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Protective Effect of 2-Deoxy-D-Glucose on Chemotherapeutic Drugs Induced Damages on Peripheral Blood Lymphocytes Exposed *in-Vitro*

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KEYWORDS Bleomycin; Mitomycin-C; chromosomal aberrations; sister chromatid exchanges

ABSTRACT The effect of 2-deoxy-D-glucose (2-DG), an antimetabolite of glucose was studied in peripheral blood lymphocytes (PBL) exposed to radiomimetic drug bleomycin and an alkylating agent mitomycin-C. The PBL were exposed to 2-DG (5 mM), 30 minutes pretreatment and with Bleomycin (10 to 80 µg/ml) and Mitomycin-C (2 to12 µg/ml) for three hours. The drug as well as 2-DG was removed by washing the cells with HBSS buffer. Then the cells were cultured for 48 hours to study chromosomal aberrations (CA), Translocations (TL) and 72 hours for micronuclei (MN) and Sister Chromatid Exchanges (SCE). Exposures of PBL to Bleomycin and Mitomycin-C showed, a concentration dependent increase in the aberration frequencies, both in the presence and absence of 2-DG. While, the regression analysis showed, that the presence of 2-DG reduced bleomycin induced TL, CA frequencies and Mitomycin-C induced CA and MN frequencies significantly ($P<0.001$) when compared to PBL treated with the drugs alone, Bleomycin induced MN frequencies and Mitomycin-C induced SCE's reduction were not significant. The difference could be attributed to the mechanism of the action of drugs on the cells. Furthermore the alteration in the cell cycle kinetics, suggest that the presence of 2-DG during drug exposure, alter the cellular environment and delay the cell proliferation and provide sufficient time to repair the damages, could resulted in the reduced aberration frequencies.

INTRODUCTION

It is well known that most of the radiation used for therapy not only controls the division of tumor cells but also mutagenic to normal cells during multiple cycles of treatment (Bleehen et al. 1974). Therefore, studies were reported on the radio protective effect of compounds like 2-Deoxy-D-gluocose (2-DG), Hoechst (Denison et al. 1992) cysteine (Pattet et al. 1949), caffeine (Franchitto et al. 1998) and calcium channel blockers (Rajeeve and Kale 1995) on normal cells. Of which the protective effect of 2-DG was extensively studied in animal models (Jain et al. 1979) and in the lymphocytes of healthy individuals (Kalia et al. 1988; Prabhu et al. 2004) as well as glioma cells followed by radiation (Dwarakanath et al. 1989). In addition to radiotherapy, chemotherapy alone or in combination with radiation plays an important role to treat malignant diseases. The combined use of chemotherapy and radiotherapy, in patients may cause a degree of injury that would be clinically toxic following either therapy alone because, both may cause toxic to the same organ by different mechanisms. Furthermore, the response of cellular systems to therapeutic agents depend on its effect on DNA, types of lesions induced, capacity of the cells to repair those lesions and cell cycle kinetics (Jain et al., 1999). As our previous study had shown a protective effect of 2-DG on radiation induced chromosomal aberrations in the PBL (Prabhu et al. 2004), the present study focus on the effect of 2-DG on radiomimectic drug bleomycin and alkylating agent mitomycin-C induced damages in PBL treated *in-vitro*. The PBL were exposed to those drugs in the presence of 2-DG at G_0 phase of the cells, which is the normal stage of majority of the cells in the body at any given time, which enables to some extent that the overall response of tumors surrounding cell to therapeutic drugs. The 5 mM concentration of 2-DG was selected get an equimolar concentration of glucose in the normal blood samples.

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MATERIALS AND METHODS

In-vitro Treatment of Peripheral Blood lymphocytes to Bleomycin and Mitomycin-C: About 10 ml of peripheral blood was collected in a heparinised sterile container from a healthy

volunteer. The blood was divided into aliquots, suspended in RPMI-1640 medium treated with bleomycin (10 to 80 μg/ml) or mitomycin-C (2 to12 μg/ml) in the presence and absence of 2-DG (5 mM, 30-min before treatment) for 3 hours at 37°C. At the end of 3 hours the drugs and 2-DG were removed from the cells by washing with HBSS (Hanks Balanced Salt Solution) buffer for 3 times.

