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EFFECT OF METHYL METHANE SULFONATE ON EHRLICH ASCITES CARCINOMA CELLS: DOSE EFFECT RELATIONSHIPS

P. VISHWAPRAKASH MAHADIMANE AND V. VASUDEV*

Department of Studies in Bioscience, University of Mysore,
PG. Center, Hemagangothri, Hassan-573220, Karnataka, India.

ABSTRACT

A well known monofunctional alkylating agent, Methyl methanesulfonate (MMS) has been employed to understand its influence on the chromosomes of Ehrlich ascites carcinoma (EAC) cells. The literature review revealed that there are no reports on this aspect. Hence, a dose range of 25 to 150mg/kg bw MMS were used and ascitic fluid was drawn from treated animals to analyze the chromosomal aberrations. The analysis of chromosomal aberrations revealed that there is significant increase in frequency of aberrations compared to controls. Further there was also a linear increase of aberrations as dose increased indicating the dose effect relationship. The results are discussed to highlight the exploitation of EAC model for clastogenicity in general and carcinogenesis in particular.

Key words: Ehrlich ascites carcinoma cells, Methyl methanesulfonate, Chromosomal aberrations, Dose effect relationships

1. INTRODUCTION

Major attention has been drawn recently by developing different chemical classes of anticancer drugs against a variety of experimental and human cancers. All of these anticancer drugs exhibit cellular cytotoxicity, and a majority of them interact directly with cellular DNA, producing a variety of genetic alterations such as base alkylation, cross-linking and DNA strand breaks, etc. (Sancar et al., 2004; Kondo et al., 2010). In view of their widespread clinical use, the clastogenic effects of anticancer drugs are of considerable research interest and concern. Alkylating agents represent one of the most important classes of anticancer agents and play a major role in the treatment of several types of cancers (Chaney and Sancar, 2003, Kondo et al., 2010). Methyl methanesulfonate (MMS), simple monofunctional alkylating agent reported to be clastogenic and mutagenic in variety of genetic systems (Brewen et al., 1975; Boyd and Setlow,1976; Sing and Guptha, 1983; Lambert et al., 1984; Edwards et al., 1993; Vasudev et al., 1997; Kaya, 2003; Doak et al., 2007; Pottenger, 2009). It is found to induce dominant lethals in *Drosophila* and mouse test systems (Partington and Bateman, 1964; Ryo *et al*,1981). Moreover its chromosomal breaking activity was reported in mouse (Riaz Mahmood et al., 1996; Guruprasad et al., 2002) and *in vitro* human lymphocyte test systems (Harish et al., 1998).

Multi effects of MMS such as mutagenic, carcinogenic, recombinogenic and clastogenic effects in normal somatic cells of variety of test systems paved the way for us to understand the influence of this agent on the Ehrlich Ascites Carcinoma (EAC) cells. EAC appeared firstly as a spontaneous breast cancer in a female mouse and Ehrlich and Apolant (1905) used it as an experimental tumor by

transplanting tumor tissues subcutaneously from mouse to mouse. In 1932, Loewenthal and Jahn obtained the ascites in liquid form in the peritoneum of the mouse and named it as "Ehrlich ascites carcinoma". Lettre et al., (1972) used this tumor as test system which is suitable for qualitative and quantitative cancer studies. However, this cancer cell line has not been fully exploited for the evaluation of clastogenic effects of anticancerous agents. Thus, as an attempt to exploit this carcinoma as a test system, the standard alkylating agent MMS has been employed to test the induction of chromosomal aberrations and the results of the same are presented in this paper.

2. MATERIALS AND METHODS

2.1 Chemicals

Alkylating agent MMS (CAS No. 66-27-3) was obtained from Sigma Co. St. Louis , MO, USA and Colchicine (CAS No. 64-86-8) from Himedia, Pvt. Ltd., Mumbai, India. Giemsa stain and other chemicals were of analytical grade commercially available.

