

Radio-Protective Effect of 2-Deoxy-D-Glucose in Cervical Cancer Patient's Lymphocytes Exposed *in Vitro* as Estimated by the Comet Assay

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ABSTRACT The main objectives of the present study was two fold, namely, first to evaluate the baseline Single-Strand Breaks (SSBs) frequency in Cervical Cancer patient's and secondly, to study the radio-protective effect of 2-deoxy-D-glucose (2-DG) on such cells when exposed to ionising radiation like that of gamma radiation. The reason for proposing such a study is that the DNA of cancer patients has a general tendency to "break", i.e. "Concept of Common Fragile Sites". This is not yet clearly understood as to whether it is the cause or effect of the pathological processes and necessitates looking into the base-line SSBs in this type of patients. Secondly, the main line of treatment of Cervical Cancer is that of radiotherapy & / or surgery, therefore there is a need to protect the normal cells surrounding the tumour tissue during the course of radiotherapy. This could be achieved by means of a radio-modulator such as 2-DG, which protects normal cells and sensitises the tumour tissue, thus acting as a double-edged sword. Hence the study was designed to evaluate the SSBs by Comet Assay (Single cell Agarose Gel Electrophoresis – SAGE) using a silver staining method, which does not require the usage of fluorescent microscopy and can be analysed with the aid of a simple light microscopy. The results showed that the Cervical Cancer patient's peripheral blood lymphocytes are not entirely fragile and the radiation exposure leads to increasing levels of DNA SSBs, which is reduced by 2-DG showing that the compound can be used as an adjuvant to radiotherapy.

INTRODUCTION

Cancer (Kardinal et al. 1979; Diamandopoulos 1996; Gallucci 1985) has become one of the ten leading causes of death in India. It is estimated that there are nearly 1.5-2 million cancer cases at any given point of time. Over 7 lakh new cases of cancer and 3 lakh deaths occur annually due to cancer. Nearly 15 lakh patients require facilities for diagnosis, treatment and follow up at a given time. Data from population-based registries under National Cancer Registry Programme (Rao et al. 1991) indicate that the leading sites of cancer are oral cavity, lungs, oesophagus and stomach among men and cervix, breast and oral cavity among women. Cancers namely those of oral and lungs in males, and cervix and breast in females account for over 50% of all cancer deaths in India.

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Cervical cancer is a disease caused by the abnormal growth and division of cells that make up the cervix, which is the narrow, lower end of the uterus (Contras et al. 1989). This is an important type of cancer in Indian population, especially the rural population and since nearly 80% of the population in India lives in villages it is very important from the health as well as the socio-economical viewpoint. The main line of treatment of this type of cancer is exclusively Radiotherapy (RT) (Sundar et al. 2005) or surgery (Harvey et al. 1974) followed by RT. Even at the beginning of the 20th century, shortly after radiation began to be used for diagnosis and therapy, it was discovered that radiation could cause cancer as well as cure it. This necessitated lower levels of fractionation of doses followed by adjuvants to reduce the effects to surrounding cells (Hall 1972). The best studied such a radio-modulator candidate is a caloric-restriction mimetic or anti-metabolite, 2-deoxy-D-glucose (2DG) (Kalia et al. 1982, 1987; Dwarakanath et al. 1987, 1991; Jain et al. 1977, 1985), works by interfering with the way cells processes glucose.

2DG protects the formation of double stranded breaks (DSBs) in normal lymphocytes from healthy volunteers and hence was selected to assess the effect of the same on lymphocytes obtained from Cervical Cancer patients, as when the cells were exposed *in vitro* to different doses of radiation. The method of assessment was by means of comet assay and the SSBs were screened as the cytogenetic end point because it is these that give rise to DSBs and are also formed more abundantly than DSBs.

MATERIALS AND METHODS

Irradiation of Blood Samples: Blood sample of about 25 ml from a Cervical Cancer patient was obtained after informed consent, divided into 10 aliquots of 1 ml each for the comet assay and 3 ml for cell viability, which was irradiated at different doses from 0.05 to 5.0 Gy, using a Co-60 teletherapy unit at Government Aringar Anna Memorial Cancer Hospital, Kancheepuram, India. The samples were irradiated at room temperature, as the duration of exposure was very short varying between 3 seconds to 5 minutes. The samples were transported to the laboratory from the hospital in an ice bath.

