A Comparative Study on the Genotoxic Effect of Pyrimethamine in Bone Marrow and Spermatogonial Mice Cells

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Pyrimethamine is an antimalarial agent widely used in clinical therapy. We aimed to compare its mutagenic potential in mammalian spermatogonial and bone marrow cells. For studying chromosomal aberrations mice were treated acutely (single treatment) with 4 dose levels of pyrimethamine (5, 10, 20 and 40 mg/kg). Pyrimethamine was found to produce a significant increase in structural chromosomal aberrations after acute treatment in bone marrow cells of mice ($p < 0.001$). It also induced chromosome abnormalities in spermatogonial cells ($p < 0.05$) at the highest dose.

Key words: Pyrimethamine, Spermatogonial Mitosis, Chromosome Aberrations

Introduction

Pyrimethamine [2,4-diamino-5-(*p*-chlorophenyl)-6-ethylpyrimidine, PYR], a folic acid antagonist, is used as an antiprotozoal agent in humans and animals for the prevention and treatment of malaria, toxoplasmosis, and leucocytozoonosis (Ono *et al*., 1997) and recently against human immunodeficiency virus (HIV)-associated *Pneumocytis carinii pneumonia* (Vijayalaxmi and Vishalakshi, 2000). It is applied in a lot of countries and exists in the list of Basic Drugs which is formed by the Community of Turkish Physicians since 1995.

An embryo-toxic effect of PYR, especially teratogenicity, has been reported in different species from laboratory animals to humans (Sullivan and Takacs, 1971; Harpey *et al.*, 1983; Hayama and Kokue, 1985; Tsunematsu *et al*., 1990). Its genotoxicity data are discrepant. Pyrimethamine is not mutagenic in *Salmonella typhimurium, E. coli*, and *Bacillus subtilis* (Ohta *et al.*, 1980), but is clastogenic in fungal test systems (Albertini, 1990) and cultured mammalian cells (Antoccia *et al*., 1991; Egeli and Erdoğan, 1991; Ono and Yoshimura, 1996) and male mouse germ cells (Aydemir and Bilaloğlu, 1996; Egeli *et al*., 1999). It was found that orally administered pyrimethamine induces micronuclei in rat bone marrow cells (Ono *et al.*, 1997) and DNA single-strand breaks in liver cells but not in bone marrow cells in the mouse comet assay (Sasaki *et al*., 1997).

The mutagenic/carcinogenic effects of chemical agents have been determined by *in vivo* and *in vitro* short-term screening tests such as chromosome aberration, sister chromatid exchange, micronuclei in somatic and germ cells. Chromosome aberrations in spermatogonia have been often used to estimate the genetic damage induced in animals exposed to mutagenic agents (Adler, 1974, 1982). Analyses of spermatogonial mitoses will be restricted, particularly for the mouse, with its uniform karyotype-to chromatid-type aberrations at first posttreatment mitoses (Adler, 1982).

In the present study, we compared the effect of PYR on mitotic cells in spermatogonia and bone marrow, in order to contribute to the elucidation of the mutagenic potential of PYR, since it is widely used in Turkey and worldwide.

Materials and Methods

Laboratory animals

Male Swiss albino mice were obtained from the Test Animals Breeding Center of Uludag University. They were 8- to 10-week-old and housed in plastic cages with a bedding of wood shavings. Animals weighed 25–30 g. They were fed with fresh standard pellet and given tap water *ad libitum*. All mice were kept under constant environmental conditions with a 12 h/12 h light/dark cycle. This study has been approved by the Ethical Commit-

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Drugs

PYR was purchased from Sigma Chemical Company (St. Louis, MO; Lot No. 88F0320). The purity of this compound was > 99%. Ethylmethane sulphonate (EMS) was also purchased from Sigma Chemical Company. PYR was dissolved in 50% ethanol. Drug solutions were prepared within 1 h prior to injection and used within 24 h to avoid drug degradation.