2.2 Animals

Male swiss albino mice weighing 25-30g of 6-8 weeks old were used and housed in polypropylene cages which were provided with standard feed pellets and water *ad libitum* under 12h of light/dark cycle. Study was approved by the Institutional animal ethical committee according to the institutional guidelines and the national animal welfare regulations.

2.3 Tumor cells

Ehrlich Ascites Carcinoma (EAC) cells were initially procured from department of Applied Zoology, Mangalore University, Mangalore, India. They were maintained by weekly intraperitoneal (*i.p.*) inoculation of 10^6 cells/ mouse.

2.4 Treatment schedule

Each animal was inoculated with 0.2ml of saline containing $1X10^6$ EAC cells and this day was taken as zero day. On 10^{th} day after inoculation 0.5 ml of saline containing MMS of different concentrations (25, 50,100,125 and 150 mg/kg bw) was injected i.p. to treatment groups and 0.5 ml of saline only for controls. 24h, 48h and 72h recovery times have been employed for all groups for chromosome analysis.

2.5 Chromosome analysis

Each animal received 0.5 ml of 0.05% colchicine by *i.p*. injection 90 minutes prior to the removal of ascites. 0.1 ml of ascitic fluid was removed at 24h, 48h and 72h recovery times. This was processed and slides were prepared by modified method of Evans et al., (1964). In brief, ascitic fluid was added to 0.4ml of 0.3% NaCl and this was incubated at 37 ^{0}c for 45 minutes. After incubation, the mixture was centrifuged at 1000 rpm for 10 minutes and the supernatant was discarded. To the pellet 5ml of fixative (3:1 V/V of Ethanol: Acetic acid) was added and mixed intensively to avoid clumping of cells. The tubes were kept at 4^0C for 30 minutes and then centrifuged at 2000 rpm for 5 minutes. The pellet was processed thrice as above. Finally the pellet was resuspended in 0.5ml of fixative dropped on to clean, non-greasy, prechilled slides and heat fixed. Coded Giemsa stained slides were screen for presence of chromosomal aberrations such as chromatid breaks, exchanges, triradials, chromosome breaks, dicentrics, rings and minutes and scored. In each treatment group a minimum of 300 well spread, non overlapping metaphase plates were scored and a minimum of three experiments were conducted for all recovery times.

2.6 Statistical analysis

The experimental data are expressed as Mean±SE. The statistical significance was assed using one way ANOVA followed by DMRT, a post-hoc test using SPSS 17.0 and judged significant if $p<0.05$.

3. RESULTS AND DISCUSSION

All most all alkylating agents including MMS have been, without any doubt, demonstrated to induce chromosomal aberrations in *in vivo* test systems such as *Vicia faba* (Rao and Natarajan,1967), *Drosophila* (Gatti et al., 1975), *P.pictus* (Vasudev et al., 1998),mouse (Frie and Vinitt*.,* 1975), and *in vitro* systems such as CHO (Natarajan et al., 1983) and human lymphocytes (Harish et al., 1998). MMS in particular has been assayed to suggest that this produces point mutations (Xiao and Samson, 1993;

