Iran. J. Radiat. Res., 2012; 10(1): 11-18

Radiosensitizing effects of gemcitabine on aerobic and chronically hypoxic HeLa and MRC5 cells *in-vitro*

E. Heshmati[1], H. Mozdarani[2*], P. Abdolmaleki[1], K. Khoshaman[3]

[1]Department of Biophysics, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, Iran
[2]Department of Medical Genetics, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran
[3]Department of Biology, Parand branch, Islamic Azad University, Saveh highway, Tehran, Iran

Background: Gemcitabine (2′, 2′-difluoro-2′-deoxycytidine, an analogue of deoxycytidine) is a relatively new drug with wide range of anti-cancer activity. In this study, radiosensitizing effects of gemcitabine was investigated on HeLa and MRC5 human originated cell lines under both chronically hypoxic and normoxic conditions using the micronucleus (MN) assay. ***Materials and Methods:*** For induction of chronic hypoxia, the cell culture flasks were saturated with N_2 gas. To evaluate the radiosensitizing effects, in the presence of the non-genotoxic concentration (1ng/ml) of gemcitabine, cells were exposed to different doses (0.5, 1, 2 Gy) of X-ray in both chronically hypoxic and normoxic conditions. ***Results:*** Results showed that there was no significant difference in MN induction under chronically hypoxic and normoxic condition when using 1 ng/ml gemcitabine alone, however in the absence of drug, MN induction was significantly different in irradiated cells ($P<0.01$). Radiosensitizing effects of gemcitabine in chronic hypoxic condition was greater than normoxic condition in both cell lines ($P<0.01$), although more pronounced in HeLa cells. ***Conclusions:*** Radiosensitizing effects and greater dose modifying factor of gemcitabine under depleted oxygen condition is not clearly understood. It might be due to depletion of deoxynocleotides pools via inhibition of ribonucleotide reductase and mismatched nucleosides incorporation into DNA after radiation exposure. **Iran. J. Radiat. Res., 2012; 10(1): 11-18**

Keywords: *Gemcitabine, radiosensitizer, hypoxia, HeLa and MRC5 cells, cytochalasin blocked micronucleus assay.*

INTRODUCTION

Gemcitabine (2′, 2′- difluoro- 2′-deoxycytidine; dFdC) is a nucleoside analogue of deoxycytidine with significant cytotoxic effect on solid tumor cell lines *in-vitro* and *in vivo* [1-4]. This drug has shown activity in various solid tumors, including non-small cell lung cancer, small cell lung cancer, head and neck squamous cell cancer, germ cell tumors, and tumors of the bladder, breast, ovary, cervix, pancreas and biliary tract [5-13] as well as some hematological malignancies [14, 15].

Complex self-potentiating mechanisms of action, made this drug so interesting, especially in combination with other agents [16]. In combination with radiation, as a radiosensitizer, gemcitabine has been the matter of ongoing investigations [17-19]. In brief, there isn't any confirmed agreement in therapeutic regimen for gemcitabine as radiosensitizer and *in-vivo* data are to some extent more limited, but *in-vitro* studies show excellent radiosensitizing effect for gemcitabine [18]. It is a fact that hypoxic microenvironment is commonly found in the central region of solid tumors. Because hypoxia in tumors is associated with poor prognosis, resistance to chemotherapy and radiation therapy, and increases metastatic potential, targeting hypoxia response pathways is of potential therapeutic value [20]. However, the effects of gemcitabine as a single agent under hypoxic condition are limited to a few investigations. Yokoi and Isaiah (2004) [21] showed that, under hypoxic condition, L3.6pl cells are resistant to apoptosis mediated by gemcitabine. In a recent study, Wouters *et al.* (2011) [19] have

Corresponding author:
Prof. Hossein Mozdarani,
Department of Medical Genetics, Faculty of Medical Sciences, Tarbiat Modares University, P.O. Box: 14115-111, Tehran, Iran.
Fax: *+98 21 88006544*
E-mail: *mozdarah@modares.ac.ir*

shown that gemcitabine retains its radiosensitizing potential under low oxygen conditions.

Using the cytochalasin B blocked micronucleus (MN) assay we studied the radiosensitizing effect of gemcitabine on HeLa and MRC5 human originated cell lines in both normoxic and chronic hypoxic conditions.

