Study of cytogenetical effects of chemotherapeutic agents Mitomycin C and Cisplatin on normal Somatic and Germ cells: An *in vivo* study

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Abstract: The chemotherapeutic drugs available in the market are not specified to eliminate only cancer cells but leads to numerous toxic physical, cytogenetical and physiological side effects. Since the common target of all anticancer drugs is to decrease the growth rate (cell division) of the cancer cells, the side effects are observed in bodily systems that naturally have a rapid turnover of cells including skin, hair, gastro-intestinal, and bone marrow. Hence the healthy, normal, proliferative cells of the body also get targeted by the chemotherapy program. In this study we have observed the cytogenetical consequence induced by two most commonly administered chemotherapeutic agents Mitomycin C and Cisplatin. We have observed that both MMC (2 mg/kg bw) and Cis (4 mg/kg bw) administered to Balb/C mice induced significant level (P < 0.001) of chromosomal aberrations and Micronucleus at both 24 and 48 hour studies. So also study in germ cells have shown that exposure to Mitomycin C and Cisplatin induce highly significant level of sperm head abnormality in the animals.
Keywords: Mitomycin C; Cisplatin; Chromosomal Aberrations, Micronucleus, Sperm head abnormality.

Introduction and Methodology:

The use and application of drugs synthesized or procured from natural or synthetic sources for cancer inhibition and cure is known as "chemotherapy" and the drugs are more commonly named as chemotherapeutic drugs. We know that Cancer is a state where cells or tissues of the body starts to divide uncontrollably and evade the normal cell cycle as a result of which progression of large tumors occur, and the tumorous cells by the mechanism of metastasis may invade the neighboring normal cells/tissues of the body causing harmful consequences. Keeping this in mind the cancer drugs has been designed to slowly act on the cancerous cells and halt their progression by suppressing them through various molecular mechanisms. The most common mechanism by which the chemotherapeutic agents act on cancerous cell is that they damage the DNA of the cancerous cell by binding to the DNA forming intrastrand/ interstrand cross-links. They also may cause single strand (SSB) or double strand (DSB) DNA breakage leading to nonsense DNA. Again certain drugs are designed to

inhibit the synthesis of new DNA by interfering with the building blocks (folic acid; heterocyclic bases; nucleotides) of DNA or the replication process of DNA. So, also some drugs are capable of inhibiting the process of mitosis and halt cancer growth. Unfortunately these drugs not only target the cancer cells but can act on the normal cells in a likewise manner causing serious cytogenetical consequences starting from faulty genome formation to faulty protein formation to development of secondary neoplasms and also passing on the faulty genome to the next generation. The present study is aimed to find the severity of the cytogenetical damage inferred to the normal proliferative cells of the mammalian system induced by two most common chemotherapeutic agents Mitomycin C (MMC) and Cisplatin (CIS).

Material and Methods:

Chemicals

MMC and CIS were obtained from Cadila Pharmaceuticals, India. Colchicine was purchased from Sigma chemicals Co. (St. Louis, MO). Giemsa stain was purchased from HiMedia Laboratories Pvt. Ltd, India. All other chemicals used were of analytical grade. The chemical solutions were freshly prepared in distilled water prior to experimentation.

Test animal

This study has clearance from Institutional Animal Ethics Committee of Assam University, India. Inbred strains of Balb/C mice were purchased from Pasteur Institute (Shillong, India). Male and female mice between 6 and 8 weeks old, weighing 25–30 g, maintained at room temperature at 25.0 ± 5.0 ∘C with 12 h dark and 12 h light cycle were used for the study. Standard food pellets and water provided ad libitum.

Dose and treatment

MMC (2 mg/kg bw) and Cis (4 mg/kg bw) was used through i.p. route in an aqueous medium.