Culture Set up for Chromosomal Aberration: The blood samples treated as mentioned above were used for the preparation of metaphase chromosome for further analysis as described by (Venkatachalam et al. 1999). Briefly, to 1.0 ml of the blood sample, 10 ml culture medium (RPMI-1640) supplemented with 7.5 % $NaHCO_3$, 20% fetal calf serum, 200 mM L-Glutamine, penicillin 100 units/ml and streptomycin 100 mg/ml, was added. 200 ml of PHA-P was added to the culture to initiate cell division. At 46 hours, the cells were blocked at metaphase stage by adding colcemid at a final concentration of 0.1 mg/ml. The culture was further incubated until 48 hours. The sample was harvested by given hypotonic treatment (20 minutes with 0.45 % KCl at 37°C), washed thrice with Carnoy's fixative (methanol and acetic acid 3:1) and cast on clean pre-cooled slides. Multiple slides were casted for each sample and used for chromosomal aberration analysis and fluorescence in-situ hybridisation.

To analyse the chromosomal aberrations the slides were stained with 10% Giemsa, air-dried and mounted with coverslip using DPX. The slides were observed under microscope to record various types of aberrations like dicentric chromosome, ring chromosome, minutes, gaps, acentric fragments and chromatid gaps were recorded as described (Technical report 1986).

Fluorescence in-situ Hybridisation: The slide with metaphase chromosomes prepared as mentioned above was denatured for 6-8 min in denaturation solution at 70°C; dehydrated in 70%, 85% and 100% ethanol for 2 min each at room temperature and air dried. The whole chromosome probe (# 2 labelled with TRITC) was mixed with hybridisation buffer and deionised distilled water, denatured at 70°C for 5 min and applied to the slide, containing denatured chromosomes. The slides were sealed with coverslip using rubber cement and hybridisation was carried out for 24 hours at 37° C in moistened hybridisation chamber. After 24 hours hybridisation, the coverslip was removed and the slides are rinsed in fromamide wash solution three times, for 10 min each, 10 min in 2 × SSC and 5 min in 2 × SSC / 0.1% NP-40 at 45° C sequentially. After air drying the slides were counterstained with DAPI (7.5 μl/slide) and covered with coverslip. The number of painted chromosomes with and without translocation was recorded for each metaphase. The genomic translocation frequency was estimated from the fraction of genome painted as suggested by (Lucas et al. 1992). In case of chromosome-2, it comprises 8.34 % of genome (Mendelshen et al. 1973), and hence 16.68 % of total exchanges can be detected.

Micronucleus Assay: The blood samples were cultured in RPMI-1640 medium, supplemented with 20% fetal calf serum and 0.2 ml PHA-M (Paul et al. 1997). Cytochalasin - B at a final concentration of 3 μg/ ml culture was added at 44 hours of culture. The cells are further incubated for 28 hours at 37° C. The cells were harvested with brief hypotonic treatment and slides were prepared by fixing the cells with Carnoy's fixative. The cell suspensions were dropped on to a clear cooled slide and stained with Giemsa. Cells with two daughter nuclei surrounded by cytoplasm were scored for the presence of MN according to criteria of (Paul et al. 1997).

Cell Cycle Kinetics and Sister Chromatid Exchange Assay (SCE): Chromosome culture was set up, as in the case of chromosomal aberration assay, except for the addition of Brdu (100 μl/ 5ml) and the culture time was 72 hours. After the preparation of slides, metaphase chromosomes were stained with Hoechst (33258) for 10 minutes, washed with Mcllvaine's buffer, mounted temporarily with coverslip and exposed to sunlight for 2 hours. The slides were stained with 2% Giemsa, mounted with DPX and the stages of cell cycle and number of exchanges in the second cell cycle metaphases alone were recorded as per (Technical Report 1986).

Modulatory Factor: The modulatory effect of 2 DG was calculated as given by (Dwarakanath and Jain 1987).

$$\text{Dose reduction factor (r)} = \frac{\text{Drug induced aberration frequency with 2-DG}}{\text{Drug induced aberration frequency without 2-DG}}$$

Statistical Analysis: The regression analysis for the aberration frequency was carried out using the SPSS/PC+Software.

