PRINT: ISSN 0972-3757 ONLINE: 2456-6360
Int J Hum Genet, 4(1): 45-49 (2004)
DOI: 10.31901/24566330.2004/04.01.05

Effect of 2-Deoxy-D-Glucose on The Induction of Chromosomal Aberrations in Lymphocytes Exposed *in vitro* to Gamma Radiation at a Dose Rate of 1.0 Gy/Minute

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KEYWORDS 2-DG; dicentrics; radioprotection

ABSTRACT The glucose antimetabolite, 2-deoxy-D-glucose (2-DG) has previously been shown to be a radio-sensitiser in certain tumours and a protector in dividing lymphocytes. Since the majority of lymphocytes under normal physiological conditions are in Go stage and could be considered to represent tumour-surrounding tissues, they were exposed *in vitro* to gamma radiation from a Co-60 teletherapy unit at a dose-rate of 1.0 Gy / minute, in the presence and absence of 2-DG. The doses studied ranged from control to 4.0 Gy. Whole blood cultures were set up and the various types of chromosomal aberrations were analysed. The results indicated that there was a decrease in the frequency of dicentric chromosomes from the dose of 0.1 Gy up to 4.0 Gy. In case of excessive acentric fragments there was reduction at the doses of 0.05, 0.5, 1.0 and 3.0 Gy. Surprisingly there was an increase of double-minute chromosomes in the doses of 0.05, and from 2.0 to 4.0 Gy. The total chromosomal aberrations showed decrease in the doses from 1.0 to 3.0 Gy. The study indicates that 2-DG could be used as a radio protectant at clinically useful dose of 2.0 Gy and the median dose-rate of 1.0 Gy / min, apart from discussing the reasons for the decrease and increase of the various chromosomal aberrations.

INTRODUCTION

High dose-rate gamma teletherapy is used in therapeutic as well as in palliative capacities. For therapeutic purposes, the radiation dose usually is around 2.0 Gy with a dose-rate varying from 0.5 to 2.0 Gy / min. However there is a necessity to maximise the dose delivered to the tumour while care is to be taken from preventing the surrounding normal tissues from the incidental radiation. The purpose of a radio-modifier is to sensitise the cancerous tissue while protecting the normal surrounding tissues. It has been shown that the frequency of chromosomal aberrations (CA) as well as that of micronuclei (MN) were decreased in PHA-stimulated peripheral human leukocytes exposed *in vitro* to X-rays in the presence of 2-DG (Kalia et al. 1982). In the present study peripheral blood lymphocytes were irradiated in the presence of 2-DG at Go phase of the cell cycle, which is the normal stage of majority of cells in the body at any given time. This helps not only to asses the radio-protective effect of the 2-DG but also enables to some extent in understanding the overall response of the tumour surrounding tissues to radiation. The concentration of 2-DG was chosen to be equimolar (5 mM) to that of glucose in normal blood samples. The experimental dose-rate was chosen to be 2.0 Gy / minute and the doses ranged from 0.05 Gy to 4.0 Gy, apart from the control samples with and without 2-DG. This paper discusses the radioprotection offered by 2-DG and its clinical relevance.

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MATERIALS AND METHOD

Collection and Irradiation of Blood Samples: Blood samples were obtained from a volunteer who was normal, healthy, non-smoking, non-alcoholic, males with no previous medical / diagnostic exposures to radiation for the past couple of years. From him about 36 ml peripheral blood was collected in a heparinised vial. The blood was divided into 18 aliquots of 2 ml each. Among these 8 aliquots of the sample were irradiated with gamma doses varying between 0.05 and 4.0 Gy at a dose-rate of 1 Gy / min. using

teletherapy unit at Government Arignar Anna Memorial Cancer Hospital, Kancheepuram, India. As the time duration of exposure was only 4 minutes even for the highest dose of 4.0 Gy the samples were irradiated at room temperature itself. The next 8 aliquots were irradiated as mentioned above with the same amount of doses, but in the presence of 2-DG at a concentration of 5 mM (0.821 mg / ml). Two aliquots of 2 ml each were set aside as normal control and the other one with 2-DG but without irradiation as positive control, in order to check for any 2-DG induced chromosomal aberrations. After irradiation the blood samples were transported to the laboratory in an ice bath and were brought to 37^{O} C before setting up of the cultures.

