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Cytosine arabinoside-induced cytogenotoxicity in bone marrow and spermatogonial cells of mice and its potential transmission through the male germline

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ABSTRACT

Cytosine arabinoside (Ara-C), a widely prescribed antineoplastic drug, especially for the treatment of acute myeloid leukaemia, is a pyrimidine analog, in which the ribose sugar of cytidine is replaced by arabinose moiety. Ara-C reportedly competes with dCTP for incorporation into DNA during synthesis and exhibits various cytogenotoxic effects. In the present study, single intraperitoneal treatment of three different doses of Ara-C, 100, 150 and 200 mg/kg b.w. of mice, selected in accordance with its human therapeutic dose, induced statistically significant ($p \leq 0.01$) and dose dependent increase in the percentages of aberrant metaphases and chromosomal aberrations (CAs) at 24 h post-treatment, and micronuclei (MN) in polychromatic erythrocytes (PCEs) at 30 h post-treatment. However, there was no significant change in the mitotic index (MI) at 24 h post-treatment, when compared to that of the control mice. In the male germline, all the three doses of Ara-C induced statistically significant ($p \leq 0.05$ or $p \leq 0.01$) and dose dependent increase in the percentages of aberrant spermatogonial metaphases and CAs at 24 h post-treatment and statistically significant ($p \leq 0.01$) and dose dependent increase in the percentages of aberrant primary spermatocytes at week 4 post-treatment. However, the induction of abnormal sperm at week 8 post-treatment was decreased, although significantly. The results indicated that Ara-C was clastogenic to both bone marrow and spermatogonial cells of mice, and some of its cytogenotoxic effects were found transmitted through the male germline, at least up to the formation of primary spermatocytes. As the drug is not target specific, the induced cytogenotoxic effects of Ara-C on non-cancerous cells of cancer patients retain possible risk of recurrence of second malignancy among the post-chemotherapeutic cancer survivors. In addition, there is every risk of transmission of some induced genetic alterations to the next generation through the gametes of the cancer survivors pre-treated with this drug. Therefore, Ara-C essentially be made target specific.

1. Introduction

Antimetabolites constitute one of the important groups of antineoplastic drugs, particularly, with respect to their efficiency is concerned [1,2]. Its folic acid analogs and pyrimidine and purine analogs are widely prescribed for the treatment of various types of cancers. These drugs have successfully cured certain cancers and, at least, have enhanced the life expectancy of cancer patients. Some of such drugs are also routinely included in the treatment regimen of non-malignant diseases like rheumatoid arthritis, psoriasis, in organ transplantations etc. [3]. Its pyrimidine analogs comprise a diverse and interesting class of drugs that have the capacity of inhibiting biosynthesis of pyrimidine nucleotides, or mimicking the natural metabolites to such an extent that they interfere with vital cellular functions, particularly, in the synthesis and function of DNA [4]. Therefore, these drugs are mostly S-phase dependent in their actions.

In addition to the use of base analogs in cancer chemotherapy, nucleotides with sugar analogs are also synthesized for the purpose. Drugs with such alterations are prescribed mostly for the treatment of acute myeloid leukaemias [5,6]. One of the important modifications in the sugar moiety that have been made successfully is the replacement of the ribose of cytidine by arabinose yielding a very useful chemotherapeutic agent, cytosine arabinoside (1-β-D-arabinofuranosyl-cytidine, Ara-C). In Ara-C the hydroxyl group is attached to the 2′-carbon in the β, or upward configuration, as compared to the α, or downward position of the 2′-hydroxyl in ribose. The 2′-hydroxyl causes steric hindrance to the pyrimidine base around the nucleosidic bond.