Chromosome aberration (CA) assay:

The CA assay was carried out as described by Krishna and Theiss [1] with minor modifications. Experimental animals were injected with 2 mg/kg b.w of colchicine1.5 h prior to sacrifice following 24h and 48h of the treatment of test chemicals. Bone marrow cells were collected by flushing with 0.56% KCl (pre-warmed at 37 ∘C) from femur bone and incubated for 20 min at 37 ∘ C. The material was centrifuged at 1000 rpm for 5 min, fixed with aceto-methanol (1:3) followed by refrigeration for 30 min. The material was centrifuged and re-suspended in fresh aceto-methanol. The slides were prepared by dropping the sample on

chilled slides and run over the flame. Staining was done in 5% buffered Giemsa stain (P^H 7.0) after 24h, air dried and covered with cover slips. At least 100 well spread metaphase plates were studied per animal for CA as indicated in **Table 1 and 2.**

Micronucleus (MN) assay:

After appropriate time of treatment, the animals were sacrificed by cervical dislocation and MN slides were prepared as described by Schmidt [2] with minor modifications. The bone marrow cells were flushed out and collected in 0.9% NaCl (pre-warmed at 37 ∘ C). Bone marrow smear was prepared, air-dried and fixed in absolute methanol for 10 minutes and stained with 5% buffered Giemsa (P^H 7.0) in the following day. The slides were mounted in DPX using cover slips. At least 2000 polychromatic erythrocytes (PCE) and corresponding normochromatic erythrocytes (NCE) were scored per animal. The PCEs stain light blue to gray and NCE's stain light pink to light yellow.

Sperm head abnormality assay

The protocol of Wyrobek and Bruce [3] was followed. Experimental animals were sacrificed by cervical dislocation after 35 days of treatment. Both the cauda epididymis were dissected out and placed in 1ml of 0.9% NaCl. The sperms were released by mechanical disruption and washing of the epididymis. The suspension was sieved through two layers of muslin cloth to remove tissue debris. A drop of the suspension was taken on a clean slide and a smear was made, air-dried, fixed in absolute methanol for 10 minutes. The slides were stained in the following day in 0.1% eosin Y for 10 minutes. One thousand sperms per animal were scored and the abnormalities categorized based on Wyrobek and Bruce categorization of various types of abnormal with many more additional types in the present study and also excluding some which were not observed in the present studies. The following types of abnormal headed sperms were considered in the present study. Normal, hookless, hooked, giant, dwarf, altered head, triangular, banana, needle, pin. head, amorphous, beaked.

Mean % aberrant Sperm values is calculated as shown below:

% Aberrant Sperms = Total number of aberrant sperm head studied / Total number of sperm head studied * 100

Statistical analysis

Student's t-test was used for comparing the level of significance between the treated and control groups as well as among the various treated groups.

Result and Discussion:
CA results:

In CA study (**Table 1 and 2),** the metaphase chromosomes of bone marrow cells of treated animals were analyzed for detecting abnormalities like chromatid breaks and gaps, isochromatid breaks and gaps , exchanges, Robertsonian translocations, sister chromatid unions, sticky chromosomes and pulverized metaphases. Total aberrations were expressed as mean value ± S.D (excluding and including gaps in separate columns). In addition to total aberrations, percent aberrant cells were also evaluated out of the total normal metaphases studied. In Table 1and 2 we see that MMC and CIS induced very significant level (P < 0.001) of CAs. The most frequent type of aberrations observed was the chromatid breaks. We observed that in both 24 and 48 hour study MMC induced CAs (excluding gaps) of 31.03 ± 1.56 and 28.33 ± 0.51 respectively which were statistically significant compared to control values of 2.30 ± 0.50. Similarly, it was also observed that at both 24 and 48 hour study of CIS treated groups induced CAs of 30.33 ± 2.25 and 19.66 ± 1.86 respectively compared to control values of 2.30 ± 0.54. A similar trend was observed with the CAs values including gaps.

Table 1 (24 hour)
Frequency of CAs in the bone marrow cells of mice induced by anticancer agent MMC and CIS

Treatment	Route	Total Cells (n)	Aberrant cells (Total)	% Aberrant cells ± S.D	Chromatid Break/Gaps		Isochromatid Breaks/Gaps		Exchanges	SCU	R.T.	Total Aberrations mean ± SD (excluding gaps)	Total Aberrations mean ± SD (including gaps)
Con	i.p	305/3	8	2.61 ± 0.48	7	1	0	0	0	0	0	2.30± 0.54	2.62 ± 0.48
MMC	i.p	306/3	88	28.75 ± 2.05	66	15	14	5	13	7	4	31.03±1.56***	37.57± 2.01***
CIS	i.p	300/3	80	26.66 ±3.14	50	4	6	1	7	12	14	30.33±2.25***	32.00±2.68***

Con: Solvent vehicle was given; MMC (2 mg/kg/bw); CIS (4 mg/kg/bw) i.p. = Intraperitoneal; SCU = Sister chromatid union; R.T. = Robertsonian translocation. n = Total number of animals; Groups bearing the any of the following symbol is significantly different from the Control groups, ***P<0.001.