RESULTS

Aberration Frequency Induced by Bleomycin: Frequencies of TL, CA, and MN obtained from PBL treated with bleomycin in the presence and absence of 2-DG are given in Figures 1-3, respectively. The base line frequencies of TL in the control (0.046 ± 0.007) are higher than that obtained for DC (0.008±0.002) and MN (0.005±0.002). It was also observed that bleomycin induced a concentration dependent increase in the frequencies of all types of aberrations. Addition of 2-DG reduced the bleomycin induced TL frequency by 21-60%. Similarly for DC and MN it varies between 21-32% and 55-84%. The dose reduction factor (DRF) for TL, DC and MN are given in the Table 1. The regression analysis between the aberration frequencies induced by bleomycin with and without 2-DG showed the regression coefficient of 174.2 x 10^{-2} and 0.326 x10^{-2} for TL, 0.836 x 10^{-2} and 0.230 x 10^{-2} for CA and 0.292 x 10^{-2} and 0.249 x 10^{2} for MN for unit increase in the concentration. While, the obtained results showed a significant reduction for TL and CA frequency ($p<0.001$) in PBL treated in the presence of 2-DG, the reduction is not significant for MN.

Aberration Frequencies Induced by Mitomycin–C: Frequencies of MN, CA and SCE obtained from PBL treated with mitomycin-C in the presence and absence of 2-DG are given in Figures 4-6, respectively. Similar to bleomycin, mitomycin-C also induced a concentration dependent increase in the frequencies of all types of aberrations. Addition of 2-DG reduced the SCE frequency by 42-89% for various concentrations of mitomycin-C. Similarly for CA and MN it varies between 23-55% and 65-86%. The dose reduction factor (DRF) for SCE, CA and MN are given in the Table 2. The regression analysis between the aberration frequencies induced by mitomycin-C

Table 1: Dose reduction factor of 2-DG on aberration frequency on peripheral blood lymphocytes exposed to bleomycin

Concentration (μg/ml)	*Trans-locations*	*Chromosomal aberrations*	*Micronuclei*
10	0.47	0.21	0.55
20	0.39	0.24	0.84
40	0.6	0.32	0.1
80	0.21	0.27	0.83

Table 2: Dose reduction factor of 2-DG on aberration frequency on peripheral blood lymphocytes exposed to mitomycin-C

Concentration (μg/ml)	*Sister chromatid exchanges*	*Chromo-somal aberrations*	*Micronuclei*
2	0.42	0.51	0.86
4	0.52	0.59	0.67
8	0.83	0.55	0.65
10	0.78	0.54	0.73

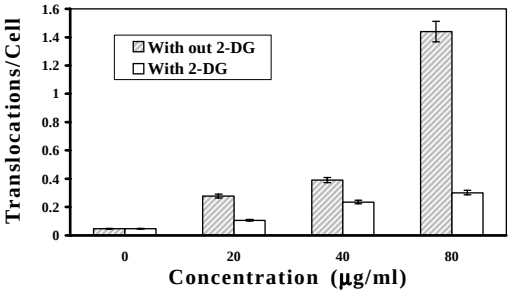

Fig. 1. Frequency of Translocations in peripheral blood lymphocytes exposed to bleomycin

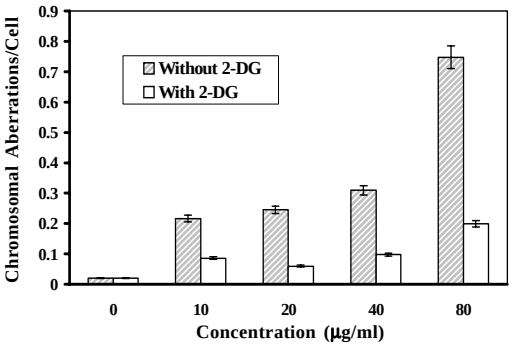

Fig. 2. Frequency of chromosomal aberrations in peripheral blood lymphocytes exposed to bleomycin

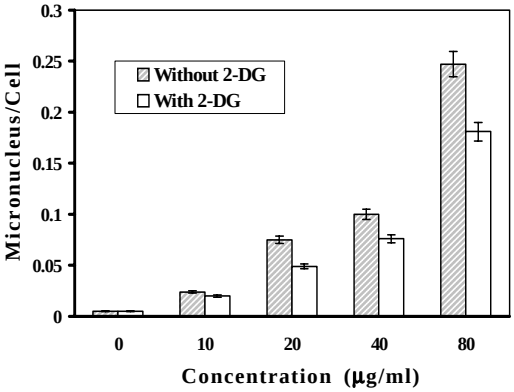

Fig. 3. Frequency of Micronucleus in peripheral blood lymphocytes exposed to bleomycin

