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RESEARCH ARTICLE

Protective Effect of Vitamin A on Methotrexate Induced Micronuclei

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ABSTRACT

Methotrexate is an antineopalstic agent widely used in low dose to treat patients with rheumatoid arthritis. It is known to induce micronuclei at multiple doses in rats. The present study investigates the effect of vitamin A on methotrexate-induced micronuclei in rat bone marrow erythrocytes. Male wistar rats were (n=5) injected with 0, 8, 16 and 20mg/kg methotrexate (single i.p dose). A group of rats received 5000 IU of vitamin A (i.p) for 4 successive days. Another group of rats received a combination of vitamin A (5000 IU of vitamin A for 4 successive days) and single dose of methotrexate (20mg/kg dose). Samples were collected at 24 hours after last methotrexate exposure in to 5% bovine albumin. Smears were obtained and stained with May-Grunwald and Giemsa. Thousand polychromatic erythrocytes were counted per animal for the presence of micronuclei and PCE% was also calculated. The percentage of micronuclei increased with increase in the dose of methotrexate (percentage of PCE decreased with increase in the dose of methotrexate). Combination of methotrexate and vitamin A therapy showed a significant decrease in micronuclei percentage and an increase in PCE% compared to rats treated with methotrexate alone. Hence the results of this study suggest that vitamin A protects against methotrexate induced genetic damage.

Keywords: *Methotrexate, Micronuclei and Vitamin A*

Methotrexate, an inhibitor of reductase of dihydrofolate is known to induce micronuclei at multiple dosing [1-2]. Methotrexate is mainstay treatment for childhood acute lymphoblastic leukemia with meningeal infiltration [3] and also for many other forms of carcinoma. Methotrexate has also become an important therapeutic alternative in the treatment of severe psoriasis [4]. Methotrexate has anti-inflammatory properties. It reduces lymphocyte proliferation, rheumatoid factor production, and leukocyte interaction. It is used intermittently at low dosage to induce remission in refractory rheumatoid arthritis [5]. Methotrexate is also used in management of ectopic pregnancy [6-7]. Methotrexate induced chromosomal aberrations in these conditions is of great concern.

Methotrexate restricts the synthesis of thymidilate and purine nucleotides by inhibiting dihydrofolate reductase and to a lesser extent, thymidilate synthatase. In cells treated with methotrexate, a progressive accumulation of strand break in mature DNA (post-replicated DNA) was detected by Li and Kaminskas [8]. They postulated that the strand break arises from spontaneous and normally repaired DNA lesions that are not repaired due to shortage of dTTP and purine nucleotides. Cytogenetic effect of methotrexate has been studied in mouse ascites tumors [9], in cultured potorous cells [10] and in human cells in vivo [11]. Methotrexate was found to be a clastogenic agent in tumor cells and in cultured mammalian cells and a micronuclei study performed by CSGMT/JEMS-MMS [2] for various chemicals revealed that only methotrexate shown positive response in single dose (16mg/kg). In single dose, micronuclei were positive only 48 hours after the treatment. The effect was clear when mice were dosed 2 or 4 times.

The objective of the present study is to evaluate the genotoxic effect of methotrexate in single dosing after 24hr sampling time. There are many reports [12-13] suggesting a role for vitamin A in protecting against micronuclei induction. Hence this study is also planned to see the effect of vitamin A on methotrexate-induced micronuclei.

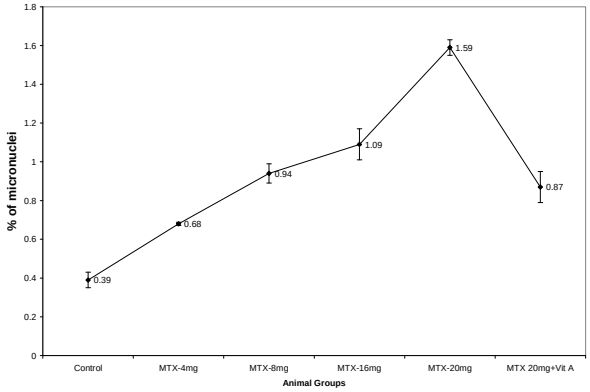

Fig 1. The dose response relationship for methotrexate-induced micronuclei in rats (single dose and 24 h sampling time) p=0.0079, when MTX-20mg group compared with MTX 20mg+Vitamin A, Error bar indicates ±SE.

