Original Article

Cytogenetic Damages from Iododeoxyuridine -induced Radiosensitivity with and without Methoxyamine in Human Glioblastoma Spheroids

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Abstract

Objective: Iododeoxyuridine-induced Radiosensitivityi (IUdR) is a halogenated thymidine analogue recognized to be effective in vitro and *in vivo* radiosensitizer in human cancers. It is reported that Methoxyamine (MX) potentiates DNA damages in cancer cells with blocking the repair pathway of IUdR damages. But studies, entirely, are restricted on monolayer culture cells from human colon cancer cells. Spheroids are 3D form of cells that aggregate and grow together which resemble *in vivo* tumor models in several aspects and the the results of such studies can be extended to tumor *in vivo*. The aim of the current study was to evaluate DNA damages from IUdR and gamma rays with and without Methoxyamine in human Glioblastoma spheroids.

Materials and Methods: The DNA induced damages in U87MG cell line were compared using alkaline comet assay method. Experiments were performed with two different sizes of spheroids (100μm and 300μm).

Results: Evaluation of the effects of IUdR with and without MX pretreatment on spheroids following ionizing radiation showed that MX increased the cell damages of IUdR with and without irradiation in both diameters spheroids. The damages were further increased in 100μm compared with 300μm diameter.

Conclusion: Comparisons of tail moments in spheroids with 100 and 300μm diameter showed that cell damages in larger spheroids, 300μm, are lesser than smaller one, 100μm. This could be due to existence of G_0 cells and cells with longer cycle which IUdR was less incorporated into them. Thus, decrease in IUdR radiosensitization and base wxcision repair (BER), results in reduction of MX activities. Using agents for Inhibiting the activities of proteins which are responsible for carrying the cells to G_0 may be beneficial in solving such problems.

Keywords: Radiosensitization, Spheroid, IUdR, Methoxyamine, BER

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Introduction

The incidence of malignant glioma has been reported to be 18,300 new cases and its mortality rate has been estimated to be approximately 13,100 patients just in 2003 in the United States. The primary management is surgery, followed by radiotherapy (1-4). Unfortunately, the irradiation effective enough to control the tumors far exceeds the tolerance of normal brain tissues (5). Thus, to avoid such unfavorable outcomes, methods which sensitize the tumor cells to ionizing radiation (IR) are used.

Iododeoxyuridine is a halogenated pyrimidine that can be incorporated into DNA instead of thymidine during DNA replication. This halogenated pyrimidine is considered as clinical radiosensitizer, where the extent of radiosensitization correlates directly with the level of halogenated pyrimidine-DNA incorporation (6-8). Although, the cellular and molecular mechanisms of IUdR radiosensitization are not clearly understood, it is presumed that IUdR sensitizes cells through enhancing

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the formation of IR-induced double-strand breaks (9-12).
Recent studies have shown that IUdR incorporated into DNA can be recognized by base excision repair (BER) proteins (13, 14). Therefore, the cellular status of tumor cells repaired by BER may alter the degree of IUdR radiosensitization. Methoxyamine (MX) is a specific chemical inhibitor of BER mediated by tight binding to AP sites generated by cleavage of BER glycosylases and rendering the phosphodiester bonds adjacent to the AP site refractory to the catalytic activity of AP endonuclease (14). Studies have indicated that MX potentiated IUdR-induced radiosensitization in human cancer cells (14, 15). Since most of the previous studies conducted on evaluating the effectivenss of the IUdR/MX radiosensitization have been confined to laboratory trials on monolayer cell culture in which the corresponding cells were extracted from human colon cancer, in the present study, the genetic damages of IUdR/MX radiosensitization were investigated in spheroid cell culture of glioblastoma cell lines.
The multicellular spheroid represents an *in vitro-in vivo* transition model which is important *in vivo* solid tumors (16). These include intimate cell-cell contact (17), individual hypoxic cell populations (18), and cycle times that range from as those of exponential monolayer rates through an essentially nondividing state (19). In brief, they combine the relevance of organized tissues with the accuracy of *in vitro* methodology (20-25). It has been suggested that some physiological differences may exist between cell growth in two dimensional contact (monolayer) and cells grown in three-dimensional contact (multicellular tumor spheroids) (26, 27). A research conducted on the growth of human glioma cells in these two systems showed different degrees of sensitivity to radioionated IUdR (28). Several authors have reported higher radioresistance of cells in spheroids compared with monolayer cultures (29-32). In this paper, we have studied the radiosensitivity of glioblastoma cell line U87MG multicellular spheroids using IUdR in combination with MX. Due to the existence of hypoxic and G_0 in larger spheroids compared with lesser one, experiments were performed with two different sizes of spheroids (100µm and 300µm in diameter).