MATERIALS AND METHODS

Cell culture and incubation conditions

HeLa cells (NCBI Code C115) and MRC5 cells (NCBI Code C125, Pasteur Institute, Tehran, Iran) were obtained from the National Cell Bank of Iran (NCBI). HeLa cells are epithelial-like cell line isolated from a carcinoma of cervix of a 31 year old patient. It has been the most widely studied cell line so far. MRC5 cell line was derived from normal lung tissue of a 14 week old male fetus. Cells were grown in alpha minimum essential medium (αMEM, Sigma) supplemented with 15% fetal bovine serum (FBS, Gibco, BRL), 1% L-glutamine and antibiotics (Penicillin 100 IU/ml, Streptomycin 100 µg/ml, Sigma). Cells were then routinely grown in 25-cm^2 flasks, and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air as normoxic condition.

To induce the hypoxic condition, closed systems were used as described previously [22]. The glass flasks were then gassed (at t=0 in the figure 1) at room temperature with filter sterilized N_2 gas for at least 15 minutes. After tightening screw caps, around the cap was sealed with parafilm. We have not measured the level of oxygen in the system and thus the level of hypoxia was not known. However, N_2 gas was present in the cellular environment at least for 16-18 hours during treatments with gemcitabine and prior to irradiation (figure 1) [22]. No change in the color of the medium was seen after filling the culture vessels with N_2.

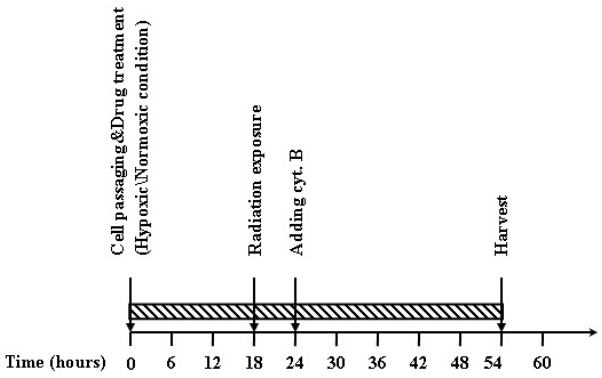

Figure 1. Experimental design of the study.

Experimental design

Exponentially growing cells were sub-cultured in glass flasks under the normoxic conditions and treatments. Flow diagram of experimental design is illustrated in figure 1.

To find the non-genotoxic concentration of gemcitabine (dFdC; Eli Lilly, Indiana, USA), cells were initially treated with various doses (0, 1, 5, 10 and 20 ng/ml) of gemcitabine. The cells were then exposed to various doses of radiation (0, 0.5, 1, and 2 Gy X-ray) in the presence or absence of the non-genotoxic concentration of gemcitabine. To evaluate the effects of hypoxia, all above experiments were repeated under chronic hypoxia condition.

Radiation exposure

Irradiations were performed using X-ray generator (Siemens, Germany) at 140 kVp and 40 mA with a 3 mm aluminum filter at 37°C. Absorbed dose rate was determined to be 0.284 Gy/s at 30 cm far away from source of radiation. Exposure time of 0.88 s delivered 0.25 Gy absorbed dose to samples.

Micronucleus assay

HeLa and MRC5 cells were incubated in the culture medium with 4 µg/ml cytochalasin B (Sigma) 24 h after drug treatment to block cells in cytokinesis. Thirty hours after cytochalasin B treatment, the cells were tripsynized, collected with centrifugation and treated with hypotonic solution containing 0.075 M KCl for 2±1 min. After centrifugation at 1000 rpm, cells

were fixed in a mixture of methanol and acetic acid (3:1 v/v) for three times. Cells were dropped onto cooled, clean blindness coded slides and air-dried. Slides were then stained in 4% Giemsa (Sigma). Micronuclei (MN) were scored in cytokinesis blocked binucleate cells (BN) using ×400 magnification. The criteria described by Fenech (1993) [23] were used to identify BNs and MN. One thousand binucleated cells were scored for each sample. Dose modifying factor (DMF) then were calculated by the ratio of mean MN induced in the presence of drug to mean MN inducted in the absence of drug for each radiation dose.

Statistical analysis

All experiments were repeated three times to minimize statistical errors. The data were analyzed using SPSS software, version 13. The significance of any inter-group differences in the number of micronuclei was statistically evaluated by Students *t-test*. The differences were expressed as significant at level of $P<0.05$.