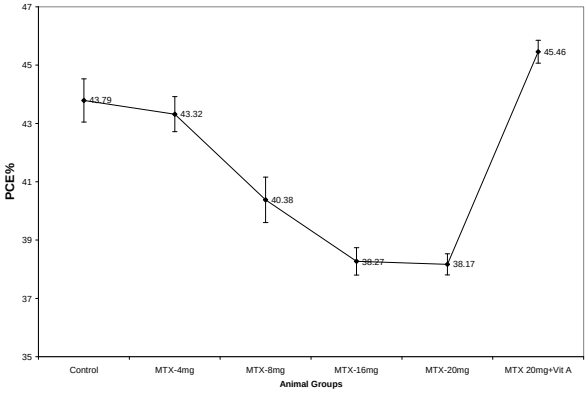

Fig 2. The dose response relationship for methotrexate-induced PCE% in rats(single dose & 24h sampling time) p=0.0079, when MTX-20mg group compared with MTX 20mg+Vitamin A group, Error bar indicates ±SE.

METHODS

Animals

Four–month-old male Wistar rats bred in house were used in the present study. Animals were maintained under controlled conditions of light (10h: light: 14h:

dark), temperature (22±3°C), and humidity (approximately 50±10%) in an air-conditioned animal house. All rats were maintained on the standard rat food and water ad libitum. The average weight of the rats was 226 g. All the experimental procedures were approved by the Institutional ethics committee.

Chemicals

Methotrexate and vitamin A were obtained from 'Biochem Pharmaceutical Industries' (Ahmedabad, India). Bovine albumin (sigma grade, 96-99% B No.140), May-Gruenwald powder and Giemsa powder were obtained from Romoli Co., (Mumbai). All other chemicals and reagents were of HPLC or analytical grade (Sigma, St. Louis, Mo.).

Animal Groups

Group 1 was control and received saline injection. Group 2, 3 and 4 were rats treated with 8, 16 and 20mg/kg-body weight of methotrexate respectively. Group 5 rats received combination of vitamin A (5000 IU for 4 successive days) and 20mg/kg body weight methotrexate (single dose). All drugs were administrated by i.p. injection. Five rats were randomly assigned to each treatment group.

Micronucleus test

In the present study Schmid's [14] standard procedure was followed however with slight modification. Instead of foetal calf serum, 5% bovine albumin was used as suspending medium to collect the bone marrow [15]. The rats were sacrificed 24h after last methotrexate treatment and femurs were trimmed and a blunt needle was pushed to pierce the marrow cavity. The marrow was flushed through a syringe with 5% bovine albumin to obtain a fine suspension. The suspension was centrifuged at 1000 rpm for 8 to 10 minutes. The supernatant was discarded and half a drop of fresh suspending medium was added and mixed thoroughly by a Pasteur pipette. A small drop of suspension was placed on one end of the slide and a smear was prepared (3-4 slides/animals). Slides were cooled and air dried overnight and fixed by methanol for 5 minutes, dried and stained with May-Grunwald and then with combination of May-Grunwald and phosphate buffer at pH 6.8 for the proper color differentiation of polychromatic erythrocytes (PSE's) and normochromatic erythrocytes (NCE's). Finally slides are stained with Geimsa and buffer at pH 6.8 for micronuclei staining. After washing with distilled water and buffer good slides were dried and mounted. Two thousand PCE's were screened/animal and micronucleated PSE's (MNPCE's) were recorded. Consequently identified normochromatic erythrocytes (NCE's) and micronucleated NCE's (MNNCE's) were also recorded. The percentage of MNPCE and PCE were calculated for each animal.

Statistical analysis

Non-parametric Mann-Whitney test (unpaired two tailed test) was employed for statistical analysis. P values < 0.05 were considered as significant.

RESULTS

Methotrexate induced significant number of micronuclei in all the doses investigated (Fig 1) when compared to the control group. It was observed that, micronuclei induction was dose dependent. At 8mg/kg it was 0.94% and at 20mg/kg it was 1.59% showing a significant increase (Fig 1). The percentage of PCE differed significantly between methotrexate treated (8 and 16 mg/kg dose) and control. The percentage of PCE declined significantly with increase in the dose of methotrexate (Fig 2).

The micronuclei percentage differed significantly (p=0.0079) between group 4 (20mg/kg dose of methotrexate) and group 5 (combination of methotrexate and vitamin A). The PCE percentage was increased signifi-

cantly (p=0.0079) from 38.17% to 45.46% (Fig 2) while comparing these groups.