The intraperitoneal (i.p.) route of application was used in all experiments. Four doses of the drug, 5, 10, 20, and 40 mg/kg, were used for the experiments. The maximum tolerated dose in mice (i.p.) is 50 mg/kg (Ono and Yoshimura, 1996). The volume of dose applications was 0.20 ml. The mice assigned to the two negative control groups received 0.20 ml of 50% ethanol, distilled water and, as a positive control, 150 mg/kg of EMS solved in distilled water separately.

Bone marrow metaphase analysis

For bone marrow metaphase analysis, each dose and week group, including negative controls, consisted of 5 male mice. 2 h before sacrification, mice were injected with 4 mg/kg b. w. colchicine.

Bone marrow metaphase slides were prepared according to Adler (1982). The slides were stained in 5% Giemsa for 10 min. The structural chromosome aberrations (CAs) were scored in 50 metaphases for each animal. The scoring and classification of aberrations were done as described by Preston *et al*. (1987). Gaps and pulverizations were both included and excluded from the total number of CAs.

Analysis of spermatogonial mitoses

For the spermatogonial metaphase chromosomal aberration study, at 22 h post treatment, 5 mice from each of the dose and week group were colchicinized intraperitoneally at the rate of 4 mg/kg body weight. After 2 h mice were sacrificed with cervical dislocation, the testes were dissected and washed thoroughly in 2% sodium citrate. Then tunica was removed and the tubules were transferred in 1% sodium citrate and left for about 20 min at room temperature. The fixed tubules were centrifuged and slides for spermatogonial metaphase chromosome preparations were made following the flame-drying (Adler, 1982). Slides were stained with 5% Giemsa solution for 10 min. About 50 well-spread spermatogonial metaphases were scanned from each animal. Aberrant spermatogonial metaphases with chromatid/chromosome gaps, chromatid/chromosome breaks, fragments etc. were recorded. Percentages of aberrant metaphases (inclusive gaps) and aberrations excluding gaps per 100 metaphases were calculated for each animal.

Statistical analysis

The mean ± SD data were calculated for each group of mice. All data were analyzed by one-way (ANOVA) analysis of variance. Differences between the control and experimental groups were analyzed by means of the Tukey HSD test. All statistical analyses were done by the SPSS 11.5 Package Programme for Windows. The limit of statistical significance was set at $p = 0.05$.

Results and Discussion

The results of bone marrow and spermatogonial metaphase chromosome analyses are showed in Table I and Fig. 1. The bone marrow metaphase chromosome aberrations both including and excluding gaps and pulverizations were significantly increased at 10, 20 and 40 mg/kg of pyrimethamine [for the total aberration, (TA), $F = 20.94$; for the TA excluding gaps and pulverizations, $F = 16.26$; $p < 0.001$].

The spermatogonial metaphase chromosome aberrations excluding and including gaps were not

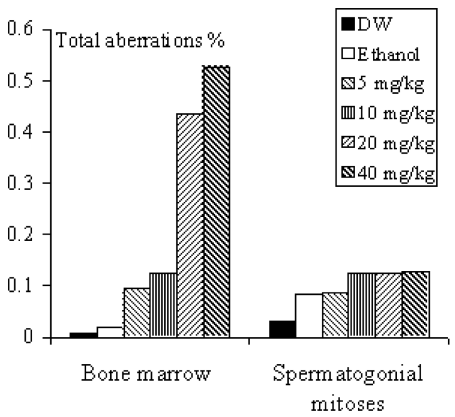

Fig. 1. Incidence of chromosome aberrations in bone marrow and spermatogonial cells of mice following an administration of four doses of pyrimethamine.

Table I. Frequencies of bone marrow and spermatogonial metaphases of male mice treated with pyrimethamine[a].

Dose [mg/kg]	No of animals	Total metaphase number	Bone marrow metaphases CAs		Spermatogonial metaphases CAs	
			Total aberrations (%) (excl. G + P)[b]	Total aberrations (%)	Total aberrations (%) (excl. G)[c]	Total aberrations (%)
DW	5	250	0.004 ± 0.011	0.008 ± 0.010	0.012 ± 0.011	0.032 ± 0.011
Ethanol	5	250	0.008 ± 0.017	0.020 ± 0.020	0.048 ± 0.030	0.084 ± 0.035
5	5	250	0.080 ± 0.042	0.096 ± 0.043	0.068 ± 0.018	0.088 ± 0.030
10	5	250	0.228 ± 0.118**	0.124 ± 0.126**	0.072 ± 0.030	0.124 ± 0.021
20	5	250	0.420 ± 0.131**	0.436 ± 0.121**	0.092 ± 0.022	0.124 ± 0.032
40	5	250	0.492 ± 0.152**	0.528 ± 0.150**	0.110 ± 0.020*	0.128 ± 0.022*
EMS	5	250	0.065 ± 0.043	0.095 ± 0.054	0.065 ± 0.015	0.080 ± 0.031

