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GENOTOXICITY OF MYTOMYCIN C- CYTOGENETIC STUDY

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Abstract: *The present study was undertaken to study genotoxic effect of Mitomycin C at different concentrations for different duration on human chromosomes in search of a dose which will be more effective and less toxic in lymphocytic culture and to find the specificity of its action on human chromosomes in lymphocyte culture. The range of concentration of Mitomycin C (0.05, 0.1, 0.5, 1.0, 2.0, 5.0 µg /ml medium) were used for different durations (24, 72, and 1hour) Genotoxicity was less for low concentration (0.05, 0.1 µg/ml) for long duration (72 hours). Mitomycin C specifically involves chromosome 9, 1 and 16 in chromosomal aberrations. Interchange exchange was the most common chromosomal aberration induced by Mitomycin C. Most commonly involved chromosome in interchange exchange was chromosome 9.*

Keywords: *Genotoxicity ,Mitomycin C, Interchange exchange*

I. Introduction

Mitomycin C has been widely used as an anticancer drug because of its selective cytotoxicity to cancer cells. Due to its wide clinical application it is a centre of biomedical research for more than 40 years. 1[15] Mitomycin c has proved effective in the frontline treatment of many solid tumors such as superficial urinary bladder cancer 2[3], gastric carcinoma 3[4], pancreatic neoplasias3[4], anal carcinomas and esophageal carcinomas4[5]. The currently practiced dose of 20 mg/m^2 of mitomycin c with peak concentration in plasma will be 0.4 ug/ml.[2] The main dose dependent toxicity of mitomycin c is myelosuppression, increasing with dosage >10 to 20 mg/m^2.5[8]

According to previous studies mitomycin c was considered both genotoxic and cytotoxic agent 1[15] Genotoxicity refers to the production of DNA adducts, part of a wide spectrum of DNA changes generated by different repair mechanisms of DNA double strand breaks by an agent or its metabolites microscopically visible as Chromosomal aberrations. The ideal antitumor drug should produce maximum inhibition of proliferating cancer cells and produce minimum side effects such as chromosomal aberrations. To achieve this, the dose of the drug in the lowest possible concentration having highest efficacy should be administered may be for longer duration1[15]As Mitomycin C is very useful in solid tumor which are resistant to radiotherapy but produces dose dependent toxicity there is need of further evaluation to find new dose regimen of Mitomycin C to enhance its therapeutic effects and minimize side effects.

Cytogenetic provide a method to study the genotoxicity and cytotoxicity of anticancer drug in living replicating human cells. Hence present study was undertaken for evaluating genotoxic effects of Mitomycin C with regards to its specific effects on human chromosomes in lymphocyte culture which will be helpful in planning a more effective regimen with less toxicity.

II. Material and Methods

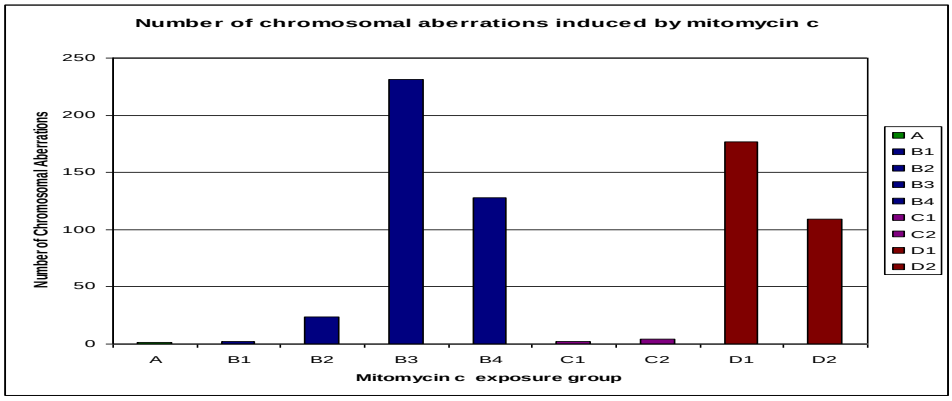
50 blood samples were taken from healthy volunteers having no history of exposure of Mitomycin C and grouped into four groups. Group A (20 samples) Control sample with No MMC exposure. Group B (10 samples) each of these sample were exposed to different range of concentration of mitomycin c (0.05, 0.1, 0.5, 1.0, 2.0, 5.0 µg Mitomycin C / ml medium) in tube B1, B2, B3, B4, B5 and B6 respectively. Group C (10samples) each sample were exposed to 0.05 and 0.1 µg Mitomycin C / ml medium in tube C1 and C2 respectively. Group D (10 samples) each sample were exposed to 0.5 and 1.0 µg Mitomycin C / ml medium in tube D1 and D2 respectively. Culture were set in RPMI media by adding 4.5ml of complete medium and 0.5 ml of heparinized blood sample. All culture tubes were incubated at 37^{0}C for 70 hours and 40 minutes. After 70 hours and 40 minutes of incubation, Colchicine were added to each sample. Slides were prepared using the pellet after harvesting procedure and stained with giemsa stain and observed under microscope. All slides were screened first under low power objective of microscope. All slides were screened under oil-immersion objective and selected metaphases were photographed using CCD camera to asses chromosomal aberrations to determine its genotoxicity

