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Evaluation of Genotoxicity of Ethyl Methanesulfonate (EMS) Using Human Lymphocytes

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ABSTRACT

Among the class of ethylating agents known, ethyl methanesulfonate (EMS) is a potential carcinogen and a mutagen. The objective of the present study is to determine the genotoxic potential of ethyl methanesulfonate in human peripheral blood lymphocytes using the gold-standard chromosomal aberration assay. Peripheral blood, collected from a healthy donor was exposed to various concentrations (0.5M, 0.05M and 0.005M) of EMS for 24 hours and 48 hours. Following exposure, the analysis revealed the presence of different types of unstable aberrations like chromatid breaks, gaps and radials. Chromosomal aberration frequency was calculated for EMS-treated cells and untreated (control sample) cells. The results were dose-dependent. From the dose-response curve it was evident that cells exposed for 24hours showed more damage compared with those exposed for 48hours. This could probably be attributed to DNA repair mechanism in the subsequent rounds of replication. This finding was consistently observed in all the three doses. Yet another important finding was that, there was no aberration documented in the sample treated with 100µl of 0.005M EMS. This particular observation therefore, helped to define the threshold molar dose below which no genotoxic effect was documented.

Keywords: *In-vitro;* Genotoxicity; Chromosomal aberration assay; EMS; Ethylating agents.

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INTRODUCTION

Ethyl methane sulfonate (EMS) is the ethyl ester of methanesulfonic acid and a member of the class of monofunctional ethylating agents [1, 2]. It is a colorless liquid at room temperature and is soluble in water [2]. When EMS is subjected to the process of decomposition it emits toxic fumes of sulfur oxides [2]. Exposure to EMS is limited to laboratory researchers. It has been identified as a trace contaminant in pharmaceutical products [3]. The results of the National Occupational Exposure Survey that was conducted from 1981 to 1983 showed that 971 workers, including 448 women, significantly were exposed to ethyl methanesulfonate [3]. According to the IARC reports, EMS is anticipated to be a human carcinogen and this was derived based on extensive evidence of carcinogenicity demonstrated in experimental animals [2]. It was found in elevated levels in nelfinavir tablets in May 2007 [8]. EMS produces random mutations in the DNA by the process of guanine alkylation. During the process of nucleotide substitution, the ethyl group of EMS reacts with guanine in DNA and results in the formation of the abnormal base O-6-ethylguanine. During DNA replication the DNA polymerases that are involved in catalyzing the process, frequently place thymine, instead of cytosine, opposite O-6-ethylguanine. Therefore following subsequent rounds of replication, the actual G: C base pair tends to become an A:T pair. This results in base-pair insertions and deletions, more specifically extensive intragenic deletions. Therefore, this acts a strong evidence that EMS has the ability to cause chromosomal breaks [1].

Several gentoxicity studies have been conducted using EMS. The first study that was conducted to assess the risk from EMS exposure and to understand the threshold dose allowed for humans was by Müller et al. The study involved MNT and MetaMouse studies inorder to understand the induction of gene mutation and chromosomal damage [8]. An *in-vivo* study on mice showed that, administration of EMS resulted in the induction of renal carcinomas in female rats and lung carcinomas, in both sexes [4]. An experiment performed on cell lines using the gold-standard in-vitro micronucleus test has yielded some significant results. Combined dose of γ-radiation and EMS showed supra-additivity in mouse lymphoma L5178Y cells [5].

Another study investigated the dose-rate effects of EMS on the survival and induction of mutations in Chinese hamster Don cells. The optimal time of exposure to EMS for reducing the surviving fraction of cells was found to be 4 hours. Any other duration that was shorter or longer was less effective. The threshold concentration of EMS giving a surviving fraction of 0.5 was found to be 0.05 mg/ml. It was also understood that the minimal effective time of exposure to EMS for causing cell death was 1 hour. From the corrected survival curves it was evident that protracted exposure times at lower dose rates of EMS had reduced cytotoxic effect when compared with shorter exposure times at higher dose rates. The experiment involving exposure of Don cells to different doses of EMS for various times, measured the frequencies of mutations resistant to 6--thioguanine (6TG). Lower mutation frequency was produced after 4 hours of exposure, rather than shorter or longer exposure times that yielded same surviving fraction of cells. After 20 hours of exposure, the highest induced mutation frequency was produced. Therefore the system using cultured Chinese hamster cells was found to be useful in detecting the mutagenic actions of chemicals in terms of sensitivity [6].