Doak et al., 2007), dominant lethals(Brewen et al., 1975), recessive lethals (Ryo et al., 1981),single strand breaks(Pascucci et al., 2005), recombination (Vogel, 1992), micronucleus (Hahn and Kim ,1979;Werner et al., 2005) and sister chromatid exchanges(Sing and Guptha 1983; Kaina , 2004) in varied test systems which are normal. All these examples give us an indication that MMS is mutagenic, carcinogenic, clastogenic and recombinogenic *in vivo* and *in vitro* model systems. In spite of the establishment of EAC as model system for cancer studies way back in 1972 by Lettre et al., it is yet to be exploited to the fullest extent as that of other cancer cell lines. Hence, it is our aim to use this model to show its utility in clastogenic studies using alkylating agents and to demonstrate that it is one of the best models now available to understand carcinogenesis. When EAC cells are exposed to low dose of MMS (25 mg/kg bw) 2.9% of chromatid aberrations are produced. On the other hand when it is interacted with the highest dose of MMS (150 mg/kg bw), along with 53.7% of chromatid aberrations, 12.9% of chromosome aberrations are induced (Table 1). From these results, it can be said that: (*i*) MMS not only produces chromosomal aberrations in normal cells but also in EAC (cancerous) cells; (*ii*) As in normal cells, MMS induces exclusively chromatid type of aberrations in EAC (cancerous) cells and thus it is S-dependent agent;(*iii*) It is clear that MMS is clastogenic in nature even in EAC cells and (*iv*) MMS produced dose dependent effect of chromosomal aberrations in both normal and EAC (cancerous) cells. These are also true to other recovery times i.e., 48h and 72h (Tables 2 and 3). These results strengthen the opinions of Rao and Natarajan (1967) who reported that MMS is clastogenic and S- dependent agent when it is exposed to *Vicia faba.* Further, Vogel and Natarajan (1982) also opined the same in *Drosophila*. Similarly, Riaz Mahmood (1993) found that 40 to 160 mg/kg bw of MMS produced dose dependent chromosomal aberrations in mouse bone marrow cells at different recovery times.

MMS was also found to induce chromosomal anomalies in meiotic cells such as spermatocytes (Moutschen, 1969; Leonard and Linden, 1972) and Oocytes (Brewen et al., 1975; Braun et al., 1986) of mouse. Meiotic chromosomal anomalies such as stickiness, clumping, bridges, laggards and fragments induced also by MMS in *P.pictus* have been demonstrated by Vasudev et al., (1998). In the present investigation an interesting point noticed is that frequency of chromosomal aberrations was found to be highest at 24h recovery time and the same decreased appreciably during later recovery times. Khynriam and Prasad (2003) and Guruprasad et al., (2002, 2012) also showed the highest chromosomal aberrations at 24h recovery time and aberrations decreased at subsequent recovery times. It is also reported that chemicals in general produces the highest frequency of aberrations in rodents at 24h after single exposure (Giri et al., 1998). The highest frequency of aberrations observed at 24h of drug treatment may be related with the fact that it roughly coincides with normal length of mammalian cell cycle time of 22-24h (Schmid, 1973). A significant decrease in chromosomal aberrations during later periods could be due to clearance of drug from body/cells (Khynriam and Prasad, 2003; Guruparasad et al., 2012). The exact mechanism is to be studied.

Similar to the present observation, clastogenicity of MMS has been proved beyond doubt in cancerous cell line. Blazak et al., (1986) and Moore et al., (1989) studied chromosomal aberrations in mouse lymphoma cells. This is on par with agents like Bleomycin (Paika and Krishan, 1973), Nitrogen mustard and Cytosine arabinoside (Wobus et al.,1978), Methylxanthine (Fingert et al., 1986), Cisplatin (Khynriam and Prasad, 2003), Hedamycin (Tu et al., 2005) and Ginger extract (Hanafy, 2010) produces similar type of aberrations in cancerous cells. Ehrlich ascites carcinoma offers many advantages such as:(*i*) EAC have high transplantable capability and original picture reproduce with high consistency due to lack of tumor specific transplantation antigen (TSTA); (*ii*) They reproduce rapidly; (*iii*) cells are suspended homogeneously in ascitic fluid and can be assumed to be under identical nutritional conditions and (*iv*) the investigated chemicals can be injected intraperitoneally, thus corresponding to a direct intra tumoral injection and being independent of the blood supply of the tumor was advantageous. Thus, it is obvious from the above advantages that EAC can be used as a test system and can be exploited to the maximum extent.