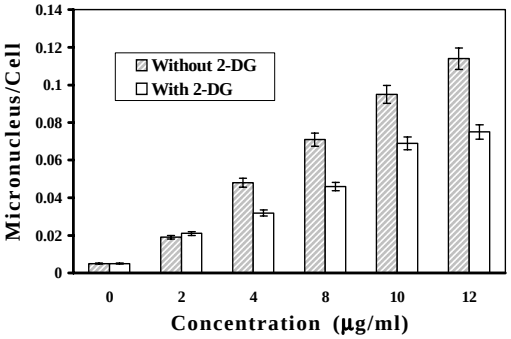

Fig. 4. Frequency of Micronucleus in peripheral blood lymphocytes exposed to mitomycin-C

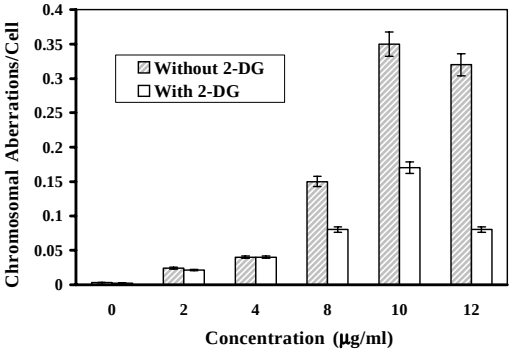

Fig. 5. Frequency of Chromosomal aberrations in peripheral blood lymphocytes exposed to mitomycin-C

with and without 2-DG showed the regression coefficient of 288.2 x 10^{-2} and 272.1x 10^{-2} for SCE, 2.927 x 10^{-2} and 1.070 x 10^{-2} for CA and 0.889 x 10^{-2}

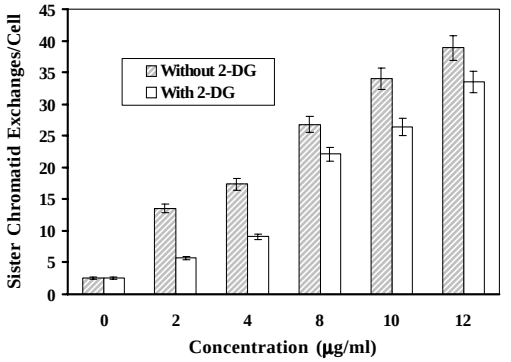

Fig. 6. Frequency of Sister Chromatid Exchanges in peripheral blood lymphocytes exposed to mitomycin-C

and 0.582 x 10^{-2} for MN for unit increase in the concentration. The results showed that 2-DG reduced the mitomycin-C induced CA and MN frequencies significantly ($p<0.001$) and the reduction is not significant for SCE.

Effect of Bleomycin and Mitomycin-C on Cell Cycle Kinetics: Both bleomycin and mitomycin-C reduces proliferation index (PI) and increase the Average Generation Time (AGT). Tables 3 and 4 show the effect of bleomycin and mitomycin-C on cell cycle kinetics. PBL treated with mitomycin and bleomycin, both in the presence and absence of 2-DG showed an increase in AGT and decrease in PI, in a concentration dependent manner. Furthermore, PBL treated with drugs in the presence of 2-DG, the AGT is more when compared with the absence of 2-DG.

Table 3: Cell cycle kinetics in peripheral blood lymphocytes exposed to bleomycin

Concentration	*Proliferative index (%)*		*Average generation time (h)*	
(µg/ml)	*With out 2-DG*	*With 2-DG*	*With out 2-DG*	*With 2-DG*
0	1.15	1.06	41.7	45.28
10	1.15	1.06	41.7	45.28
20	1.14	1.04	42.1	46.15
40	1.12	1.0	42.9	48.00
80	1.02	1.06	47.1	45.25

Table 4: Cell cycle kinetics in peripheral blood lymphocytes exposed to mitomycin – C