Another study that involved exposure of human peripheral blood lymphocytes to varying concentrations of EMS showed that, the percentages of chromosomal fragmentations in EMS-treated and control cells were found to be statistically significant [2]. The choice of sample that one has to consider while performing a genotoxicity test is critical. Lymphocytes are thought to be a good sample due to the ease in culturing them and also their high levels of sensitivity to radiation, in comparison with other types of cells. Several classes of mitogens like Phytohemagglutinin (PHA-C, M, P or W), Concanavalin-A (Con-A), Lectin, Pokeweed Mitogen (PWM), Lipopolysaccharides (LPS), Wheat Germ Agglutinin (WGA) and Soybean Agglutinin (SBA), are available to stimulate the lymphocytes to undergo the process of cell cycle. Once stimulated, these cells can be arrested at various stages of cell cycle, by the use of thymidine or methotrexate (for S-phase), colchicine, or colcemid (for M-phase) and in cytokinesis with cytochalasin-B. In addition to this, lymphocytes, generally, remain in G_0 stage of the cell cycle. This specific property of lymphocytes is helpful in retaining unstable aberrations for a longer period. Since circulating lymphocytes carry various information from all parts of the body, they tend to form a homogenate population of mutated cells which may has received insults by various clastogens [7].

Several types of genotoxic assays have been exploited to understand the nature of clastogens. However certain tests are regarded as gold-standard assays due to their degree of sensitivity and specificity. When cells are treated with DNA-damaging agents, they can result in irreparable lesions in both strands of DNA. This causes chromosome breakage. As with the case of cultured cell lines such as CHO and CHL, the problem that arises is that, cultured cell lines tend to lose and gain chromosomes spontaneously. This results in a high and unpredictable rate of chromosome aberrations. However, with primary cultures of human lymphocytes their chromosomes are far more stable and therefore they are recommended [7]. Hence the chromosomal aberration assay was chosen for this particular experimental study.

The present study aimed to investigate, (i) the genotoxic potential of the well known mutagen EMS, using the chromosomal aberration assay and (ii) to understand the dose that will act as the threshold molar concentration above which significant genotoxic changes are to be observed and below which no aberrations are to be seen, based on the doses chosen for this particular study.

MATERIALS AND METHODS

Chemical Reagents

Ethyl methanesulfonate was purchased from Sigma Aldrich (M0880-5G). The culture medium, Roswell Park Memorial Institute 1640 (RPMI 1640), Fetal Bovine Serum (FBS) and phytohaemagglutinin, M-form (PHA-M) were purchased from Gibco. Methanol and glacial acetic acid were supplied by Merck. Potassium Chloride, sodium thiosulphate, colchicine and ethydium bromide (EtBr) were supplied by HiMedia. For staining, giemsa Stock solution was sourced from Karyomax.

Preparation of the Drug for Exposure

From the stock solution of EMS, three different concentrations of working solution were prepared. The three concentrations were 0.5M, 0.05M and 0.005M of EMS prepared in 50% ethanol. The working solutions were stored at 4ºC. After completion of preparation, the tips that were used were discarded after immersion in 10% sodium thiosulfate for the purpose of neutralizing the toxicity from EMS.

Collection, Culture Maintenance and *In-vitro* Drug Exposure of Peripheral lympHocytes

7ml of peripheral blood was collected from a healthy donor. Seven culture flasks were taken and labelled as mentioned below (Table 1). To each flask 8ml of RPMI 1640 medium, 2ml of FBS, 300µl of PHA and 1ml of blood was added. The flasks were incubated for 72 hours at 37ºC and 5% CO_2. Based on the designation given in Table 1, EMS was added at 24th hour and 48th hour respectively.