Culture Set up for Chromosomal Aberrations: Whole blood culture was set up as described elsewhere (IAEA, 1986). Briefly to 0.5 ml of the blood sample, 5 ml culture medium (RPMI-1640) supplemented with 7.5 % $NaHCO_3$, 20 % foetal calf serum, 200 mM L-Glutamine, penicillin and gentamycin of 100 units/ml each and streptomycin 100 mg/ml, was added. 0.2 ml of phytohemagglutinin-M (PHA-M, GIBCO) was added to the culture to initiate cell division. At 46 hours, the cells were blocked at metaphase stage by adding colcemid to a final concentration of 0.1 mg/ml (150 ml). The culture was incubated for another two hours. The sample was harvested by centrifuging at 400 g (1000 rpm for 10 minutes) and the cell pellet was given hypotonic treatment with 0.45 % KCl for 20 minutes at 37^{O} C. The cells were washed with a mixture of methanol and acetic acid (3:1, Carnoy's fixative) for three times. After final wash with Carnoy's fixative, the cell pellet was suspended in a known volume of fixative (0.5 ml). Three drops of the cell suspension was then allowed to fall on a pre-cooled slide from a height of 10 cm. Two slides were casted for each sample. The slide was stained with 10% Giemsa and mounted with DPX. The slide was examined under 10 X 100 oil immersion and scored for the various chromosomal aberrations.

Statistical Analysis: The distribution of aberrations in cells was studied by the method described by Papworth and adopted by Savage (1975). This was done by the standard u test using the formula

$$u = \frac{d - (N-1)}{\sqrt{(\text{var } d)}}$$

Where N is the total number of cells scored, d is the coefficient of dispersion (N-1) s^2/Y, where Y is the mean number of observed aberrations, s^2/Y is the relative variance and var. d is the variance of d given by 2(N-1) (1-1/NY). This method makes use of the fact that variance(s) equals the mean (Y). The variance divided by mean is equal to 1, so that u = 0. p-values were calculated by unpaired Student "t" test using software named 'INSTAT'.

RESULTS

Table 1 gives the comparison between the frequencies of dicentric chromosomes obtained in the absence and presence of 2-DG. The frequency of DC chromosomes ranged from 0.0017 / cell to 1.05 and 0.62 in the absence and presence of 2-DG respectively. The distribution pattern was Poisson for all the doses studied. The control samples, with and without 2-DG gave the same frequency of DC chromosomes. The percentage decrease in the DC frequency ranged

Table 1: Comparison of dicentric chromosomes induced in peripheral blood lymphocytes on *in vitro* exposure to gamma rays at a dose-rate of 1.0 Gy / min. the presence and absence of 2-DG

S.No.	*Dose (Gy)*	*DC / Cell (±SE) (-2-DG) (TCS)*	*Dist Pat.*	*DC / Cell (±SE). (+2-DG) (TCS)*	*Dist. Pat.*	*% Decrease in DC*	*p-value*
1	0.0	0.0017 ± 0.0017 (600)	-	0.0017 ± 0.0017 (582)	-	-Nil-	-
2	0.05	0.0050 ± 0.0040 (400)	PD	0.0057 ± 0.0040 (350)	PD	-NA-	-
3	0.10	0.0100 ± 0.0070 (200)	PD	0.0057 ± 0.0057(175)	-	43	< 0.1
4	0.25	0.0200 ± 0.0141 (100)	PD	0.0100 ± 0.0100 (100)	-	50	< 0.1
5	0.50	0.0500 ± 0.0224 (100)	PD	0.0160 ± 0.0113 (125)	PD	68	< 0.1
6	1.0	0.1200 ± 0.0346 (100)	PD	0.0600 ± 0.0245 (100)	PD	50	< 0.1
7	2.0	0.3200 ± 0.0566 (100)	PD	0.1200 ± 0.0346 (100)	PD	63	< 0.001
8	3.0	0.5000 ± 0.0707 (100)	PD	0.2800 ± 0.0529 (100)	PD	44	< 0.01
9	4.0	1.0500 ± 0.1025 (100)	PD	0.6200 ± 0.0787 (100)	PD	41	< 0.001

DC – Dicentric Chromosomes TCS – Total Cells Scored
SE – Standard Error NA – Not Applicable
PD – Poisson Distribution

from 41% to 68% with the clinically relevant dose of 2 Gy showing a decrease of about 63% with highly significant decrease as indicated by the p-value (< 0.001). Significant decrease was also observed at the doses of 3.0 and 4.0 Gy.

