial species (1). Trimethoprim is a folic acid antagonist by inhibition of dihydrofolate reductase which catalyses the conversion of dihyrofolate to tetrahydrofolic acid. So trimethoprim affects the biosynthesis of DNA (2,3). Trimethoprim has been used clinically in the treatment of bacterial infections, particularly the common urinary tract infection (4). Deficiency of folic acid may have important role in carcinogenesis (5). The mechanism of promoting carcinogenesis may be through genetic damage associated with folic acid deficiency (6).

Results obtained from reports about genotoxicity of trimethoprim are controversial. Positive and negative effects were reported in the same test system with both bacterial and mammalian cells (7,8). The aim of this work is to provide additional information about the effects of trimethoprim in mammalian cells trough *in vivo* study.

Material and Methods

Animals:

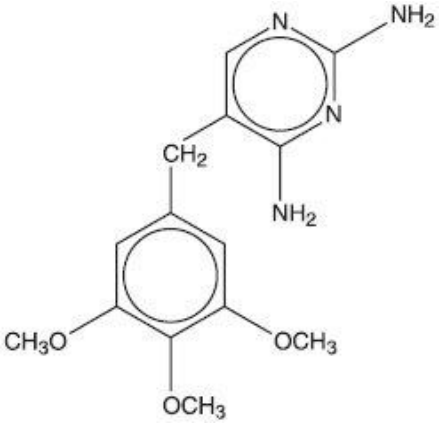
White Swiss mice were used in all experiments. The animals were obtained from a closed random bred colony in the State company for Drugs Industry and medical appliances, Samarra- Iraq (SDI),

maintained under controlled conditions of temperature and humidity and received food and water ad labium.

Test chemical:

Trimethoprim is 5 - [(3,4,5-trimeth-oxyphenyl)methyl] – 2 , 4 - pyrimidinediamine molecular weight 290.32) was obtained from (SDI).

Formula: Structural



(Baccnari, 1995)

Treatments:

For bone –marrow the experimental design is based on the study of the effect of trimethoprim by oral gavages for one week, 6 groups of five mice with different dose levels 15.35, 3.70 mg/kg b.wt. which stand for half value of LD_{50} and 1/10 LD_{50} respectively and 0.96 and 1.92 mg/kg b.wt which represent curing and double curing dose respectively. Cyclophosphamide was used as positive control while another group without any treatment used it as negative control. Mice were sacrificed at 22h after treatments.

Micronucleus Test:

Bone-marrow preparations were made and stained according to the method described in schmid (9). The presence of micronucleated polychromatic erythrocytes was visually scored (at least 1000 per mouse)by optical microscopy using a Leica bright field microscope. Cells were considered to be micronucleated when they contained neatly defined chromatin corpuscles with diameter of less than one-

third the diameter of the cell nucleus and stained equal or denser than the nucleus of the cell from which the micronucleated cell had developed (9). The experimental and control micronucleus frequency for each specimen within and between different mice groups were compared using the paired two-tailed t-test. Chromosome aberration

Chromosomes from bone- marrow cells were prepared following the method of Yoshida and Amano (10) 100 well spread metaphases were analyzed per each animal for scoring different types of aberration.

Comet Assay:

For single cell gel electrophoresis (comet assay) was used the method described by Singh (11) with some modifications. Samples of liver and bone marrow were minced with mincing solution prepared from HBSS and EDTA. 10µL of cell suspension was mixed with 0.5% of low melting point agarose (LMP) (sigma) and spread on fully frosted microscope slides pre coated with 0.6% normal melting point agarose (NMP). The slides were immersed in a jar containing cold lysing solution (2.5 NaCl, 100mM EDTA, 10 mM Tris, 1% triton X-100 and 10% DMSO were added fresh) at 4ºC over night. The cells were exposed to alkali solution (300 mM NaOH and 1 mM Na2EDTA) for 20 minutes to allow DNA unwind and express the alkal-labile sites. The electrophoresis was applied in an alkaline buffer (300 mM NaOH and 1 mM Na2EDTA) for 20 minutes, using electric current of 25 V and 300 mA. After the electrophoresis, the slides were neutralized with 0.4 M Tris buffer (pH 7.5), stained with 200 µL ethidium bromide (2µL/mL), and analyzed using Leitz Orthoplan fluorescence microscope equipped with a 515-560 nm excitation filter. The images of 100 randomly selected cells were analyzed from each sample. Each nucleoid was assigned to one of the specific damage categories(0-4) based on the tail size relative to the head general appearance, modified from Avishai *et al* (12), and previously reported (Urbina-Cano *et al* (14) as follows: 0 = nucleoids without tail or undamaged, 1 = tail length equal to or shorter than nucleus head diameter, 2 = tail length 1.1 to 3.5 times more than the head diameter, 3 = tail length more than 3.5 times the head diameter; and 4 = absence of head (all DNA migrates to the tail).