DISCUSSION

The positive results with methotrexate in the bone marrow micronucleus test indicated that the chemical is capable of inducing cytogenetic effect. In the present study, maximum frequency of micronuclei was observed at 20mg/kg dose (Fig 1). The micronuclei frequency in NCE was high; this may be due to the maturation of micro nucleated PCE to NCE. The percentage of PCE declined significantly at higher doses of methotrexate, suggesting the percentage of PCE at higher doses indicates the extent to which the erythrocyte production was suppressed. Thus it gives an idea about the toxic effects of the chemical. The decreased PCE% also indicates the extent of cell death (Fig 2). Formation of micronuclei indicates that considerable amount of genetic information is no longer available to the cell; it is reasonable to assume that induction of micronuclei could pave the way for cell death. Midander and Revesz [16] who worked on Chinese hamster cells after radiation exposure supported this hypothesis. The cell death is a consequence of chromosome breakage in the various nucleated cells; this creates a void in the bone marrow canal. The empty space may then be filled with blood. Freshly produced erythrocytes remain in the marrow after maturation instead of entering the peripheral blood stream [17].

People with low or high micronuclei yield show a low or high frequency of chromosome aberration [18]. Hence micronucleus assay is often used to detect the frequency of chromosomal aberrations. The drug induced chromosomal aberration or mat distribution of chromosomes can lead to certain genetic diseases [19]. These chromosomal changes can also involve in causation of tumors [20]. According to this hypothesis, a patient with high score of micronuclei is prone to chromosome breakage that it can lead into a tumor. Stich et al [21] presented evidence that micronuclei may serve as predictors of carcinogenic risk. He demonstrated that, after exposure to certain chemicals, tissues that are at elevated risk for cancer show markedly enhanced number of micronuclei.

Methotrexate penetrates the bone marrow cells and binds with ‘dihydrofolate reductase’ enzyme. Then it completely inhibits the activity of this enzyme. The continuous inhibition of DHFR activity might cause an imbalance in the dNTP pools due to shortage of thymidylate and purine nucleotides and, as a consequence, lead to DNA lesions [8]. Complete inhibition of DHFR might produce severe suppression of PCE generation from erythroblasts in bone marrow. In the present study, though methotrexate showed micronuclei induction, it was high only with higher dose (20mg/kg). Hence, it reinforces the need to adjust the accepted dose.

The protective effect of vitamin A against genotoxicity and cancer chemoprevention was studied by many workers. Slamenova et al [12] observed that dietary intake of vitamin A has reduced micronuclei induction in rat hepatocytes against different carcinogens. Similar results were reported by Alaovi-Jamali et al [22]. Stich et al [13, 23, 24] observed decreased micronuclei frequency in buccal mucosa of betel nut/tobacco chewers.

Vitamin A through its antioxidant function, immunomodulatory effects and control of intercellular messages via gap junctions blocks the carcinogenic process [25]. In the present study rats treated with a combination of methotrexate and vitamin A had significantly reduced micronuclei frequency when compared to methotrexate (20mg/kg) alone treated rats. The percentage of PCE was also increased in rats treated with combination vitamin A and methotrexate. Hence our study confirms that vitamin A minimizes the genotoxic and cytotoxic effects of methotrexate. One has to bear in mind the inherent dangers while prescribing methotraxate, especially in non-cancerous condition like rheumatoid arthritis and resistant psoriasis. Hence it requires an acceptable dose of methotrexate, which shows a lower level of micronuclei frequency.