Table I

Frequency of chromosomal aberrations in MMS treated EAC cells at 24h recovery time

Chromosomal aberrations									
Conc. mg/kg bw	**Exp. No**	**B'**	**B"**	**RB'**	**RB'B"**	**Minutes**	**Dic**	**Rings**	**Total No. of Breaks**
0	**1**	5	0	0	0	0	0	0	5
	2	8	0	0	0	0	0	0	8
	3	5	0	0	0	1	0	0	6
	Mean±SE	**6.00±1.00**	**0**	**0**	**0**	**0.33±0.33**	**0**	**0**	**6.33±0.88** [a]
25	**1**	7	2	0	0	2	0	0	15
	2	8	3	0	0	4	0	0	20
	3	6	2	0	0	3	0	0	17
	Mean±SE	**7.0±0.58**	**2.33±0.33**	**0**	**0**	**3±0.58**	**0**	**0**	**15.33±1.45** [a]
50		15	6	2	0	2	3	1	41
		19	7	1	0	3	5	2	52
		12	3	3	0	1	4	1	35
	Mean±SE	**15.33±2.03**	**5.33±1.20**	**2±0.58**	**0**	**2±0.58**	**4±0.58**	**1.33±0.33**	**42.67±4.98** [b]
100	**1**	43	8	2	2	10	8	2	99
	2	52	5	1	0	7	3	3	83
	3	48	4	3	0	12	5	3	90
	Mean	**47.67±2.60**	**5.67±1.20**	**2±0.58**	**0.67±0.67**	**9.67±1.45**	**5.33±1.45**	**2.67±0.33**	**90.67±4.63** [c]
125	**1**	75	10	5	7	20	15	7	190
	2	89	11	6	2	15	11	3	172
	3	66	11	3	4	22	13	2	158
	Mean±SE	**76.67±6.69**	**10.67±0.33**	**4.67±0.88**	**4.33±1.45**	**19±2.08**	**13±1.15**	**4±1.53**	**173.33±9.26** [d]
150	**1**	100	12	7	10	26	20	9	252
	2	106	13	10	8	33	18	8	261
	3	98	16	8	12	24	16	4	248
	Mean±SE	**101.33±2.40**	**13.67±1.20**	**8.33±0.88**	**10.00±1.15**	**27.67±2.73**	**18.00±1.15**	**7.00±1.53**	**253.67±3.84** [e]

Note: Data of 3 independent experiments: 3 animals per experiment were used; 100 cells per animal scored, and a total of 900 cells scored per dose. B'- Chromatid break, B"-Isochromatid break, RB'-Chromatid exchange, RB'B"-Triradials, Dic-Dicentrics. Values with same superscripts are not significantly different where as values with different superscripts are significantly different from one another.

Table II

Frequency of chromosomal aberrations in MMS treated EAC cells at 48h recovery time

Dose mg/Kg bw	Exp. No	Chromosomal aberrations		Total no. of breaks
		Chromatid aberrations	Chromosome aberrations	
0	1	4	0	4
	2	6	0	6
	3	6	0	6
	Mean±SE	**5.33±0.667**	**0**	**5.33±0.67** [a]
25	1	8	0	9
	2	8	0	10
	3	10	0	12
	Mean±SE	**8.67±0.67**	**0**	**10.33±0.88** [a]
50	1	22	0	32
	2	24	2	36
	3	23	2	33
	Mean±SE	**23.00±0.58**	**1.33±0.67**	**33.67±1.20** [b]
100	1	49	7	82
	2	41	3	61
	3	48	1	56
	Mean±SE	**46.00±2.51**	**3.67±1.76**	**66.33±7.97** [c]
125	1	91	14	156
	2	94	9	139
	3	90	9	139
	Mean±SE	**91.67±1.20**	**10.67±1.67**	**144.67±5.67** [d]
150	1	122	20	198
	2	117	18	197
	3	114	16	188
	Mean±SE	**117.67±2.33**	**18.00±1.16**	**194.33±3.18** [e]

Note: Data of 3 independent experiments: 3 animals per experiment were used; 100 cells per animal scored, and a total of 900 cells scored per dose.