Separation of Lymphocytes: Lymphocytes were separated from peripheral blood by density gradient method using Histopaque-1077. Three ml Histopaque was taken in a conical test tube and 3 ml of blood sample was carefully layered on the above solution. This was centrifuged at 2200 rpm for 30 minutes. Erythrocytes and the granulocytes sedimented at the bottom of the centrifuge tube, while lymphocytes formed a buffy coat over the Histopaque layer. The upper layer contained plasma. The buffy coat was carefully aspirated with a Pasteur pipette and carefully transferred to a conical tube and the plasma was used as serum supplement in all the experiments. The lymphocytes obtained were washed with 6 ml PBS and centrifuged at 1200 rpm for 10 minutes. The cell pellet was again washed with PBS for another 4 times as above. For the comet experiments, 1 ml of the blood sample was over laid on 1 ml of Histopaque solution and lymphocytes were separated as above.

Comet Assay (Or) Single-Cell Agarose Gel Electrophoresis (SAGE): The single-cell agarose electrophoresis (SAGE), popularly known as the comet assay is a simple, versatile and visually

pleasing technique that is widely being used in detecting and quantifying DNA damage and repair. Ever since Singh et al. (1988) standardised the SAGE assay, different types of DNA damage, caused by a variety of chemical and physical mutagens have been studied (Tice 1995).

The present investigation was undertaken to check whether the compound 2-DG acts as a radio-protective agent in human peripheral lymphocytes obtained from cervical cancer patient's blood samples. Accordingly the comet assay was employed to study the rate of DNA SSBs induced in the lymphocytes after their exposures (as whole blood samples) to different doses, from 0.0 (control) to 5.0 Gy at a dose-rate of 1.0 Gy / minute. The method of Singh et al. (1988), modified by Gedik et al. (1992) with silver staining was used in this study (Kizilian et al. 1999).

Preparation of Slides: 75 ml of 1% normal agarose in PBS at 65°C was dropped gently on to a fully frosted micro-slide, covered immediately with a cover slip and placed over a frozen ice pack for about 5 minutes. The cover slip was removed after the gel had set. The lymphocyte suspension from a particular exposure-dose was mixed with 1% low melting agarose at 37°C in a 3:1 ratio. 75 ml of this mixture was applied quickly on top of the gel coated over the micro-slide and allowed to set as before. A third coating of 75 ml of 1% low melting agarose was given on the gel containing the lymphocyte suspension and allowed to set. Similarly, slides were prepared in duplicates for each lymphocyte fraction.

Cell Lysis: After solidification of the agarose the cover slip was removed and the slides were immersed in ice-cold lysis solution and placed in a refrigerator at 4°C for at least 1 hr. All the above operations were performed in low lighting conditions in order to avoid additional DNA damage.

Electrophoresis: After removing the slides from the lysing solution, were placed horizontally in an electrophoresis tank. The reservoirs were filled with electrophoresis buffer until the tanks were just immersed in it. Slides were allowed to stand in the buffer for about 20 minutes to allow DNA unwinding, after which, electrophoresis, was carried out at 0.6 V / cm for 15 min.

After electrophoresis the slides were removed and washed thrice in neutralisation buffer and gently dabbed to dry. A few drops of the working

solution of the silver stain was added in a dark room followed by washing in the destaining solution after a 15 min. exposure, as stated in the silver staining kit, CometAssay™ Silver, RnD Systems, U.S.A. The stained DNA in the cells was examined at 20x and 40x magnification. The lengths of DNA migration (comet tail) in these cells were measured directly by fixing an ocular micrometer in one of the eyepieces of the microscope. About 150 comets were scored per point.

The cells display the typical comet appearances. The undamaged nuclear core and the damaged DNA is extended as a tail, resembling a comet. In the absence of a strand break(s) the DNA is unable to unwind and hence does not move in an electrostatic field thus resulting in the absence of a tail formation, indicating an intact nuclear material.

Calibration of Ocular Micrometer: Comet's tails observed under the microscopes were measured using an ocular micrometer (OM) and a stage micrometer (SM). Each SM division was considered to be equal to 10 microns. The initial and the first coincidences of the divisions on the OM and SM were noted. The total units covered by the OM and SM for a specific length were read for a given set of ocular and objective lens as:

One OM div. =

$$\frac{\text{Total no. of units covered by SM}}{\text{Total no. of units covered by OM}} = \times 10\mu$$

RESULTS

The various irradiation points and the relevant comet formation were tabulated. The tests yielded large amount data in form of comet tail lengths with regard to increasing dose. The overall view present by the experiment was that as dose increase the damage, namely, single stranded breaks, increase in a directly proportional way and reached a peak value of damage at the LD50 dose of 4.0 Gy beyond which the damage slightly decreased at 5.0 Gy dose.