Table 1: **Designation of Culture Flasks According to Dose of EMS**

Flask Name	Concentration of Ems(µl)	Duration of exposure(hours)
CONTROL	Ethanol only	-
0.5M	500	24
0.5M	500	48
0.5M	100	24
0.5M	100	48
0.05M	500	24
0.05M	500	48
0.05M	100	24
0.05M	100	48
0.005M	500	24
0.005M	500	48
0.005M	100	24
0.005M	100	48

Harvesting of Cells

At the 66.5 hour 100µl of ethidium bromide was added to all the cultures. At the 67th hour 100µl of colchicine was added to all the cultures. After an hour of incubation the contents of the flask was transferred into sterile centrifuge tubes with appropriate labels and subjected to centrifugation at 1000rpm for 10 min. The supernatant was discarded and the pellet was gently tapped and re-suspended in 8ml of pre-warmed hypotonic solution (0.075M potassium chloride prepared in sterile water). This was incubated for 20 min at 37ºC. After incubation the samples were centrifuged at 1000rpm for 10 min. The supernatant was discarded and pellet

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was gently tapped. The pellet was re-suspended in 8ml of pre-chilled Carnoy's fixative (methanol: glacial acetic acid at a ratio of 3:1) and incubated at room temperature for 20 min. The pellet was stored at 4ºC until further use.

Slide Preparation and Analysis

The samples were spun and re-suspended in fresh fixative. The pellet was dropped on clean, grease free, pre-chilled glass slides, from an optimal height and placed on the slide warmer. Once dried, the slides were stained in 2% giemsa solution and subjected to analysis under the bright field microscope.

RESULTS

Peripheral blood lymphocytes were subjected to exposure to EMS and 100 metaphases were scored and analyzed under each concentration (Figure 1). From the analysis the chromosomal aberration frequency was calculated and a dose-response curve was plotted (Figure 2 and 3) in the following manner:

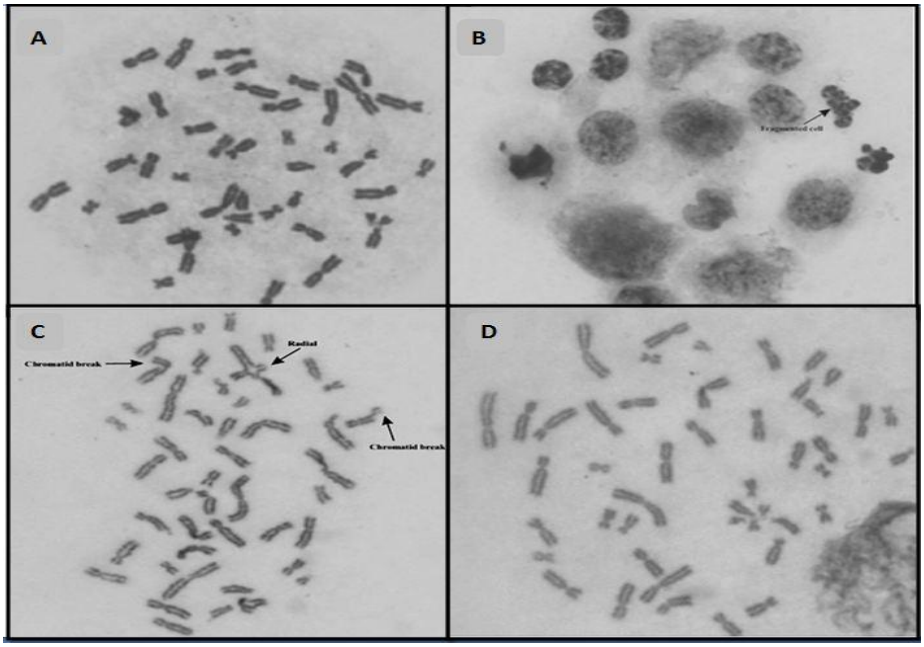

Fig.1. Results of Chromosomal Aberration Assay, where Cells were Exposed to Different Concentrations of EMS (A), Control (B), 0.5M EMS (C), 0.05M (D) 0.005M EMS