Values with same superscripts are not significantly different where as values with different superscripts are significantly different from one another.

Table III

Frequency of chromosomal aberrations in MMS treated EAC cells at 72h recovery time

Dose mg/Kg bw	Exp. No	Chromosomal aberrations		Total no. of breaks
		Chromatid aberrations	Chromosome aberrations	
0	1	3	0	3
	2	4	0	4
	3	6	0	6
	Mean±SE	4.33±0.88	0	4.33±0.88 [a]
25	1	7	0	8
	2	6	0	6
	3	6	0	6
	Mean±SE	6.33±0.33	0	6.67±0.67 [a]
50	1	19	0	29
	2	19	1	27
	3	14	0	20
	Mean±SE	17.33±1.67	0.33±0.33	25.33±2.73 [b]
100	1	27	1	45
	2	28	2	37
	3	34	2	50
	Mean±SE	29.67±2.17	1.67±0.33	44.00±3.79 [c]
125	1	49	6	87
	2	63	6	97
	3	53	5	104
	Mean±SE	55.00±4.16	5.67±0.33	96.00±4.93 [d]
150	1	92	11	148
	2	82	8	125
	3	79	10	123
	Mean±SE	84.33±3.93	9.67±0.88	132.00±8.02 [e]

Note: Data of 3 independent experiments: 3 animals per experiment were used; 100 cells per animal scored, and a total of 900 cells scored per dose. Values with same superscripts are not significantly different where as values with different superscripts are significantly different from one another.

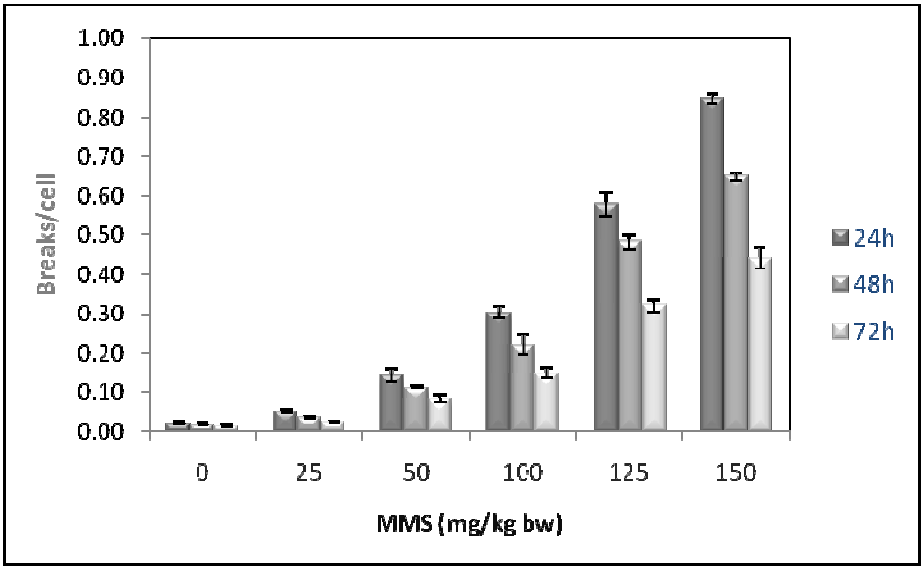

Figure 1
Frequency of total number of breaks/cell after MMS treatment in EAC cells at different recovery times

4. CONCLUSION

MMS induces chromatid type of aberrations in EAC cells which demonstrate that it is S-dependent agent. As MMS also produces dose dependent effects of chromosomal aberrations at different recovery time, it is concluded that MMS is clastogenic in nature. It is obvious that EAC has been exploited to a maximum extent in carcinogenesis due to its many advantages. These advantages can be used in mutagenicity studies, as has been proved in the present studies and further experiments make EAC to exploit as the best in vivo test system

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