In case of excessive acentric (ExAC) fragments the distribution pattern was Poisson for 0.5 and 1.0 Gy doses above that it was over dispersed up to 4.0 Gy in the absence of 2-DG. In the presence of 2-DG it was Poisson only in the dose of 2.0 Gy while for the doses of 1.0, 3.0 and 4.0 Gy it was over dispersed. Even though the frequency of ExAC decreased from 14% to 60% in the presence of 2-DG for various doses of 0.05, 0.5, 1.0 and 3.0 Gy doses significant decrease (p-value<0.05) was seen in 3.0 Gy dose only (Table2).

Double-minute chromosome (DM) is actually seen to increase in the presence of 2-DG (Table 3). The samples without 2-DG showed Poisson distribution for the doses from 2.0 to 4.0 Gy. In the samples with 2-DG the Poisson distribution was seen in 0.05 and 4.0 Gy doses, while it was over dispersed in 2.0 and 3.0 Gy doses. The percentage increase of DM chromosomes was from 57% to 88%, with the higher doses of 3.0 and 4.0 Gy showing highly significant (p-value < 0.0001) increase.

Table 4 shows the total chromosomal aberrations (TCA), which included all of the three types of chromosomal aberrations studied herein, obtained in the absence and presence of 2-DG. The TCA followed Poisson distribution for all the doses from 0.05 to 3.0 Gy while it was under dispersed at the highest dose of 4.0 Gy. Consolidated values for all the aberrations showed a decrease in their frequencies to be from 17% to 56%, while significant (p-value < 0.1) decrease was seen in the doses of 1.0 and 3.0 Gy and highly significant (p-value < 0.0001) decrease was seen in 2.0 Gy dose.

Table 2: Comparison of excessive acentric fragments induced in peripheral blood lymphocytes on *in vitro* exposure to gamma rays at a dose-rate of 1.0 Gy / min. the presence and absence of 2-DG

S.No.	*Dose (Gy)*	*ExAC / Cell (±SE) (-2-DG) (TCS)*	*Dist. Pat.*	*ExAC / Cell (±SE) (+2-DG) (TCS)*	*Dist. Pat.*	*% Decrease in DC*	*p-value*
1	0.0	0.0017 ± 0.0017 (600)	-	0.0017 ± 0.0017 (582)	-	-Nil-	-
2	0.05	0.0025 ± 0.0025 (400)	-	0.0029 ± 0.0029 (350)	-	14	< 0.1
3	0.10	0.0005 ± 0.0005 (200)	-	0.0000 ± 0.0000 (175)	-	-NA-	-
4	0.25	0.0100 ± 0.0100 (100)	-	0.0100 ± 0.0100 (100)	-	-Nil-	-
5	0.50	0.0200 ± 0.0141 (100)	PD	0.0080 ± 0.0080 (125)	-	60	< 0.1
6	1.0	0.0300 ± 0.0173 (100)	PD	0.0200 ± 0.0141 (100)	OD	34	< 0.1
7	2.0	0.1000 ± 0.0316 (100)	OD	0.1200 ± 0.0346 (100)	PD	-NA-	-
8	3.0	0.1500 ± 0.0387 (100)	OD	0.0600 ± 0.0245 (100)	OD	60	< 0.05
9	4.0	0.2600 ± 0.0509 (100)	OD	0.2400 ± 0.0489 (100)	OD	-NA-	-

ExAC- Excess Acentrics
PD – Poisson Distribution
OD-Over Dispersion
TCS – Total Cells Scored
SE – Standard Error
NA – Not Applicable

Table 3: Comparison of double-minute chromosomes induced in peripheral blood lymphocytes on *in vitro* exposure to gamma rays at a dose-rate of 1.0 Gy / min. the presence and absence of 2-DG