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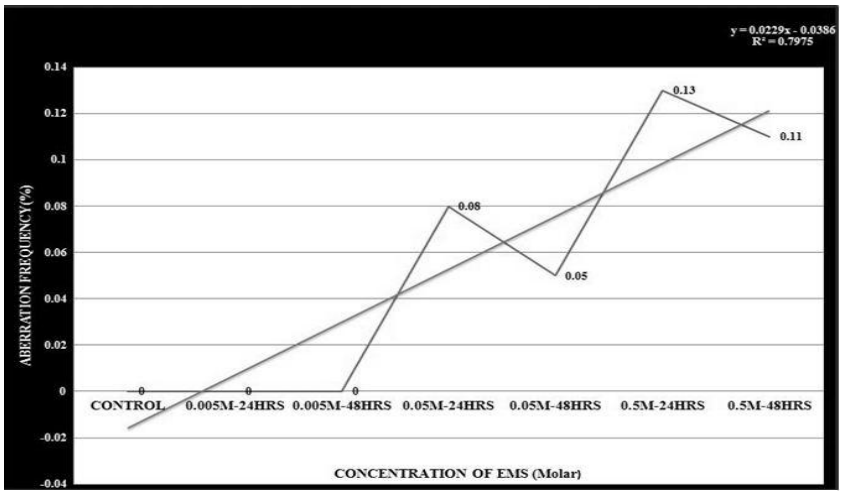

Fig.2. Chromosomal Aberration Frequency of Lymphocytes Exposed to 100µl of EMS

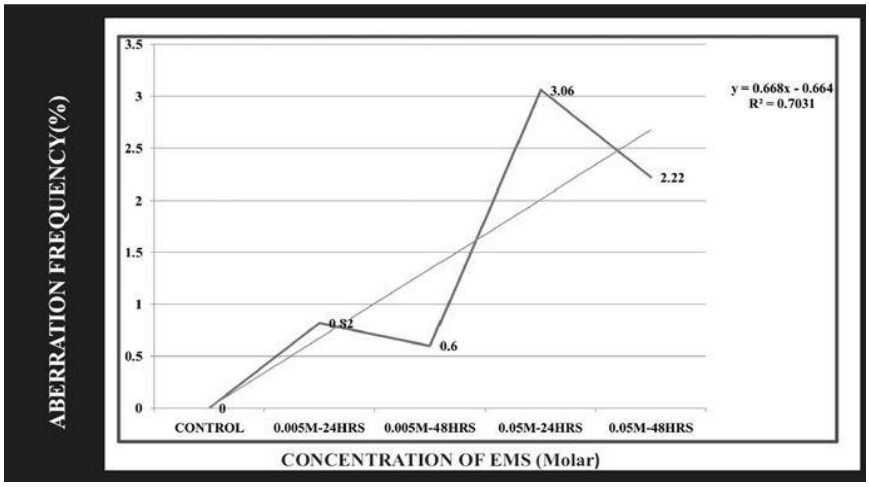

Fig.3. Chromosomal Aberration Frequency of Lymphocytes Exposed to 500µl of EMS

Formula:

Chromosomal aberration frequency = $\frac{\text{Number of aberrations scored}}{\text{Total no of metaphases scored}}$

Standard error = $\frac{\sqrt{\text{Number of aberrations scored}}}{\text{Total no of metaphases scored}}$

The data obtained from chromosomal aberration assay revealed that the damage occurred was in a dose-dependent manner (Table 2).

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Table 2. Summary of Observations Documented from Chromosomal Aberration Assay.