In the absence of any radiation, the lymphocytes of the cervical cancer patient's showed very good integrity as nearly 90% of the cells had a diameter ranging from 20 to 40 mm, which means that their DNA were intact without any SSBs and no significant tail formation was seen. The remaining 10% showed very little damage as only tail lengths of about 40 to 60 mm were seen. This might be due to the environmental factors inducing the SSBs as well as the cancer patient's cells predisposition to form spontaneous breaks, indicating the concept of "Fragile Genome". Beyond that point the cells exhibited different tail lengths indicating the various degree of damages (Table 1). Similar set of experiment was carried out for all the doses in the presence of 2-DG and the data obtained is given in Table 2.

Table 1: Different doses and relative comet lengths on exposure to gamma radiation

DNA Migration [Comet tail length in μm] & Grading	Radiation dose (Gy)									
	0.0 (Control)	0.05	0.10	0.25	0.50	1.0	2.0	3.0	4.0	5.0
Excellent(20-40 μm)	90%	80%	70%	60%	50%	40%	30%	20%	10%	25%
Good(41-60 μm)	10%	10%	10%	10%	10%	10%	10%	10%	10%	10%
O.K.(61-80 μm)	-	10%	10%	10%	15%	20%	15%	10%	10%	10%
Damaged(81-100 μm)	-	-	10%	15%	20%	25%	30%	40%	45%	50%
Highly Damaged(101-120 μm)	-	-	-	5%	5%	5%	15%	20%	25%	5%

Table 2: Relative comet lengths obtained in the presence of 2-DG

DNA Migration [Comet tail length in μm] & Grading	Radiation dose (Gy)									
	0.0 (Control)	0.05	0.10	0.25	0.50	1.0	2.0	3.0	4.0	5.0
Excellent(20-40 μm)	95%	95%	80%	70%	60%	50%	40%	30%	20%	35%
Good(41-60 μm)	5%	5%	10%	20%	20%	20%	25%	20%	35%	15%
O.K.(61-80 μm)	-	-	10%	10%	15%	20%	20%	25%	25%	20%
Damaged(81-100 μm)	-	-	-	-	5%	10%	10%	25%	20%	30%
Highly Damaged(101-120 μm)	-	-	-	-	-	-	5%	5%	10%	5%

DISCUSSION

The main objectives of the present study was two fold, namely,

- i. First to evaluate the baseline SSBs frequency in cervical cancer patient's and
- ii. Secondly, to study the radio-protective effect of 2-DG on such cells when exposed to ionising radiation likes that of gamma radiation.

The reasons for proposing such a study is that it is well known that the DNA of cancer patients have a general tendency to "break", i.e. the double bands, the cross links and other such forces that hold the DNA together tend to be disrupt automatically without any external stimuli. This is called as the "Common Fragile Site Syndrome" (Arlt et al. 2003; Hecht et al. 1984; Murthy et al. 1985; Reidy 1988; Sevankaev et al. 1974) and has been studied extensively using several techniques as well as by many researchers. Earlier study (Venkatachalam 1999) has shown higher levels of chromosomal aberrations and micronuclei in Cervical Cancer patient's lymphocytes. The reason is not yet clearly understood, even the fact that whether this is the cause for cancer predisposition or because of cancer such fragility arises is also not yet clearly known. However the fact remains that these people (Cancer patients) tend to develop several syndromes associated with genetic and biochemical disorders. Thus the basic aim was to characterize the baseline damages in such patients.

The second objective was that when cancer patients are given radiation treatment as in the case of cervical cancer, which ranks as the second most important cancer in women of this world and the primary cancer in rural India, the treatment itself induces several damages in the surrounding cells when the tumour gets irradiated, leading to "Treatment Induced Tumours" or "Secondary Tumours". In order to avoid this and also to enhance the killing of tumour tissue effective, certain compounds like 2-DG (Kaplan et al. 1990; Purohit et al. 1982, 1983; Singh et al. 1990; Jain 1993) have been tried out and these tend to sensitise the tumour while the normal surrounding cells are protected, thereby better treatment and latter good prognosis of the disease are obtained. Hence this aspect deals with the application part of the study.

The control values obtained by the comet

assay for those samples with and without 2-DG incubation were interesting because in the presence as well as in the absence of 2-DG the cells were mostly (~90%) intact with no obvious comet formation. This indicates primarily that the cancer patient's lymphocytes do not show much SSBs even though they are said to be fragile. Secondly the presence of 2-DG did not enhance the level of baseline damage seen in the cells, even though 2-DG is an anti-metabolite, by reacting with the cellular glycolysis pathway and reducing anaerobic production of ATP molecules. This is vital because if 2-DG itself induces such damages then it may not be a suitable compound to be considered as an adjuvant to radiotherapy of tumours like that of cervix.