RESULTS

Table 1 shows the oxygen enhancement ratio (OER) calculated for MRC5 and HeLa cells exposed to various doses of X-rays in the presence of O_2 and N_2. As seen, there was a significant differential effect for radiation when used alone at two different O_2 and N_2 conditions for both cell lines for a dose of 2 Gy; OER was 1.76 for MRC5 and 1.61 for HeLa cells; clearly indicate presence of low oxygen tension in N_2 filled culture vessels treated with radiation in combination with gemcitabine and similar treatment conditions for both cell lines. The effects of different doses of gemcitabine in MN induction both in normoxic and chronically hypoxic conditions for HeLa and MRC5 cells are summarized in table 2. It was found that the MN induction was dose dependent in both treatment conditions. Although, for both cell lines treated at normoxic and chronic hypoxic conditions, there was no significant difference between effect of gemcitabine at 0 and 1 ng/ml concentration; but treatment with higher concentrations of gemcitabine (5, 10 and 20 ng/ml) showed significantly different effects ($P< 0.05$). We consequently chose 1 ng/ml of gemcitabine as non-genotoxic concentration for treatment of both cell lines and this concentration was used in conjunction with radiation to show the radiosensitizing effects of gemcitabine in both normoxic and

Table 1. Mean OER calculated for the effect of different doses of radiation at normal and depleted oxygen tensions for MRC5 and HeLa cell lines. Data are mean values obtained from three independent experiments. ± indicates standard deviation of mean values.

Radiation dose (Gy)	MRC5 cells			HeLa cells		
	Normoxic	Hypoxic	OER	Normoxic	Hypoxic	OER
0	9.7±2.1	10 ±3.0	0.96 ± 0.3	10.3 ± 4.2	9.3 ± 2.5	1.11 ± 0.14
0.5	42 ± 7.2	36.3 ± 16.3	1.32 ± 0.59	45.7 ± 10.7	37 ± 9.6	1.33 ± 0.53
1	132 ±21.7	95.7 ±24	1.41 ± 0.24	145 ± 22.6	109 ± 18.4	1.33 ± 0.05
2	434 ± 47.3	246.7 ±31.7	1.77 ± 0.17	454.3 ± 48.6	282.3 ± 27	1.63 ± 0.31

Table 2. Effects of different concentrations of dFdC on MN induction in MRC5 and HeLa cells under normoxic and hypoxic conditions. Values are mean values obtained from three independent experiments and ± shows standard deviation of mean values.

dFdC concentration (ng/ml)	MN induction/1000 BN (Mean±SD)			
	MRC5 cells		HeLa cells	
	Normoxic	Hypoxic	Normoxic	Hypoxic
0	8.00±3.61	9.00±3.00	11.33±2.08	9.33±1.53
1	13.00±2.65	11.00±4.58	15.67±3.06	12.00±2.64
5	44. 67±14.01	21.67±5.51	48.67±9.61	25.33±4.163
10	75.67±14.15	39.33±9.50	86.00±15.87	43.66±7.50
20	229.00±33.81	107.33±16.17	238.00±37.32	111.00±17.52

chronically hypoxic conditions.

The effects of different doses of radiation in the presence and/or absence of 1 ng/ml gemcitabine in both chronically hypoxic and normoxic conditions is shown in figure 2 for HeLa (panel A) and MRC5 (panel B) cells respectively. As seen in figure 2, in the absence of gemcitabine, the frequency of MN reduced significantly in chronically hypoxic condition (dashed line in both panels) compared to normoxic condition (solid line) ($P<0.01$). Treatment of cells with gemcitabine in normoxic condition has led to a significant increase in the frequency of radiation induced MN in a dose dependent manner (figure 2 dot-dash line). Treatment of cells with gemcitabine in chronically hypoxic condition also led to an increase in the frequency of MN, significantly different with cells irradiated at hypoxic condition alone ($P<0.01$). This effect was more pronounced in HeLa cells than MRC5 cells, so that for HeLa cells the effect was as much as the effect seen for treatment of cells in normoxic condition (figure 2 dot-dash line in panel A) whereas for MRC5 cells the effect was increased at the level of normoxic only condition (figure 2 solid line in panel B). For better comparison of data, dose modifying factor (DMF) of gemcitabine for both cell lines in normoxic and chronic hypoxic conditions is calculated and presented in table 3. Also results of *t-test* analysis between data obtained for irradiated cells in normoxic and hypoxic conditions in the absence and presence of gemcitabine indicate that there exists statistically significant difference between normoxic – dFdC and normoxic + dFdC for all doses for both cell lines ($P<0.01$); between hypoxic – dFdC and hypoxic + dFdC for all doses in HeLa cells ($P<0.01$) and for doses of 1 and 2 Gy MRC5 cells ($P<0.01$); between hypoxic – dFdC and normoxic –dFdC for the dose of 2 Gy for both cell lines ($P<0.01$) and between hypoxic + dFdC and normoxic + dFDC in MRC5 cells ($P<0.05$). For the rest of treatments, there was no statistical difference between the effect of gemcitabine at normoxic and chronically hypoxic conditions.