Concentration of EMS(µl) and duration of exposure	Total no of metaphases scored	Total no of aberrations scored	Aberration per cell ± standard error
Control	100	0	0.00
0.005M-100-24hrs	100	0	0.00
0.005M-100-48hrs	100	0	0.00
0.05M-100-24hrs	100	8	0.08±0.0282
0.05M-100-48hrs	100	5	0.05±0.0223
0.5M-100-24hrs	100	13	0.13±0.0360
0.5M-100-48hrs	100	11	0.11±0.0331
0.005M-500-24hrs	100	82	0.82±0.0905
0.005M-500-48hrs	100	60	0.60±0.0774
0.05M-500-24hrs	100	306	3.06±0.1749
0.05M-500-48hrs	100	222	2.22±0.1489
0.5M-500-24hrs	**0**	**HCT**	**HCT**
0.5M-500-48hrs	**0**	**HCT**	**HCT**

Note: **HCT-** highly cytotoxic (due to which there was complete fragmentation of cells and thereby documentation of aberration and calculation of CA frequency was not possible for the dose 0.5M-500µl for both 24 and 48 hours.

DISCUSSION

The present study evaluated the genotoxicity of EMS by exploiting the gold-standard assay namely the chromosomal aberration assay as the *in-vitro* end-point. To understand the degree of genotoxic potential that EMS possesses, the study was initiated from the *in-vitro* front and therefore the chromosomal aberration assay was performed on human peripheral blood lymphocytes(due to its high sensitivity to clastogens). The concentrations were chosen based on studies conducted by Songul et al, 2010 [2].

Lymphocytes were exposed to three different concentrations of EMS; 0.5M, 0.05M and 0.005M for a period of 24hrs and 48hrs under each concentration. The control sample that had ethanol showed no aberrations. Similarly, cells exposed to 0.005M, with a final volume of 100µL for 24hrs and 48hrs also demonstrated absence of aberrations (Figure 2). However, at a volume of 500 µL, both 24hr and 48hr cultures showed aberrations with a frequency of 0.82% and 0.60% (Figure 3). With an increase in the dose to 0.05M; both 100 µL and 500 µL exposures for 24hrs and 48hrs showed a sharp increase in the aberration frequency and the values were found to be 3.06% (0.05M-500 µL-24hrs) and 2.22%(0.05M-500-48hrs). The types of unstable chromosomal aberration that was documented were dicentrics, acentrics, chromatid breaks, chromatid gaps and radials. While chromatid breaks and gaps were the most repeating and consistent type of aberration seen; the dicentrics was found only at a dose of 0.05M (500 µL), in 24hr culture. Beside this, there was a significant decrease in the aberration frequency in all the doses in 48hr culture when compared with the 24hr culture. This decrease shall be attributed to the fact, that these aberrations are unstable in nature and could have possibly been repaired during subsequent cell cycles. At the highest dose of 0.5M (100 µL), aberrations were documented. However, in 24hr and 48hr cultures exposed to 500 µL of 0.5M EMS, high degree

of cytotoxicity was depicted by the drug and this was evident, by the fragmented appearance of cells leaving no intact interphase nuclei nor metaphases to be scored. This finding was relevant based on the condition that was documented by Katsura et al, 1978, who observed complete cell death in CHO cells within 1 hour [6].

An important finding that was recognized in the process of constructing a dose-response curve was that, the damages have occurred in a dose-dependent manner. There was no aberration found in both 24hr and 48hr cultures exposed to 100 µL of 0.005M EMS and this was consistent in all 100 metaphases that were scored. This observation stood as an evidence, to identify the threshold molar concentration above which there was a significant increase in the chromosomal aberration frequency and below which no aberrations were documented.

CONCLUSION

In conclusion, we have demonstrated the genotoxic potential of EMS by exposing peripheral blood lymphocytes to three different concentrations of EMS and comparing the effects with the control. From the study it was evident that, (i) the results were dose-dependent, (ii) exposure of cells at all concentrations for 48hrs showed less damage compared to that of 24hrs exposure, which may be attributed to DNA repair mechanism and finally, (iii) the dose at which there was no aberrations documented was 0.005M (100µl). Therefore, from the *in-vitro* study conducted, it may be concluded that the safe dose of EMS is 0.005M (based on the concentrations chosen for this study). However further explorations and comprehensive studies are required to validate this finding prior to experimentation on *in-vivo* models.

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