The mere induction of SSBs when the cells were irradiated to different doses of gamma radiation clearly indicated that these cells were predispose to such damage and then the rise in damage in a directly proportional to the incurred dose showed a linear dose response (Hecht et al. 1984; Reidy 1988). This reached a peak when the cells reached a dose of 4.0 Gy, while the dose was further increased the total cells without damage slightly increased. This might be contra indicatory but if the concept of LD50 is invoked it could be easily explained. The cells are constantly damage using radiation, while after reaching the peak levels of damage by 4.0 Gy the remaining cells are "resistant" and hence increase in dose does actually reduce the level of damaged cells but the overall viable cells decrease, due to spontaneous cell death or apoptosis arising out of radiation injury and insult by free radicals. Thus this part high lights that there is a peak (4.0 Gy) up to which the radiation injures the cells and the a slight reduction in damage occurs not due to increase cell resistivity but because vulnerable cells are eliminated and only metabolically hardy cells survive at the highest dose studied (5.0 Gy). This is a good finding because then the concept of LD 50 dose is once again validated from a radiobiological viewpoint as well as from the physiological aspect also. Moreover another aspect is that the cancer cells also have a subpopulation of cells that are somewhat resistant to radiation itself and may even be these cells if developed into cancerous ones, could be difficult to eliminate by means of radiotherapy.

Since benign tumours like cervix carcinoma are treated either by surgery or by radiotherapy enabling the later to be more effective helps us in using non-invasive procedures for better treatment modalities in this era of fatal iatrogenic (physician induced) disease especially in an AIDS scenario. Since as seen in the previous section, hardy cancerous cells can give radiation a hard time, induction of a radio-modulator was suggested and 2-DG was selected (Kalia et al. 1982, 1987; Dwarakanath et al. 1987, 1991; Jain et al. 1977, 1985, 1993; Kapllan et al. 1990; Purohit et al. 1982, 1983; Singh et al. 1990; Karthikeya et al. 2004). The action of 2-DG is as follows:

- i. inhibits glycolysis and thereby reduces the ATP molecules synthesis,
- ii. activates the Poly Adenosine Ribosyl Phosphatase (PARP) dependent repair enzyme cascade by enhancing the cellular levels of NADP⁺ and NADPH
- iii. *in vitro* as well as *in vivo* activation of the cyclic AMP (cAMP) system is possible by means of a positive feed back loop, concerning the loss of glucose when 2-DG enters the cell.

All these enable the 2-DG to protect normal cells and sensitise the cancerous cells thereby acting as a radio-modulator.

The effect is utilised in the present study to protect the peripheral blood lymphocytes and the results do show that such protection is indeed observed by the reduction in the comet tails seen in 2-DG pre-incubated lymphocytes. The most commonly used dose of treatment in case of majority of cancer is the 2.0 Gy dose. It is important that any radio-protective compound acts well at this dose level as this is also the threshold beyond which the LD 25 and LD 50 levels of 3.0 and 4.0 Gy doses, respectively. In this regard very interesting results have been observed in the present study as the dose of 2.0 Gy in the absence of 2-DG yielded nearly 60% of cells to be damaged while in the presence of the radio-protectant 65% of the cells were protected and fell in the category of excellent of good cells. This clearly indicates that the reversal of the position i.e. from 60% damaged to 65% viable cells indicates the efficacy of the compounds, 2-DG's radio-protective nature to be highly significant as seen from the Student "t" test value of $p < 0.001$, for this dose point.

CONCLUSION

The following salient features emerge from the present study:

- a) Cervical cancer patient's peripheral blood lymphocytes are not entire fragile, as the baseline frequency of SSBs is only 10% in the observed lymphocytes, prior to radiation exposure
- b) The radiation exposure leads to increasing levels of DNA SSBs
- c) LD 50 of 4.0 Gy dose gives the maximum damage to the cells
- d) Dose of 5.0 Gy shows the presence of radio-resistant cells
- e) 2-DG acts as a radio-protector and hence reduces the SSBs
- f) The level of reduction by 2-DG is high showing that the compound can be used as an adjuvant to radiotherapy.

The present study summaries that the basic tenets of radiation biology as well as the oncology are amenable to intelligent applications like using 2-DG. It would be also appropriate to show that such research activity would lead to the development and validation of good techniques for enabling the betterment of cancer patient's lives.

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