Table 2 (48 hour)
Frequency of CAs in the bone marrow cells of mice induced by anticancer agent MMC and CIS

Treatment	Route	Total Cells (n)	Aberrant cells (Total)	% Aberrant cells ± S.D	Chromatid Break/Gaps		Isochromatid Breaks/Gaps		Exchanges	SCU	R.T.	Total Aberrations mean ± SD (excluding gaps)	Total Aberrations mean ± SD (including gaps)
Con	i.p	305/3	8	2.61 ± 0.48	7	1	0	0	0	0	0	2.30±0.54	2.62 ± 0.48
MMC	i.p	300/3	82	27.33± 1.86	65	15	5	0	5	7	3	28.33±0.51***	33.33±1.03***
CIS	i.p	300/3	56	18.66± 1.03	32	5	6	0	5	7	9	19.66±1.86***	21.33 ± 2.87***

Con: Solvent vehicle was given; MMC = Mitomycin C (2 mg/kg/bw); CIS = Cisplatin (4 mg/kg/bw); i. p = Intraperitoneal; SCU = Sister chromatid union; R.T = Robertsonian translocation. n = Total number of animals; Groups bearing the following symbol is significantly different from the Control groups, ***P<0.001.

Table 3 (24 and 48 hour)

Treatment	Route	Total PCE studied/n	Total PCE with MN	PCE with MN Mean ± SD	Total NCE studied	PCE/NCE Mean ± SD
24 Hour study:						
Con	i.p	6005/3	37	0.61± 0.06	4135	1.45± 0.01
MMC	i.p	6002/3	750	12.4± 0.08[***]	2870	2.09±0.00[***]
CIS	i.p	6000/3	257	4.28 ±0.22[***]	2705	2.21±0.18[***]
48 Hour study:						
Con	p.o	6005/3	37	0.61 ± 0.06	4135	1.45 ±0.01
MMC	i.p	6010/3	474	7.88± 0.08[***]	3623	1.70±0.00[***]
CIS	i.p	6000/3	198	3.30 ±0.20[***]	3623	1.65±0.07[***]

Con: Solvent vehicle was given. MMC = Mitomycin C (2 mg/kg/bw); CIS = Cisplatin (4 mg/kg/bw). PCE = Polychromatic Erythrocytes; NCE = Normochromatic Erythrocytes; n = Total number of animals; i.p: intraperitoneal; Groups bearing the following symbol, ***$P<0.001$ is significantly different from the Control groups.
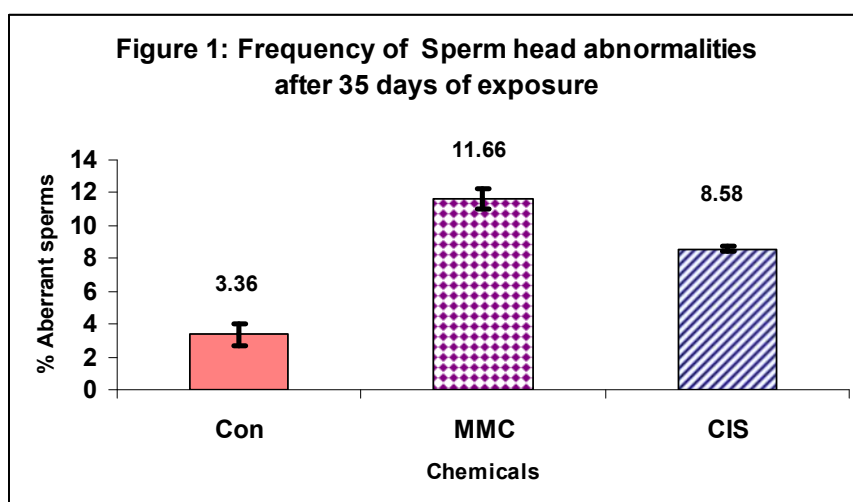
MN Results:

Micronucleus study was conducted based on the frequency of incidence of micronucleated polychromatic erythrocytes (PCE) out of the total 2000 PCEs' studied and cytotoxicity study was based on the ratio of polychromatic erythrocytes to normochromatic erythrocytes (PCE/NCE ratio). In this study in **Table 3**, it was observed that MMC (2 mg/kg/bw) and CIS (4 mg/kg/bw) induced significant level ($P < 0.001$) MN in polychromatic erythrocytes at both 24 and 48 hour study. It was also observed that there was a decline in the PCE/NCE ratio which was found to statistically significant when compared to the untreated control value.
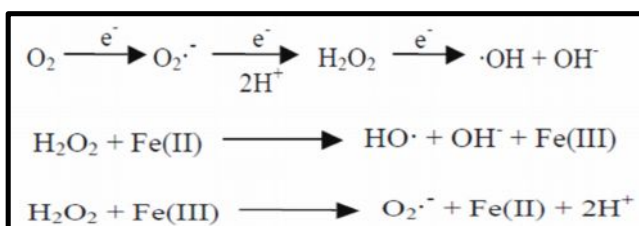
SHA Results:

Analysis of sperms after 35 days of exposure to MMC and CIS treated groups for aberrant head morphology represented in Figure 1 show significant rise ($P < 0.001$) when compared to control groups. Aberrant head morphology like amorphous, beaked, hooked, hookless, altered head, triangular, banana, pin-headed, giant, dwarf, double-headed etc were observed. The amorphous form was found to be the most frequent type of abnormality over all the other types in MMC and CIS treated groups.

Figure 1: Frequency of Sperm head abnormalities after 35 days of exposure

Discussion:

The chemotherapeutic drugs MMC and CIS employed in the experiments were chemicals with properties of known clastogenecity. In the present work, it was observed that these two test chemicals had induced significant level of genotoxicity (P < 0.001) in CA assay (Table 1 and 2), MN frequency (Table 3) and SHA assay (Figure 1) studied after 24 and 48 hour (in the case of CA and MN study) and after 35 days (in the case of SHA) of exposure when compared to untreated control groups.
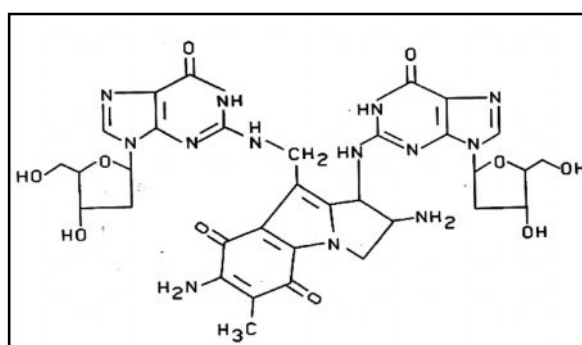
MMC which has been efficiently used in the treatment of various cancer like gastric cancer, pancreatic cancer, breast cancer, non-small cell lung cancer, cervical cancer, prostate cancer and bladder cancer [4]   was stated to possess a quinone chemical structure which through a cascade of bio-reductive process generates OH˙ radical of high reactivity which was considered to have potential to directly damage the DNA [5, 6] as well as other biomolecules of cell. Since, free radicals are highly reactive and termed as reactive oxygen species (ROS), they are prone to undergo reduction by oxidation of surrounding molecules (DNA, lipids, proteins). Oxidation (loss of electrons) and reduction (gain of electrons) are always coupled, and are termed as redox reactions and the damage brought to the biological molecules by such reactions leads to numerous pathophysiological disorders including aging and cancer [7, 8]. The major harmful ROS responsible for oxidative DNA damage in cells appears to be hydroxyl radicals (OH˙) generated by various reactions of hydrogen peroxide (H_2O_2). The hydrogen peroxide can be produced from the superoxide radical anion by either spontaneous or superoxide dismutase (SOD) mediated dis-proportionation. The hydrogen peroxide on reaction with the transition metal ions [9] like iron etc. gives rise to hydroxyl ion or superoxide radical. Such reactions are commonly known as the fenton reactions. Few reactions which can illustrate the mechanism of production of hydroxyl radical and superoxide radical are as follows:

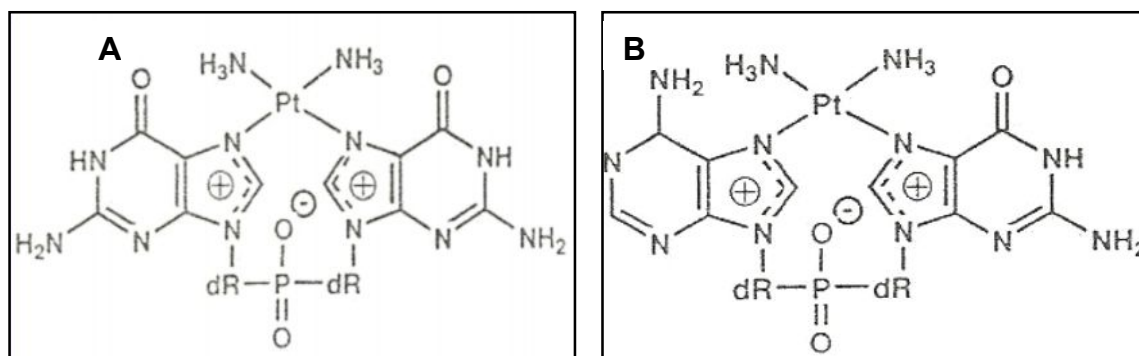**Figure 2: Free radical formation mechanism**

Other species may also contribute to DNA damage, including metal–peroxide complexes, singlet oxygen, and peroxynitrite [10, 11].   Since, MMC is a bioreductive alkylating agent, it also damages DNA by cross-linking bases in the same or adjacent strands of DNA principally at the N_2 position of the guanine (G) forming monofunctionally and biofunctionally alkylated G-MMC monoadducts and G-MMC-G interstrand and intrastrand cross-links at CpG and GpG sites, respectively [12] which may eventually lead to apoptotic cell death [13]. Various DNA-adducts formed by MMC has been identified and isolated in different cell types (one example is given in Figure 3). MMC has also been reported to activate caspase-3, caspase-8 and caspase-9 mediated apoptosis as well as necrosis [14].

Figure 3: The structure of a cross-link between Mitomycin C and DNA

Cisplatin, also called Cisplatinum and Cisdiamminedichlorido-platinum(II), is a platinum-based cancer chemotherapeutic drug, which has been used in the treatment of various types of cancers, including carcinomas, sarcomas, lymphomas and some germ cell tumors, (e.g., seminomas and germinomas). CIS was the first member of a platinum-based class of therapeutic agents, which now has other members too. It has the powerful property of being an antitumor agent and exerts its effect via interaction with DNA to produce cross-linked DNA adducts that activate checkpoint signaling pathways and thereby induce apoptosis [15]. As early as 1986, East-man suggested DNA to be the major target of CIS. Pinto and Lippard in 1985 [16] forwarded the concept that CIS causes inhibition of DNA synthesis by acting on DNA template rather then on DNA polymerase. The Cis configuration of CIS favors the formation of intra-strand crosslinks in DNA [16, 17]. Upon entering the cell, CIS is spontaneously hydrolyzed to a strongly electrophilic, bifunctional agent that platinates DNA through the positions occupied by chlorine atoms to the N7 position of deoxyguanosine and deoxyadenosine [16].

Figure 4: The major intrastrand dinucleotide DNA adducts [between two adjacent guanine (A) and between adjacent adenine and guanine (B)] formed by Cisplatin.

The major DNA adducts are the intrastrand cross-links that form between two adjacent guanines and between an adjacent adenine and Guanine (Figure 4). In addition, small quantities of monofunctional adduct, DNA-protein cross-links and DNA-interstrand cross-links are also produced [18]. Free radicals formation may be another important mechanism in the development of Cisplatin induced toxicity [19, 20]. It is considered and accepted that the toxicity of the drug CIS is contributed by the platinum metal present in its structure.

Conclusion:
Considering the severe cytogenetical consequences generated by the chemotherapeutic agents MMC and CIS, it can be concluded that application of alternative cancer therapy like application of natural products in the form of various antioxidants is far superior and better option over chemotherapy.

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