III. Observation

Observation was done under following parameters.

1. Number of Chromosomal Aberrations Induced By Mitomycin C

Mitomycin c exposure at high concentration such as 0.5 and 1.0 µg/ml for short duration (1 hour in group D-1-2) induced significantly more number of chromosomal aberrations than its exposure of low concentration such as 0.05 and 0.1 µg/ml for long duration (72 hours in group C-12).Group-B-3 (0.5µg/ml) mitomycin c for the last 24 hours showed significantly higher number of chromosomal aberrations than group-D-1 (0.5µg/ml) and was most suitable for finding types of chromosomal aberrations. Group-B-4 (1.0 µg/ml) showed significantly higher number chromosomal aberrations than group-D-2.



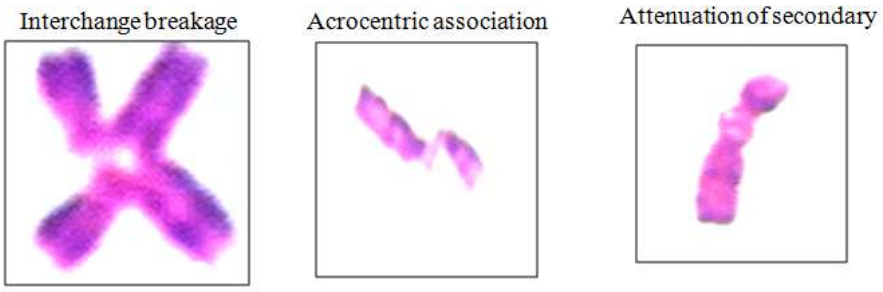
Number of chromosomal aberrations induced by mitomycin c

MCA: Mean Chromosomal Aberrations # For each sample, 100 metaphases were analyzed to find total number of aberrations $Very few metaphases to score. MMC-mytomycin c, SD-standerd deviation

This indicated that production of chromosomal aberrations by mitomycin c was depended on duration its exposure.

2. Types of Chromosomal Aberrations

Group B-3 exposed to the concentration 0.5 µg/ml for last 24 hours was the most suitable group for examining the effect of mitomycin c on human chromosomes as this group had shown less mitotic inhibition and very high number of chromosomal aberrations. Most commonly found aberrations were interchange breakages (61.6%) and second most common aberrations were satellite association (17.75%). Attenuation of secondary constrictions was 5.7% while others were 14.95% .Thus it was observed that mitomycin c had affinity for satellite and secondary constriction regions of chromosomes and most common induced aberration was interchange breakages.