The bases of poly-arabino-nucleotides fail to stack normally. Ara-C is also converted to 5′-monophosphate nucleotide (Ara–CMP),

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being catalysed by deoxycytidine kinase. This Ara–CMP can then react with appropriate nucleotide kinases to form diphosphate and triphosphate nucleotides like Ara–CDP and Ara–CTP. Accumulation of such Ara–CTP competes with dCTP for incorporation into DNA during synthesis [4–7]. Interestingly, a low concentration of Ara–CTP also inhibited DNA elongation [8]. Thus, on its incorporation, it blocks the elongation of DNA strand and also in its template function. It acts as a competitive inhibitor of DNA polymerases [7,9,10], blocks not only semi-conservative DNA synthesis but also polymerisation during excision repair pathways, induces abnormal duplication of DNA [11] leading to gene amplification in addition to breaks on DNA strand and fragmentation on chromosomes [12–17], and inhibits ligation of DNA strand breaks [18]. Although the precise mechanism of cell death caused by Ara-C is not known, fragmentation of DNA and apoptosis were observed in Ara-C treated cells [19].

Ara-C was reportedly enhancing the formation of topo-I cleavage complexes by being incorporated at the +1 position (immediately 3′) relative to a unique topo-I cleave site [20]. Its incorporation is known to alter base stacking and sugar puckering at the misincorporation site and at the neighbouring base pairs. Thus, the observed inhibition of religation at the Ara-C site suggests the importance of alignment of the 5′-hydroxyl end for religation with the phosphate group of the topo-I phosphotyrosine bond. Therefore, it was suggested that topo-I poisoning is a potential mechanism for Ara-C cytotoxicity [20]. Whatever may be the mechanism(s) involved, Ara-C induced non-random distribution of chromosomal aberrations (CAs) in human lymphocyte chromosomes [21] and micronuclei (MN) in mouse bone marrow [22,23]. Dose dependent induction of MN by Ara-C was shown both in peripheral blood reticulocytes and bone marrow of CD-1 mouse [24], and delay in cell cycle progression [25]. Ara-C-induced genotoxicity was also assessed from multiple parameters in mice both *in vivo* and *in vitro* [26]. It was reportedly an inhibitor of DNA synthesis, including repair synthesis [27–30]. The high gene density chromosome 1 was found more sensitive to repair inhibition by Ara-C than chromosome 4 [31]. Besides, depending upon the stage of cell cycle at the time of exposure to Ara-C, different types of CAs were induced [32–34]. However, a slight mutagenic effect for Ara-C was reported in one study [35].

Induction of homologous recombinations in high frequencies, which is an important mechanism in the etiology of cancer [36], by Ara-C [5,37], implicates a possible risk of induction of secondary malignancy in post-chemotherapeutic cancer survivors. Moreover, induction of aggressive tumour cell phenotypes in human melanoma cells has been reported after transient exposure to cytostatic concentration of Ara-C [38]. Therefore, detail assessment of the cytogenotoxicity of Ara-C is warranted. Although several earlier reports on its genotoxicity are available, they have their own limitations of being fragmentary, that to tested with unusually high doses and/or after chronic exposures. Whereas, low dose (in accordance with the human therapeutic dose) single exposure is more appropriate for cytogenotoxic assessments, both qualitatively and quantitatively. Besides, reports on the effects of Ara-C on germline cells and study on the potential transmission of such effects are hardly available. However, such studies are essential in mitigating the cytogenotoxic potential of the drug, in determining a suitable treatment schedule and in its dose escalation. Therefore, from extrapolation point of view, we assessed the Ara-C-induced cytogenotoxicity from three distinct end points from the somatic cell line and one from the male germline of mice, along with the assessment of potential transmission of such effects through the male germline, after a single intraperitoneal treatment of three different doses, selected in accordance with its human therapeutic dose.

2. Materials and methods

2.1. Test animals

Swiss albino mice (*Mus musculus*) of 8–10 weeks old were procured from M/s Ghosh Enterprises, Kolkata, India, and were acclimatized to the animal house of the department, maintained to a temperature of 24 ± 2 °C, 30–60% relative humidity and 12 h light/dark cycle, at least for a period of 4 weeks prior to their employment in the tests. The mice were fed with balanced diet and tap water *ad libitum*.

2.2. Test chemicals

Cytarine®, a cytosine arabinoside (Ara-C) injection, manufactured by Dabur India (Pharmaceutical Division) Ltd., New Delhi, was used as the test chemical. Each ml of the therapeutic preparation contains 100 mg of Ara-C and water for injection. Chemically, Ara-C is 1-β-D-arabinofuranosylcytosine with the empirical molecular formula $C_9H_{13}N_3O_5$.