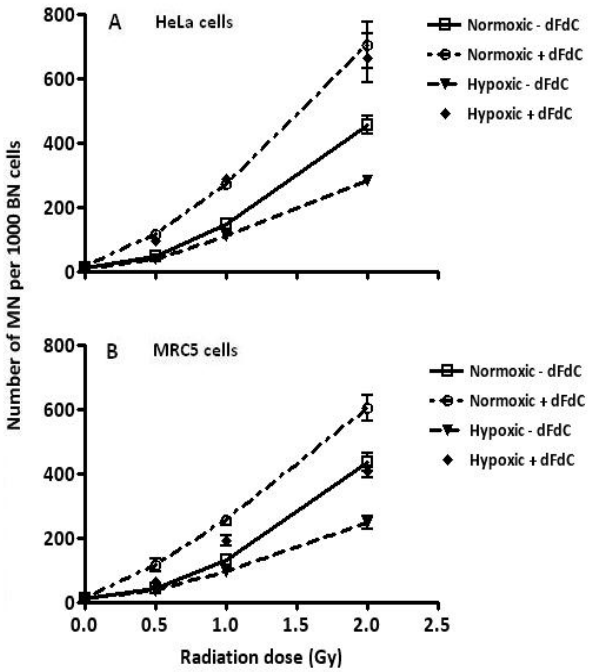

Figure 2. Effects of various doses of radiation in the presence or absence of gemcitabine (1 ng/ml) under normoxic and chronically hypoxic conditions on MN induction in HeLa (A) and MRC5 (B) cells. NB: Overlapped data are shown with one line; panel A, for HeLa cells, data shown with symbols ○ and ♦ are shown with dot-dash line; and panel B for MRC5 cells, symbols ♦ and □ are shown with solid line. Error bars indicate standard deviation (SD) of mean values obtained from three independent experiments.

Table 3. Mean dose modifying factor (DMF) of gemcitabine calculated for MRC5 and HeLa cells irradiated under normoxic and chronic hypoxic conditions. Data are mean values obtained from three independent experiments. ± indicates standard deviation of mean values.

Radiation dose (Gy)	MRC5 cells		HeLa cells	
	Normoxic	Chronic hypoxia	Normoxic	Chronic hypoxia
0.0	1.47 ± 0.89	1.19 ± 0.1	1.91 ± 1.17	1.28 ± 0.28
0.5	2.69 ± 0.54	1.90 ± 0.85	2.57 ± 0.34	2.74 ± 0.84
1.0	2.05 ± 0.44	2.19 ± 0.87	1.89 ± 0.18	2.70 ± 0.55
2.0	1.39 ± 0.18	1.67 ± 0.27	1.55 ± 0.24	2.39 ± 0.73

DISCUSSION

Radiosensitizing effect of gemcitabine in normoxic condition has been previously reported both *in-vitro* and *in-vivo* [18], but in hypoxic condition, there are very few studies in this area [19, 24]. In this study 1 ng/ml of drug, as non-genotoxic concentration, showed radiosensitizing effect that is in support of previous studies [18].

Our results showed that the frequency of radiation induced MN decreased in chronically hypoxic condition for both cell lines compared to normoxic condition (figure 2 panels A and B, dashed lines). Surprisingly it was observed that treatment of chronically hypoxic cells with 1 ng/ml gemcitabine significantly led to an increase in X-ray induced MN (figure 2 solid lines). It seems that combination of gemcitabine and radiation overcome the effects of hypoxia in MN induction. As seen in table 2 presence of N_2 in cellular environment (low oxygen tension) led to a remarkable decrease in MN formation when gemcitabine was used alone with higher doses (5-20 ng/ml) ($P<0.05$). However this effect was not significant when gemcitabinbe was used at a dose of 1 ng/ml ($P>0.05$).

The main action of gemcitabine is assumed to be competitive incorporation of gemcitabine diphosphate and triphosphate dFdCTP with deoxycytidine triphosphate into DNA [25, 26], after which DNA polymerase is able to add only one more nucleotide, leading to DNA fragmentation and cell death. Cytotoxic activity of gemcitabine has also been correlated with dFdCTP formation leading to its incorporation into DNA, and its inhibition of DNA synthesis [17, 26-28]. Other effects of metabolites of gemcitabine include inhibition of ribonucleotide reductase and dCMP deaminases enhance the incorporation of dFdCTP into DNA [29].