S.No.	*Dose (Gy)*	*DM / Cell (±SE) (-2-DG) (TCS)*	*Dist. Pat.*	*DM / Cell (±SE) (+2-DG) (TCS)*	*Dist. Pat.*	*% Increase in DC*	*p-value*
1	0.0	0.0017 ± 0.0017 (600)	-	0.0000 ± 0.0000 (582)	-	-NA-	-
2	0.05	0.0025 ± 0.0025 (400)	-	0.0086 ± 0.0049 (350)	PD	71	< 0.1
3	0.10	0.0000 ± 0.0000 (200)	-	0.0000 ± 0.0000 (175)	-	-NA-	-
4	0.25	0.0000 ± 0.0000 (100)	-	0.0100 ± 0.0100 (100)	-	-NA-	-
5	0.50	0.0000 ± 0.0000 (100)	-	0.0000 ± 0.0000 (125)	-	-NA-	-
6	1.0	0.0100 ± 0.0100 (100)	-	0.0100 ± 0.0100 (100)	-	-Nil-	-
7	2.0	0.0200 ± 0.0141 (100)	PD	0.0600 ± 0.0245 (100)	OD	57	< 0.1
8	3.0	0.0200 ± 0.0141 (100)	PD	0.1700 ± 0.0412 (100)	OD	88	< 0.0001
9	4.0	0.0500 ± 0.0224 (100)	PD	0.2800 ± 0.0529 (100)	PD	82	< 0.0001

DM - Double - Minute Chromosomes
SE - Standard Error
PD - Poisson Distribution
OD - Over Dispersion
TCS - Total Cells Scored
NA - Not Applicable

Table 4: Comparison of total chromosomal aberrations induced in peripheral blood lymphocytes on *in vitro* exposure to gamma rays at a dose-rate of 1.0 Gy / min. the presence and absence of 2-DG

S.No.	*Dose (Gy)*	*DC / Cell (±SE) (-2-DG) (TCS)*	*Dist. Pat.*	*DC / Cell (±SE) (+2-DG) (TCS)*	*Dist. Pat.*	*% Decrease in DC*	*p-value*
1	0.0	0.0050 ± 0.0029 (600)	PD	0.0034 ± 0.0024 (582)	PD	- NA-	-
2	0.05	0.0100 ± 0.0050 (400)	PD	0.0200 ± 0.0076 (350)	PD	- NA-	-
3	0.10	0.0015 ± 0.0087 (200)	PD	0.0057 ± 0.0057 (175)	-	- NA-	-
4	0.25	0.0300 ± 0.0173 (100)	PD	0.0300 ± 0.0173 (100)	PD	-Nil-	-
5	0.50	0.0700 ± 0.0265 (100)	PD	0.0240 ± 0.0139 (125)	PD	56	-
6	1.0	0.1700 ± 0.0412 (100)	PD	0.0800 ± 0.0283 (100)	PD	53	< 0.05
7	2.0	0.4500 ± 0.0671 (100)	PD	0.2300 ± 0.0479 (100)	PD	49	< 0.001
8	3.0	0.6900 ± 0.0831 (100)	PD	0.4800 ± 0.0693 (100)	PD	31	< 0.05
9	4.0	1.4000 ± 0.1032 (100)	UD	1.1800 ± 0.1086 (100)	UD	17	< 0.1

TCA - Total Chromosomal Aberrations SE - Standard Error PD - Poisson Distribution
UD - Under Dispersion TCS - Total Cells Scored NA - Not Applicable

DISCUSSION

In the present work the effect of 2-DG on normal peripheral human blood lymphocytes exposed to *in vitro* to a dose-rate of 1.0 Gy / minute, Co-60 gamma radiation was analysed using chromosomal aberration assay. The initial step was to optimise the concentration of 2-DG as it was crucial for its effect on energy metabolism, cell proliferation kinetics, radio protective action and its cellular response to radiation damage. It has been shown in literature that the various concentration of 2-DG have been used for uses as diverse as radioprotection of normal cells (Kalia et al. 1982) to radio sensitisation of tumour cells (Jain et al. 1985). Since the concentration of glucose in normal human beings happens to be around 5 mM (1.2 mg / ml) a similar concentration of 2-DG (0.821 mg / ml) was chosen to test first its effect on incubation with normal human blood. It was also known that 2-DG could inhibit growth of cells (Jain et al. 1977), which could be reversed even after 50 hrs. of incubation or contact with 2-DG at equi-, double- or even treble molar concentrations with that of glucose (2-DG / G = 1,2,3), in case of Ehrilich Ascites Tumor cells. Similar results have also been reported for human cell cultures like He La cells derived from human carcinoma (Dwarakanath and Jain 1987). Moreover the equimolar concentration was preferred in the present study as the effect of 2-DG could be easily reversed by just adding a few mg of glucose, as it was done when the cells were initiated into culture by transferring 0.5 ml of irradiated blood into 4 ml of medium, when 2-DG concentration in the total cultured components would fall by about 10 folds to about 0.5 mM compared to that of the glucose concentration in the culture medium at about 5 mM. It was also seen that the incubation of 2-DG alone with the lymphocytes without any irradiation resulted in no significant (p-value < 0.1) increase in the frequency of any chromosomal aberration studied. This indicated that 2-DG incubation alone per se did not give rise to any type of aberrations and hence the aberrations obtained by irradiation were only due to the dose delivered at least in the equimolar concentration of 2-DG. This observation has vital bearings on the further usage of 2-DG because the action of 2-DG, may not be interfering with normal metabolism of cell at the genetic level during incubation / irradiation and later while the cultures were being initiated.