Statistical Analysis:

The significance of the experiment from control data was calculated using two-tailed t-test. Significant values P≤ 0.05 and highly significant P≤ 0.01.

Results:

Micronucleus Test

Table 1 shows the number of micronuclei in polychromatic erythrocytes (MNPCEs) in mice bone- marrow 22hr after successive oral administration of methoprim 5 days. The doses used were 0.96, 1.92, 3.07 and 15.35 mg/kg.bw. and the control groups.

Table 1. Number of micronuclei in polychromatic erythrocytes (MNPCEs) in mice bone- marrow 22hr after successive oral administration of methoprim 5 days.

T\D mg/kg.bw.	No. of animals	No. of examined cells	MNPCEs Mean ± S.E.
D.W.	5	1000	2.8 ± 1.31
CP 20	5	1000	34.20± 3.87**
Trimethoprim			
0.96	5	1000	4.00 ± 1.70
1.92	5	1000	5.20 ± 0.86*
3.07	5	1000	14.00 ± 1.41**
15.35	5	1000	21.60 ± 1.80**

*** Significant at p≥ 0.05. ** Significant at p≥ 0.01 (t-test)**

Table 2 :shows percentage of polychromatic erythrocytes (PCEs) in mice bone- marrow 22hr after successive oral administration of methoprim 5 days. The doses used were 0.96, 1.92, 3.07 and 15.35 mg/kg.bw. and the control groups.

Table 2. Percentage of polychromatic erythrocytes (PCEs) in mice bone- marrow 22hr after successive oral administration of methoprim 5 days.

T\D mg/kg.bw.	No. of animals	No. of examined cells	PCEs N0	%
D.W.	5	1000	764	76.4
CP 20	5	1000	2125	21.25
Trimethoprim				
0.96	5	1000	942	9.42
1.92	5	1000	1022	10.22
3.07	5	1000	1510	15.10
15.35	5	1000	`1771	17.71

Bone-Marrow Chromosomes:

Table 3 and 4 show chromosomal aberrations in mice bone- marrow cells 22hr after successive oral administration of methoprim 5 days. The doses used were 0.96, 1.92, 3.07 and 15.35 mg/kg.bw. and the control groups.

Table 3. Total chromosome aberrations in mice bone-marrow cells after successive oral administration of methoprim 5 days with 0.96, 1.92, 3.07 and 15.35 mg/kg.bw.

T\D mg/kg.bw.	No. of animals	No. of examined cells	Total Chromosome aberrations Mean ± S.E.
D.W.	5	1000	3.20± 1.11
CP 20	5	1000	32.20± 4.92**
methoprim			
0.96	5	1000	6.60 ± 186*
1.92	5	1000	14.00 ± 1.33**
3.07	5	1000	18.80 ± 3.61**
15.35	5	1000	23.60 ± 3.63**

*** Significant at p≥ 0.05. ** Significant at p≥ 0.01 (t-test)**

T able 4. Types of chromosome aberrations in mice bone-marrow cells after successive oral administration of methoprim 5 days with 0.96, 1.92, 3.07 and 15.35 mg/kg.bw.

T/D	gap		break		frag		del		del+ frag		trans		aneuploid		euoploid	
mg/kg.bw	No	%	No	%	No	%	No	%	No	%	No	%	No	%	No	%
D.w.	-	-	2	0.4	5	1	3	0.6	3	0.6	1	0.2	1	0.2	1	0.2
CP 20	11	2.2	40	8	26	5.2	25	5	24	4.8	10	2	8	1.8	42	8.4
methoprim																
0.96	4	0.8	17	3.4	13	2.6	11	2.2	14	2.8	3	0.6	2	0.4	18	3.2
1.92	8	1.8	12	2.4	20	4	19	3.8	21	4.2	8	1.8	6	1.2	25	5
3.07	1	0.2	2	0.4	6	1.2	5	1	5	1	1	0.2	1	0.2	11	2.2
15.35	6	1.2	9	1.8	17	3.4	16	3.2	18	2.6	6	1.2	3	0.6	20	4

Table 5 and 6 show the number of cells with damaged DNA in liver and bone- marrow of mice 22hr after successive oral administration of methoprim 5 days. The doses used were 1.92 and 15.35 mg/kg.bw. and the control groups.