REFERENCES

1. Yoshinori Kasahora, Yasuharu Nakai and Daishiro Miura. Mechanism of induction of micronuclei and chromosome aberration of micronuclei and chromosome aberrations in mouse bone marrow by multiple treatments of methotrexate Mutat Res 1992; 280: 117-128.
2. CSGMT/JEMS-MMS. Collaborative study group for the micronucleus test. Single versus multiple dosing in the micronucleus test: the summary of the fourth collaborative study by CSGMT/JEMS-MMS. Mutation Research 1990; 234:205-222.
3. Toyoda Y, Manabe A, Tsuchida M, Handa R, Ikutta K, Okinoto Y. Six months maintenance chemotherapy after intensified treatment for acute lymphoblastic leukemia of childhood. J of Clinical Oncol. 18(07): 1508-1516.
4. Clark CM, Kirby B, Morris AD, Davison S, Zaki I, Emerson R, Saihan EM, Chalmers RJ, Barker JN, Allen BR, Griffiths CZ et al. Combination treatment with methotrexate and cyclosporine for severe recalcitryant psoriasis. British journal of Dormatol 1999; 141(2): 279-282.
5. Hoffmeister RT. Methotrexate therapy in rheumatoid arthritis: 15 years experience. Am J Med 1983; 30: 75(6A): 69-73.
6. Tulandi, T. Treatment of ectopic pregnancy by transvaginal intratubal methotrexate administration. Obstet Gynecol 1991; 77; 627-630.
7. Brown DL. Serial endovaginal sonography of ectopic pregnancies treated with methotrexate. Obset Gynecol 1991; 77:406-409.
8. Li J and Kaminskas. Accumulations of DNA strand break and methotrexate cytotoxicity. Proc Natl E Acad Sci (USA, 1980; 81(18): 5694-5698.
9. Murica C and Nombela J, Cytological aberrations produced by methotrexate in mouse ascities tumours. Mutation Res 1972; 14: 405-412.
10. Hittelman W. The type and time of occurrence of aminopterine-induced chromosome aberrations in cultured potorous cells. Mutation Res1973; 18(1): 93-102.
11. Jensen M and Nyfors A. Cytogenetic effects of methotrexate on human cells in vivo. Mutation Res 1979; 64(5): 339-343.
12. Slamenova D, Chalupa I, Robichova S, Gabelova A, Farkasova T, Hrusovska L, Bacova G, Sebova L, Eckl P, Bresgen N, Zeitheim P, Schneider P, Wsolova L, Barancokova M, Kazimiova A, Navarova J, Brezek S. Effect of dietary intake of Vitamin A or E on the level of DNA damage, chromosomal aberrations and micronuclei induced in freshly isolated rat hepatocytes by different carcinogens. Nutr Cancer, 2002; 42(1): 117-24.

13. Stich HF, Stich W, Rosin MP, Vallejera MO. Use of the micronucleus test to monitor the effect of vitamin A, beta-carotene and canthaxanthin on the buccal mucosa of betel/tobacco chewers. Int J Cancer, 1984: 15:34(6): 745-50.

14. Schmid W. The micronucleus test for cytogenetic analysis. In: Hallaender A (Ed). Chemical mutagens, principles and methods for their detection, vol.4. Plenum Press: New York 1976; pp.31-53.

15. Narayan K, D'Souza UJ, Rao KPS. The genotoxic and cytotoxic effects of ribavirin in rat bone marrow. Mutat Res, 2002; 521: 179-85.

16. Midander J and Revesz L: The frequency of micronuclei as a measure of cell survival in irradiation cell populations. Int J Radiat Biol 1984; 38(2): 237-242.

17. Von Ledebur M and Schmid W. the micronucleus test: Methodological Aspects. Mutat Res 1973; 19: 109-117.

18. Norman A, D Bass, D Roe, Screening human populations for chromosome aberrations. Mutat Res 1985; 143; 155-160.

19. Muller Wu and Streffer C. in: Advances in mutanenesis research-5.n Edited by Obe G. Springer-Verlag, 1994; pp. 76-78.

20. Sandberg AA, A chemosomel hypothesis of oncogenesis. Cancer Genet Cytogenet 1983; 8; 277-285.

21. Stich HF, Acton AB, Palcic B: Towards an automated micronucleus assay as an internal dosimeter for carcinogenic exposed human population groups Rescent Results Cancer Res 1990; 120; 94-105.

22. Alaoui-Jamali MA, Rossignol G, Castonguay A. Protective effect of vitamin A against the genotoxicity of NNK, a nicotine-derived N-nitrosamine. Carcinogenesis, 1991:12(3): 379-84

23. Stich HF, Mathew B, Sankaranaryana R, Nair MK. Remission of precancerous lesions in the oral cavity of tobacco chewers and maintenance of the protective effect of beta-carotene or vitamin A. Am J Clin Nutr, 1991: 53(1suppl): 298S-304S.

24. Stich HF, Rosin MP, Vallejera MO. Reduction with vitamin A and beta-carotene administration of proportion of micronucleated buccal mucosal cells in Asian betel nut and tobacco chewers. Lancet, 1984, 2:1(8388): 1204-06

25. Toma S, Losardo PL, Vincent M and Palumbo R. effective of beta carotene in cancer chemoprevention. Eur J cancer Prev 1995; 4(3): 213-223.

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