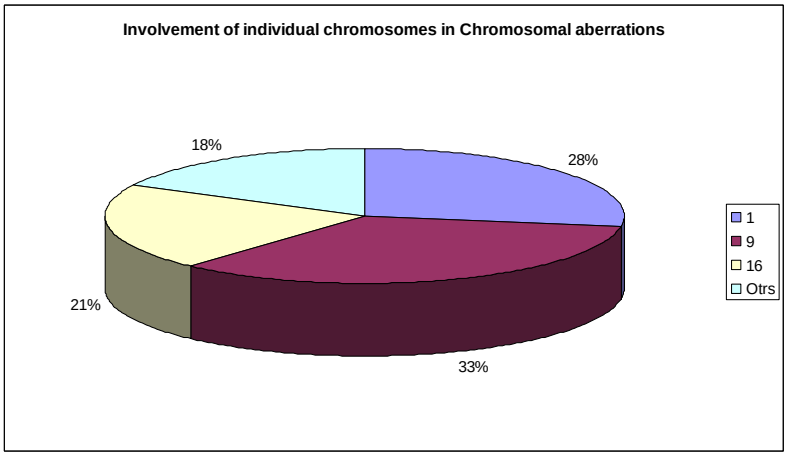


Types of chromosomal aberrations

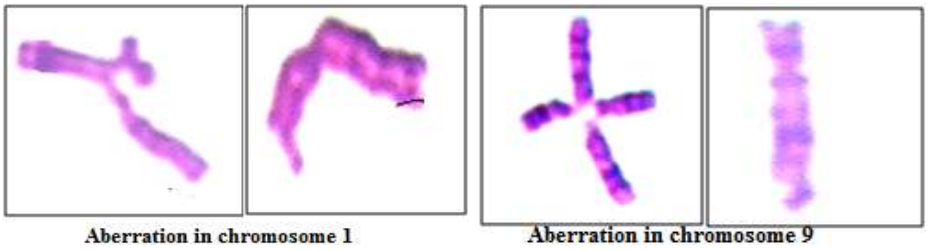
Type of chromosomal aberration	Interchange breakage	Satellite association	Secondary constriction attenuation	Others
Number of aberrations per 200 aberrations	123.2	35.5	11.4	29.9
S.D.	2.65	1.84	1.17	1.37
%	61.6%	17.75%	5.7%	14.95%

3. Involvement Of Individual Chromosomes In Chromosomal Aberrations

100 subsequent involved chromosomes in chromosomal aberrations from each sample of group-B-3 were identified and numbered to find the involvement of individual chromosomes. Excess of aberrations in chromosomes 1, 9 and 16 were observed than other chromosomes on comparison with expected involvement as per the human genome information of Sanger Institute[15]. In chromosomal aberrations, most commonly involved chromosome was chromosome 9 (34.1%) followed by the involvement of chromosome 1(27.6%) and least involvement of chromosome 16(20.7%) among these three chromosomes. These observations indicated that mitomycin c did not induce chromosomal aberrations randomly; it more commonly affect selected chromosomes. These observations also indicated affinity of mitomycin c for specific chromosome

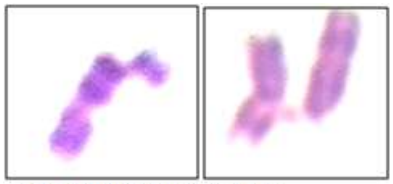


Involvement of individual chromosomes in Chromosomal aberrations



Aberration in chromosome 1

Aberration in chromosome 9



Aberration in chromosome 16

IV. Discussion

According to previous studies mitomycin c was considered both genotoxic and cytotoxic agent[1]. Genotoxicity refers to the toxic damage to DNA or the production of DNA adducts, by an agent or its metabolites. Chromosomal aberrations are the microscopically visible part of a wide spectrum of DNA changes generated by different repair mechanisms of DNA double strand breaks.

The observations of the present study suggested that mitomycin c inhibit mitosis and induce chromosomal aberrations.

1. Numbers of chromosomal aberrations were higher when exposed to concentration 0.5µg/ml hence it was most suitable for studying the types of chromosomal aberrations**,**
2. Mitomycin c induced chromosomal aberrations specifically involves chromosome 1, 9, 16 and acrocentric chromosome .
3. Frequently occurring chromosomal aberration were interchange breakage, satellite associations and attenuation of secondary constriction.
4. Interchange breakages was the most common type of chromosomal aberrations which frequently involves chromosome 9.