[a] All dose groups were only compared with ethanol as control.
[b] Excluding gaps and pulverizations.
[c] Excluding gaps.
* $p < 0.05$; ** $p < 0.001$.

significantly increased by the pyrimethamine treatment except for the 40 mg/kg treatment ($p < 0.05$; $F = 3.442$). Both in bone marrow chromosome aberration and in spermatogonial metaphase chromosome tests the most common aberration types were chromatid breaks and acentric fragments.

The bone marrow chromosome aberration test, regardless of the rationale for their conduct, can serve as an effective gateway to germ cell mutagenicity tests. There is no apparent reason to conduct germ cell mutagenicity tests with chemicals that do not give positive test results in the bone marrow (Shelby, 1996). In a previous study, PYR induced both structural CAs and micronuclei in mice bone marrow for long-term exposure (Tunca *et al.*, 2002) and it also induced a strong clastogenic effect in mouse bone marrow in the present study. PYR induced CAs only at the highest dose in spermatogonial cells of mice and it produced chromatid-type breaks and acentric fragments both in bone marrow and spermatogonial cells. Induced chromosome breakage in spermatogonia causing cell killing may result in temporary sterility and changes in the cell population of the tubules in testes (Vega *et al*., 1988; Ogawa *et al*., 2004; Sun *et al*., 2006).

The mechanism by which PYR induces chromosomal aberrations is elucidated (Sutherland *et al*., 1985; Fasumon and Uwaifo, 1989). PYR specifically binds to DHFR, an essential co-enzyme in the biosynthesis of the nucleotide thymidine (Sutherland *et al.*, 1985). Consequently, the inhibition of thymidine formation leads to the inhibition of DNA synthesis and folic acid deficiency, resulting in damages of the chromosomes (Aydemir and Bilaloğlu, 1996). It is also known that, as a result of folic acid absence, a decrease may occur in the deoxynucleotide triphosphate (dNTP) pool, and thus DNA synthesis may be affected (Liu *et al.*, 1989; Tucker *et al*., 1993; Aydemir and Bilaloğlu, 1996; Vijayalaxmi and Vishalakshi, 2000).

The possible causes of lower CA induction by PYR in spermatogonia than in bone marrow may be due to some properties of spermatogonial cells. It is well established that aberration production by chemical mutagens depends on DNA synthesis, and the majority of the cells in spermatogonial stem cells will be in less sensitive stages like G_1 and G_2 because the relative duration of the S phase is rather short (Adler, 1982). Thus the affected cell population may be lower than in bone marrow cells. Only a few chemical mutagens induce gene mutations in spermatogonial stem cells. Most chemical mutagens are effective in the differentiating germ cells, namely spermatocytes and different stages of spermatids (Russell, 1991; Adler, 2000). Several studies have suggested that PYR affects differentiating germ cells in mouse spermatogenesis (Cosentino *et al.*, 1990; Awoniyi *et al.*, 1993; Aydemir and Bilaloğlu, 1996; Egeli *et al*., 1999).

As a result, we showed that PYR has increased the number of bone marrow CAs for all doses and that of the spermatogonial CAs for the highest dose, significantly. It is interesting to know which

cell type(s) of each organ is/are affected on insult of various DNA-damaging chemicals. Almost all frequency distributions of DNA migration in comet presented in the study of Sasaki *et al.* (1997) showed heterogeneous distributions after PYR treatment, which may indicate differences in the sensitivity among the cell types. Our results suggested that bone marrow cells are more sensitive than spermatogonial cells in mice. In this respect, our findings are in good agreement with earlier studies.

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