It is known that the main critical target for radiation is cellular DNA through the formation of free radicals directly or indirectly causing DNA strand breakage including double-stranded DNA breaks. Furthermore, it is known that presence of molecular oxygen will increase radiation-induced DNA damage through the formation of oxygen free radicals that act to inflict "indirect" damage beyond the "direct" effects of radiation on DNA [30]. In the present study, MN is considered as reflection of chromosomal aberrations due to unrepaired DNA double strand breaks. There is also substantial evidence that tumor hypoxia induces genomic changes with subsequent up-regulation of genes that are linked to radiation resistance [31]. Our results somehow proved that hypoxia decreased the effects of radiation on MN induction in comparison with normoxic condition in a dose dependent manner (dashed lines in figure 2 panels A and B).

Hypoxia influences signaling pathways such as those controlling cell proliferation, angiogenesis, and apoptosis [20]. It has been shown that hypoxia has association with resistance to both chemotherapy and radiation therapy [32]. Previous studies showed that the mechanism of radio sensitizing effect of gemcitabine is somehow different from its mechanism as a single agent. As a single agent its incorporation into DNA and cell cycle redistribution is the main mechanisms, but as a radiosensitizer, its effect on nucleotide pools is more important [17]. The obtained results are in line with recent report of Wouters *et al.* (2011) [19] showing that gemcitabine retains radiosenzitizing properties in hypoxic cells using colonogenic, apoptotic and cell cycle end points. As a single agent (in cytotoxic concentration) hypoxic condition reduced the effect of gemcitabine (table 2) but when non-toxic concentration of gemcitabine (1 ng/ml) was used, hypoxia did not affect its radiosensitizing effect (figure 2 A and B). It is clear that presence of low oxygen tension in cellular environment lead to reduced initial DNA breaks, but from results it seems that in the presence of both chronic hypoxia and gemcitabine the frequency of radiation induced MN is as high as normoxic condition for HeLa cells treated with

gemcitabine (figure 2 A) and not significantly different from the normoxic condition for MRC5 cells (figure 2 B) (P>0.05). This can be related to some unknown mechanism through which the radiation-induced DNA breaks remain unrepaired. Also the reason for enhanced potentiating effect of gemcitabine on radiation induced MN in chronically hypoxic HeLa cells compared to MRC5 (normal) cells remains unknown. It might be attributed to the malignant nature of HeLa cells with under layer genome instability making these cells more susceptible to damage. However, in *in vivo* condition this differential effect might be beneficial because the main target for cancer chemo- and radio-therapy is overcoming hypoxic cells in solid tumors and presence of hypoxia will spare normal tissue from radiation effect, although it is known that this condition rarely happens in normal tissue.

Probst *et al.* (1995) [33] and Brischwein *et al.* (1997) [34] showed that hypoxia induced imbalance in dNTP pool specially depletion of dCTP. Chimploy *et al.* (2000) [35] showed depletion of dCTP mediated through inhibition of ribonucleotid reductase that is also the target of gemcitabine even in radiosensitizing concentration [29]. According to the results presented (figure 2), we suppose that reduction of dCTP pool induced by chronic hypoxia may increase the probability of gemcitabine or other mismatched nucleosides incorporation into DNA after radiation exposure. Since this drug acts as DNA chain terminator, if incorporated into areas where DNA repair has occurred after irradiation, it can cause more MN induction; thus leading to greater dose modifying factor (DMF) in hypoxic condition in comparison with normoxic condition (table 3). As an example for HeLa cells the DMF at specific absorbed dose of 1 Gy was 2.64 at chronically hypoxic condition which it is 1.41 times greater than the DMF at normoxic condition; so that, the overall observable MN in chronically hypoxia and normoxic conditions show no significant differences. However, results presented (table 3) show that the effect of gemcitabine under aerobic and chronic hypoxia might be different on normal cells and cells with malignant origin such as HeLa especially at higher doses of radiation. There is no explanation for this differential effect until more research being done with other cell lines.

However interaction of different agents makes it difficult to characterize the exact nature of their interrelations. The exact mechanism by which gemcitabine serves to sensitize cells to ionizing radiation damages is not well defined [36]. Further investigations should be performed to clarify mechanisms attributed to the effects of gemcitabine. Finally these results suggest that gemcitabine might be a good candidate for radiosensitizing agent at least at chronically hypoxic conditions which may highlight the possible application of gemcitabine as a therapeutic agent for solid tumor therapy.

ACKNOWLEDGEMENTS

This work was supported by The Research Department of the Tarbiat Modares University, Tehran, Iran.

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