Very few investigators have studied the effects of Mitomycin C on human chromosomes.

1. Frequency Of Chromosomal Aberration With Mytomycin C :

Revell SH (1955)[6] found that, higher the concentration of nitrogen mustard (alkylating agent); shorter the time before chromosomal aberrations appear. It therefore appears as though the dose of nitrogen mustard was the product of the concentration and the time of treatment. This was not so in the case of mitomycin c. Observation of the present study shows that although the dosage of mitomycin c was very important factor for the induction of chromosome aberrations, the critical time for treatment was also necessary for chromosome breakages. In our study chromosomal aberrations were mostly found at the concentration of 0.5µg/ml for 24 hr duration Chromosomal aberrations on exposure of 24 hour were higher than one hour with same concentration of 0.5µg/ml. thus the observation that induction of chromosomal aberration by Mitomycin C was dependent on duration of exposure was consistent with .

Cohen MM and Shaw MW (1964)[7] found maximum chromosomal breakages at concentration 1.0 µg/ml of Mitomycin C for 24 hour exposure. Nowell PC (1964)[8] used a same concentration and time to treat human leucocytes but failed to produce chromosome breaks. This discrepancy was probably due to the difference in the individual samples or experimental errors

Tchun TS (1976)[9] and Sontakke YA (2008)[1] also found highest frequency of chromosomal aberrations with the concentrations of 0.5µg/ml for 24 hr of cultural incubation. Tchun TS (1976)[9] had not provided the statistical analysis of the data while Sontakke YA (2008)[1] studied the effect of mitomycin c in 100 samples and provided the statistical analysis of his data and found that cultures with mitomycin c exposure to concentrations of 0.5µg/ ml for 24 hours showed higher number of chromosomal aberrations than the cultures exposed to same concentration for 1 hour indicating that the frequency of chromosomal aberration was dependent on duration of exposure.

Assuming that mitomycin c acts at a specific time during the replication cycle, it was possible that in short term leucocytes cultures some chromosomes or portions of chromosomes may have completed replication before the addition of the drug. If so, one might expect varying distributions of breaks dependent upon exposure times. Observation of the present study shows different frequency of induction of chromosomal aberration for same dose at different time period and points toward the same. Renzi L et al (1996)[10], observed that the amount of chromosome damage induced by mitomycin c in mouse splenocytes differ in relation to the proliferative status of treated cells. Nowell PC (1964)[11] reported that cultures treated with mitomycin c shortly before or during DNA synthesis showed much less susceptibility to chromosome damages. However when cells were not synthesizing DNA and subsequently dividing, numerous chromosome aberrations were produced. Doi O et al (1967)[12] studied the effects of mitomycin c on HeLa cells at the various stages of division cycle reported that latter half of the G_1 phase and the first half of the S phase was highly sensitive to mitomycin c.

Djedjevict B (1968)[13]studied the effect of mitomycin c in different stages of cell cycle in HeLa cells and observed that toxicity of mitomycin c was greatest during early G_1 phases of the cell cycle. Sognier MA and Hittelman WN (1986)[14] suggested that chromosome breaks induced by mitomycin c were the results of cells reaching mitosis without having fully completed DNA replication. Abdel-Halim HI et al (2005)[15] showed that pairing of chromosome 9 heterochromatic regions occurred in G0/G1 and S-phase cells as a result of an active cellular process initiated upon mitomycin c treatment.Though in present study, effect on various stages in division cycle were not studied but the observation of present study point towards the specificity of action of mitomycin c and can be correlated with the result of the above studies.