Generally, two standard dosage schedules are recommended to patients for administration of Ara-C, (i) rapid i.v. injection of 100–200 mg/m^2 every 12 h for 5–7 days, or (ii) continuous i.v. infusion of 100–200 mg/m^2 daily for 7 days [2]. In the present study three different doses of Ara-C, 100, 150 and 200 mg/kg b.w. were selected for treatment to mice. The doses were selected in accordance with its human therapeutic dose after taking the body weight and surface area of the mouse compared with those of a human being into consideration.

Cyclophosphamide (CY), in Endoxan-Asta injection, manufactured by Khandelwal Laboratories Ltd., Mumbai, India, was used as the positive control chemical. CY, chemically 2-[bis(β-chloroethyl) amino]-1-oxa-3-aza-2 phosphocyclohexan-2-oxide monohydrate, the widely used positive control chemical in chemical mutagenesis tests, was tested here as the positive control chemical, at the rate of 40 mg/kg b.w. of mice. 0.9% sodium chloride was used as the vehicle/negative control. Maintaining the required concentrations, dilutions of the chemicals were made in such a way that in each treatment the mice received an intraperitoneal injection of the diluted chemical uniformly at the rate of 1 ml/100 g b.w.

2.3. End points studied

From somatic cell line, chromosomal aberrations and mitotic index (MI) were studied from the bone marrow cells at 24 h post-treatment and micronucleus test (MNT) was undertaken from polychromatic erythrocytes at 30 h post-treatment. From the male germline cells, metaphase chromosomal aberrations were studied from spermatogonia at 24 h post-treatment, primary spermatocytic chromosomes were analysed from diakinesis/metaphase-I stages at weak 4 post-treatment and sperm morphology assay was undertaken at weak 8 post-treatment.

2.4. Experimental protocol

Healthy mice, of approximately 20 g b.w. each, were selected and divided randomly into 5 groups of 30 each (6 females and 24 males). One group, the vehicle/negative control group, was treated with 0.9% sodium chloride at the rate of 1 ml/100 g b.w. Another group, the positive control group, was treated with CY at the rate of 40 mg/kg b.w. The rest three groups of mice were treated with Ara-C at the rate of 100, 150, or 200 mg/kg b.w. of mice.

At 24 h post-treatment 6 mice (3 females and 3 males) from each group were employed in CA and MI study from bone marrow cells. Another 6 male mice from each group were employed in spermatogonial metaphase CA study. At 30 h post-treatment 6 mice (3 females and 3 males) from each group were employed in MNT. From each group, 6 male mice at week 4 and the rest 6 at week 8 post-treatment were employed in primary spermatocytic chromosome analysis and sperm morphology assays, respectively.

2.5. Procedures

For mitotic metaphase CA study, slides were prepared from bone marrow of mice following the colchicine–sodium citrate hypotonic–methanol, glacial acetic acid-flame drying-Giemsa technique, described earlier [39]. From each animal 150 well-spread metaphases were scanned randomly and metaphases with normal chromosome complements and aberrant metaphases with CAs, like chromatid and chromosome gaps and breaks, fragments, minutes and exchanges, were recorded and some representative metaphases were photomicrographed. Percentage of aberrant metaphases (inclusive of metaphases with only gaps) and aberrations (excluding gaps) per hundred metaphases in them were calculated. From the same slides, more than 1000 cells from each animal were taken into consideration for MI study. The number of dividing and non-dividing cells in them was recorded and the percentages of dividing cells were calculated. For MNT from PCEs at 30 h post-treatment, a simple technique described earlier [40] was followed. About 2000 tinge-blue coloured PCEs were scanned from each animal and PCE with MN and MN per thousand PCEs were calculated.

For spermatogonial metaphase CA study at 24 h post-treatment, the flame drying-Giemsa technique reported earlier [41] was followed. More than 200 well-spread spermatogonial metaphases from each group of mice were examined