Table 5. Cells with damaged DNA in the liver of mice after oral administration of different doses of methoprim for five successive days

T\D mg/kg.bw.	No. of animals	No. of examined cells	Cells with damage M ± S.E.
D.w.	5	500	5.60 ± 0.40
CP	5	500	3.20** 40.0 ±
Methoprim			
1.92	500	500	20.0 ± 0.55**
15.35	500	500	37.40 ± 0.77**

** Significant at p≥0.01 (t-test).

Table 6. Cells with damaged DNA in bone-marrow of mice after oral administration of different doses of methoprim for five successive days

T\D mg/kg.bw.	No. of animals	No. of examined cells	Cells with damage M ± S.E.
D.w.	5	500	4.20 ± 0.38
CP	5	500	0.97** 36.80 ±
Methoprim			
1.92	500	500	17.80 ± 0.86**
15.35	500	500	24.60 ± 0.87**

Significant at p≥0.01 (t-test)

Table 7 and 8 show Percentage of cells with damaged DNA in the liver and bone marrow of mice after oral administration of different doses of methoprim for five successive days.

Table 7. Percentage of cells with damaged DNA in the liver of mice after oral administration of different doses of methoprim for five successive days

T\D mg/kg.bw.	No. of animals	No. of examined cells	Cells with damage	
			No.	%
D.w.	5	500	26	5.6
CP	5	500	191	38.2
Methoprim				
1.92	500	500	100	20
15.35	500	500	133	26.6

Table 8 Percentage of cells with damaged DNA in the liver of mice after oral administration of different doses of methoprim for five successive days

T\D mg/kg.bw.	No. of animals	No. of examined cells	Cells with damage	
			No.	%
D.w.	5	500	21	4.2
CP	5	500	179	35.8
Methoprim				
1.92	500	500	89	17.8
15.35	500	500	123	24.6

Discussion:

The antimicrobial drug, methoprim, is an antifolic agent and it has been reported to affect DNA synthesis (2,3). This drug is a potent inhibitor of the enzyme, dihydrofolate reductase, which is responsible for the reduction of the enzyme, dihydrofolate to tetrahydrofolate that promotes biosynthesis of DNA (16). Thus, DNA synthesis inhibition has been suggested to be an indirect mechanism of genetic damage (17,18). The probable mechanism of cytogenetic damage induced by this antifolic agent might be attributed to misincorporation of uracil into DNA and has postulated that, during repair, niks are formed that could lead to chromosome breaks (19). Further, folate deficiency also leads to hypomethalation of DNA. This hypomethaltion may increase the susceptibility of DNA to nuclease attack and provide another mechanism for the production of DNA strand breaks (5). Folate deficiency is known to induce chromosomal aberrations (CA) and frafile sit expression, and can cause dramatic increases in the frequency of micronucleated erythrocytes in Crohn´s disease patients (20). On the other hand, it has been reported that the use of low folic acid culture medium increases the frequency of MN and CA in human lymphocytes cultures (18). Furthermore, methoprim was positive for induction of CA and sister-chromatid exchanges (SCE) frequencies in Chinese hamster ovary (CHO) cells treated at doses that caused cell cycle delay with and without metabolic activation (20,21). In addition, a significant increase in the MN and SCE frequencies has been reported in human lymphocytes treated with different concentrations of trimethoprim (8). This would support that this antibacterial drug inducing primary DNA damage. Thus, the genotoxicity of methoprim is confirmed by the results obtained in the comet assay test in our current study. Due to its sensitivity for measuring DNA damage at the individual cell level and its potential application to virtually all eukaryotic cell types, the Comet assay has been adopted as a very useful short term test in genotoxicity studies (23). In conclusion, we suggest that our results indicate that methoprim appears to have a significant clastogenic activity, which is in agreement with the data obtained in the in vivo micronucleus test, metaphase chromosome test and Comet assay test. This study suggests the usefulness of concurrent assessment of cytogenetic damage and DNA alterations.

(1) The mammalian *in vivo* micronucleus test is used for the detection of damage induced by the test substance to the chromosomes or the mitotic apparatus of erythroblasts by analysis of erythrocytes as sampled in bone marrow and/or peripheral blood cells of animals, usually rodents.

(2) The purpose of the micronucleus test is to identify substances that cause cytogenetic damage which results in the formation of micronuclei containing lagging chromosome fragments or whole chromosomes.

(3) When a bone marrow erythroblast develops into a polychromatic erythrocyte, the main nucleus is extruded; any micronucleus that has been formed may remain behind in the otherwise nucleated cytoplasm. visualization of micronuclei is facilitated in these cells because they lack a main nucleus. An increase in the frequency of micronucleated polychromatic erythrocytes in treated animals is an indication of induced chromosome damage (15).

Hence, it is therefore advisable to be careful of the potential hazards of this antibacterial drug as they may become capable of attacking the genetic material.

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