2. **Involvent Of 9,1,16 Chromosome In Chromosomal Aberrations Induced By Mitomycin C:**

In human chromosomes the secondary constrictions appears most frequently in chromosome 1 and 9[16] and also include the five pairs of acrocentric chromosomes with nucleolar organizing regions on each of the short arm as satellite.[17]Chromosomal breaks induced by chemical treatment were frequently localized in the prominent heterochromatic segments, the nucleolar organizing regions, secondary constrictions and pericentric heterochromatin[18] but the pattern of chromosome aberrations produced by mitomycin c were quite different from other well-tested chemicals and those of X-ray . Mitomycin c breaks showed a non-random distribution at very specific sites of chromosomes, while the breaks of an X-rays experiment were randomly scattered.**[19]** As like Mitomycin c, 5-Brdu (5-bromodeoxyuridine) increases the frequency and intensity of secondary constrictions in chromosome 1 and 9.**[20]**

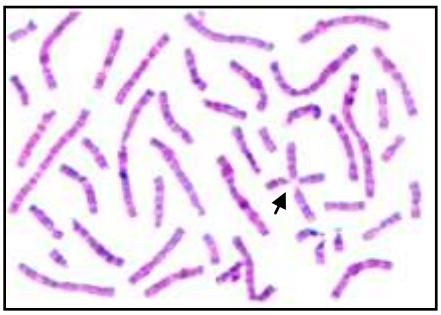
Shaw MW et al (1965)[21] suggested that attenuation of secondary constriction of chromosome 9 induced by mitomycin c increased the probability of identification of chromosome 9 approximately by 10 folds. Cohen MM et al (1964)[12] found that Chromosome 1, 9 and 16 possessing prominent secondary constrictions, appeared to be focal points of mitomycin c activity. and chromosome 9 was most commonly involved chromosome in chromosomal aberrations. These studies were carried out when techniques of G-Banding for chromosomal identification were not available though the finding goes with the present study. Tchun TS et al (1976)[14] also observed the frequent involvement of chromosome 1, 9 and 16 in mitomycin c induced chromosomal aberrations with more affinity for chromosome 9.

Sontakke YA (2008)[15] found that genotoxicity of mitomycin c specifically involve dark band and secondary constriction region of chromosome 1, 9 and 16 along with acrocentric chromosomes by using G banding technique for chromosomal identification while Morad M (1973)[22] analyzed mitomycin c induced chromosome aberrations in cultured human lymphocyte by the quinacrine mustard chromosome banding technique and found an over representation of interchange aberrations especially the secondary constrictions of chromosomes 1, 9 and 16**.**

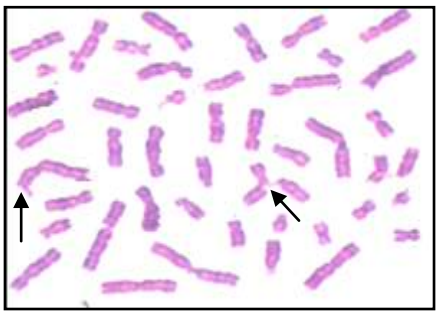
In present study mitomycin c most commonly affect chromosome 9 in chromosomal aberration.

This observation of the present study was supported by Fauth E et al (2000)[23] who showed that mitomycin c exposure induced an increase frequency of undercondensation preferentially at the pericentric heterochromatin block at 9q12 and to a much less extent at the 1q12 and 16q12. The specific decondensation of 9q12 by mitomycin c treatment corresponded well with a general sensitivity of this region to drugs which induce undercondensation and breakage like, Idoxyuridine (Fauth E, 1999)[24], and 5-Azacytidine (Haaf T, 1995)[25].

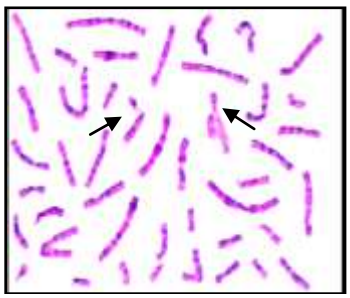
Fauth E et al (2000) concentrated his study on mitomycin c induced micronuclei by using fish analysis with whole chromosome painting probes for all human chromosome. He revealed the preferential occurrence of chromosome 9 material, which strongly correlated with preferential undercondensation of heterochromatin of chromosome 9. Most of the investigators concentrated on chromosomes 9,1,16 and had not commented on the frequency of involvement of other autosomes and sex chromosomes in mytomycin c induced chromosomal aberrations. Hovhannisyan GG (2008)[26] however studied specifically the involvement of chromosomes 7, 18 and X in mitomycin c induced micronuclei and shown that X-chromosomal material was over represented in female and underrepresented in male-derived micronuclei and speculated about a preferred inclusion of the inactive female X-chromosome into micronuclei . Abdel-Halim HI et al (2005)[15] on FISH analysis of chromatid interchanges involving chromosomes 1 and 9, and their similar sized chromosomes 2 and 8 revealed that mitomycin c induced exchanges were not homogeneously distributed over the genome. About 50% of all exchanges induced in the genome involve chromosomes 1 or 9, although the DNA content of these two chromosomes only amounts to about 13% of the genome. In sharp contrast, chromosomes 2 and 8, also with a DNA content of about 13%, participated in only about 1%