The results showed that in the dicentric chromosomes except for the lowest dose of 0.05 Gy, there was a decrease in the DC aberrations frequency from 0.1 to 4.0 Gy doses and highly significant reduction is seen in the 2.0 Gy dose. This indicates that the chief double strand damage induced by radiation, namely dicentric chromosomes are indeed reduced in the presence of 2-DG and since the clinically important dose of 2.0 Gy shows the maximum reduction from the statistical point of view.

In case of excessive acentrics the occurrence was seen to vary between the doses studied and even though there was the general trend of reduction (14% to 60%) in their frequencies when irradiated in the presence of 2-DG a series of values did not emerge. This might be due to the fact that they form only about 20% to 40% of the DC chromosome frequency.

An interesting observation was seen in case of the double-minute chromosomes, which are by themselves rare but seen in the higher dose of 2.0, 3.0 and 4.0 Gy. In the highest doses of 3.0

and 4.0 Gy the actually increased in the samples irradiated in presence of 2-DG as opposed to its absence. Superficially they might contraindicate the protective effect of 2-DG in normal cells exposed to radiation, but they could actually indicate a repair mechanism, probably non homologous end joining, as they have been shown to increase in low dose-rate irradiation experiment (Karthikeya Prabhu et al. 2003) were when other types of aberrations like DC chromosomes come down DM chromosomes actually increases. Thus the occurrence of increased frequency of DM chromosomes in the higher doses points to the fact that 2-DG is indeed acting as an efficient radio protectant

The total chromosomal aberration frequency included the major aberrations like dicentrics, excessive acentrics and double-minute chromosomes only. Even though aberrations like centric and acentric rings were seen they were less compared to the other major aberration types and also were not seen in all the doses to account for any statistically relevant conclusion. The results were particularly interesting because even though the DC chromosomes, which form the bulk of aberrations showed reduction in all doses above 0.05 Gy the total chromosomal aberrations derived from them were significantly (p-value < 0.05) reduced only in 1.0 and 3.0 Gy doses, while it was highly significant in the dose of 2.0 Gy. This might be due to the fact that its decrease, which increases up to 2.0 Gy and then comes down to not significant levels (p-value < 0.1) at the dose of 4.0 Gy, probably indicates the dose level up to which radio protection could be offered by this compound. It is also clinically significant because 2.0 Gy is the dose at which the fractionated radiotherapy is carried out.

Finally this cytogenetic study involving 2-DG and high dose-rate gamma radiation has once again confirmed the emerging concept that the increase of radio resistance in cells, caused by radio protectors like 2-DG which has been tried and testified as a radio sensitizer for neoplastic cells (Kalia and Jain 1987; Purohit et al. 1983; Purohit and Pohilit 1982) has another application, a rather drastic one, i.e., it can also be exploited as a radio protectant in normal cells, like that of the peripheral blood lymphocytes as exemplified by the present study.

ACKNOWLEDGEMENT

Authors wish to acknowledge Dr. V. S. Jeyaraman, M.D., D.M.R.D., Director, Arignar Anna Memorial Govt. Hospital and Research Institute, Kancheepuram for granting permission to use the infrastructure facilities of that centre. Thanks are also due to the radiographers who helped in irradiating the blood samples. This study was partially funded by the Extended Senior Research Fellowship awarded by Council of Scientific and Industrial Research, Government of India.

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