Concentration	*Proliferative index (%)*		*Average generation time (h)*	
(µg/ml)	*With out 2-DG*	*With 2-DG*	*With out 2-DG*	*With 2-DG*
0.0	2.30	2.30	31.30	31.30
2	1.85	2.16	38.91	33.30
4	1.94	1.80	37.11	39.80
8	1.97	1.70	36.54	41.90
10	2.03	1.36	35.54	52.94
12	1.93	1.60	37.30	45.00

DISCUSSION

The damage induced by therapeutic agents as well as mutagens, which leads to the loss of proliferative capacity in mammalian cells, is known to be located in the nucleus. Type of changes in DNA produced during the exposure, depending on the nature of cytotoxic agents. The study on the cytogenetic effects of therapeutic agents is of interest, because, the types of lesions and repair to the DNA are different among individuals and among tissues and response of patients to therapy. As the capacity to repair damages caused by cytotoxic agents is an important characteristic as well cellular system. Repair processes, which act in response to attack by cytotoxic agents profoundly, influence survival, growth, development and transformation of cells. Furthermore all those process are energy dependent events. Our earlier study on the PBL exposed to gamma radiation, showed a reduction in the frequencies of aberrations in the presence of 2-DG (Prabhu et al. 2004). To explore further, the effect of glucose antimetabolite 2-DG on bleomycin and mitomycin-C induced damages on PBL and cell cycle kinetics were studied.

Exposure to both drugs increased chromosomal exchanges, chromatid alterations and MN significantly in all the concentrations studied. Bleomycin induced chromosome as well as chromatid type exchanges, which showed a linear relation to the concentration. Whereas, mitomycin-C induced mainly chromatid type rather than chromosome type exchanges, because of its mechanism of action. Earlier reports showed bleomycin induces predominantly chromosome type aberration in Chinese hamster cells after treated *in vitro* (Kurten and Obe 1990) and in peripheral blood lymphocytes of treated patients (Schnizel and Schmid 1976). In the present study also, treatment with bleomycin induced predominantly chromosome type aberrations, which was evidenced with 90% of DC and rings were associated with fragments as well as translocations. Though the presence of 2-DG, reduces all type of aberrations, only bleomycin induced TL, CA and mitomycin-C induced CA and MN, showed a significant decrease in all the concentration studied. Similar reduction in the aberration frequencies was observed in normal lymphocytes (Kalia et al. 1988) mice (Jain et al. 1979) and tumor cell lines (Jain et al. 1979; Dwarakanath and Jain 1989) exposed to radiation in the presence of 2-DG. However, the bleomycin induced MN and mitomycin-C induced SCE frequencies were not reduced significantly of unknown reason. Generally, it is believed that SCE's is a measure of mutagenesis and therefore, 2-DG may not reduce the mutation frequency in normal lymphocytes.

General mechanism proposed for the reduction of damages on normal cells during radiation treatment is (I) Protecting the DNA against the induction of damage, (II) interference with the progression of cells in cell cycle and (III) modifications of repair of damages. It was shown that ATP synthesis and oxidative metabolism were essential for the repair of damages induced by radiation (Dwarakanath and Jain 1989). 2-DG, inhibitor of ATP synthesis through glycolytic pathway in cells, was observed to decrease the manifestation of radiation in yeast and human tumour cells (Jain et al. 1979; 1985). Cellular manifestation of radiation /chemotherapy depends on cellular dynamics like competitive process of DNA repair and fixation of lesions, which might show different energy dependence following treatment. Repair is facilitated under sub-optimal growth conditions and fixation is usually associated with cell proliferation. 2-DG reduces the proliferation-linked fixation of DNA lesions and permits the cells to repair the damages. It was reported that exposure to radiation induce a transient block in cell cycle progression, presumably permit the error free repair of DNA damages before the cell initiate the synthesis or mitosis. Similarly, in the present study also both drugs alter the proliferation kinetics of normal PBL by increasing the AGT. The delay or transient block in cell cycle by the drugs and decreased level of ATP maintain a sub-optimal conditions, favor more error free repair, there by reduction in the damages when cells are exposed to the drug in the presence of 2-DG. Therefore it has been suggested that 2-DG in combination with radiotherapy/ chemotherapy could lead an improvement in tumor therapy by inhibiting the repair process and enhance the damage by fixating the lesions.

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