Aberration involving chromosome 9 from Group B



Aberration involving chromosome 1 and chromosome 9



Involvement of acrocentric chromosomes in aberration from Group D

3. Interchange exchange by Mitomycin c:

Observation of the present study shows that interchange exchange of chromosome 9, 1, 16 and acrocentric association were the most common chromosomal aberrations.

Cohen MM et al (1964)[7] found that 50% total exchanges induced by mitomycin c were homologus and selective for chromosome 1 and 9 and Morad M (1973)[27] found an over representation of interchange aberrations involving the C-bands of homologous chromosomes especially the secondary constrictions of chromosomes**,** 1, 9 and 16. Observation of the present study was fully correlated with the findings of Tchun TS et al (1976)[9] who observed that more than half of aberration induced by mitomycin c were interchange breakage and 60 to 70% of which involve the secondary constriction region. Sontakke YA (2008)[1] found that interchange exchange was the most common chromosomal aberration induced by mitomycin c and other chromosomal aberrations were associations of acrocentric association and attenuation of secondary constriction and agrees with the present study. Latt SA (1974)[28] observed significant increase in the SCE (sister chromatid exchange) with increase in concentration of mitomycin c. Finding of the above researchers corresponds with the present study. Brogger A (1973)[25] revealed a prevalence of attenuations, lateral extensions and exchanges in the secondary constrictions of chromosomes 1, 9, and 16 in the mitomycin c treated material and not with methylmetanesulphonate MMS. This may be due to a specific gathering effect of mitomycin c upon this particular chromatin and his finding also corresponds to the present study.

Two mechanisms have been proposed to explain the chromosome basis of mitomycin c induced exchanges. First, pairing in interphase might provoke the formation of exchanges between the paired chromosomes.[25, 27, 21] Secondly, it has been suggested that the excess of homologous exchanges might result from recombination-associated repair between matching repetitive DNA sequences [29]**.**

In man, the association of satellite chromosomes[30] and the association of acrocentric chromosomes with the paracentric region of chromosome1[31] as well as other chromosomes bearing heterochromatic secondary constrictions[16] suggested that ectopic pairing also occurs. The present study also supports these findings.

Mitomycin c induces interstrand cross-links in the DNA. When left unrepaired these interstrand cross-links, form impenetrable blocks for DNA replication and are highly cytotoxic. The repair of interstrand cross-links is a complex process involving proteins belonging to nucleotide excision repair (NER), homologous recombination and translation synthesis pathways[32]**.**

Abdel-Halim HI et al (2005)[15] provided evidence that in untreated S-phase cells, pairing was not elevated when compared with G0/G1 fibroblasts and treatment with mitomycin c was able to induce pairing of homologous chromosomes 9 in a subset of cells independently of the cell cycle.

Rothfuss A et al, 2004 [33] presented a refined model in which interstrand cross-links are recognized and incised by ERCC1/XPF independently of DNA replication The incised interstrand cross-links are then processed further in S-phase where misrepair leads to chromosome exchanges and breaks.

It was possible that the exchanges reported in the present study represent relics of interphase homologues pairing wherein mitomycin c had not only induced breaks and rearrangements but also interfered with the normal separation of homologues.

Maximum chemotherapeutic drugs are first studied in animals but present study which is a cytogenetic study involve culture of human cells by which action of drug can be assessed in living and multiplying cells. Hence observations of present study may be applicable in planning and carrying out clinical trials as well as regimen of chemotherapy in cancer patients.

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