Materials and Methods

Cell Line

Human glioblastoma cell line, U87MG, were provided from Pastor Institute of Iran. This cell line was maintained in MEM (GIBCO) supplemented with 10% fetal bovine serum (FBS) (GIBCO), 500U/ml of penicillin (SIGMA) and 200 mg/lit of streptomycin (JABEREBN-HAYAN).

Monolayer culture

Cells were cultured as monolayer at a density of 10^4 cells/cm^2 in T-25 tissue culture flasks (NUNC). Cultures were maintained at 37°*C* in a humidified atmosphere of 5% CO_2. Cultures were propagated or cells were harvested by trypsinizing cultures with 1mM EDTA/ 0.25% Trypsin (w/v) in Phosphate Buffer Saline (PBS).

Spheroid Culture

Spheroids were initiated using the Liquid Overlay technique (26). A medium including 10^5 U87MG cells were seeded into 100 mm dishes coated with a thin layer of 1% agar (Bacto Agar, Difco, Detroit, MI) with 10ml of MEM supplemented with 10% FBS. The plates were incubated at 37 °*C* in a humidified atmosphere of 5% CO_2. Half of the culture medium was replaced with fresh medium twice per week.

Spheroid Growth curve

After 3 days of culture, spheroids were moved into multiwell plates (24 wells/plate) (NUNC) coated with a thin layer of 1% agar with 10ml of MEM supplemented with 10% FBS, whereas one spheroid was located into one well. The multiwells were incubated at 37°*C* in a humidified atmosphere of 5% CO_2. for one month, two perpendicular diameters of spheroids were measured and their volume calculated according to the following equation:
V (Volume) = $a \times b^2 \times \pi/6$ (1), in that, (a) and (b) are lesser and greater in diameters, respectively. Then the volume growth curve

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were calculated according to the duration.
In the logarithmic phase of curve, spheroids follow the equation:

$V=V_0 \times e^{kt}$ (2).

Where (V_0) is primary spheroids volume, (V) equals the spheroids volume after the duration (t) and k shows the gradient of the logarithmic phase of the curve. The volume doubling time of spheroids is achieved according to gradient of logarithmic phase of the curve.

Drug-IR Treatment

IUdR (1µM) and MX (6mM) in U87MG spheroids were treated for 67h in MEM containing 10% FBS. After 67 h, drug-containing medium was removed, the cultures were washed with PBS and the spheroids were immediately irradiated (2Gy). IR was irradiated using ^{60}Co at a dose rate of 1.14 cGy/s.

Comet assay

The induction of DNA damages in U87MG cell populations by IUdR alone (1µm for 67 h) or in combination with MX 6 (mM for 67h) were determined by SCGE (comet assay). This assay was performed according to the Singh et al protocol (33). Comet tail moment was determined by measuring the fluorescence intensity using the Comet scor software.

Statistical analysis

Data were given as mean values±SEM, with n denoting the number of experiments. Student's t test was considered appropriate and applied. A value of $p \leq 0.05$ was considered to be significant.

Results

The U87MG cells were able to from spheroids in liquid overlay cultures. Fig 1 shows the phase contrast micrographs of these spheroids in two different diameters.
The growth curve of U87MG cells in spheroid culture is shown in Fig 2.
The volume doubling time calculated from this curve was approximately 67±0.91 hours which was applied as drug treatment time consequently.
Comet assay were used for evaluation of DNA damages after drug treatment and ionization radiation. Experiments were performed with two different sizes of spheroids (100µm and 300µm in diameter) and for evaluation of MX effects on IUdR radiosensitization, spheroids were treated with IUdR±MX for one volume doubling time. Figs 3 (A-H) and 4 (A-H) shows the microphotographies of the cells from spheroids during comet assay between the treatment and the control groups. The photos were analyzed by comet score software and the tail moments were calculated as a parameter related to DNA damages.

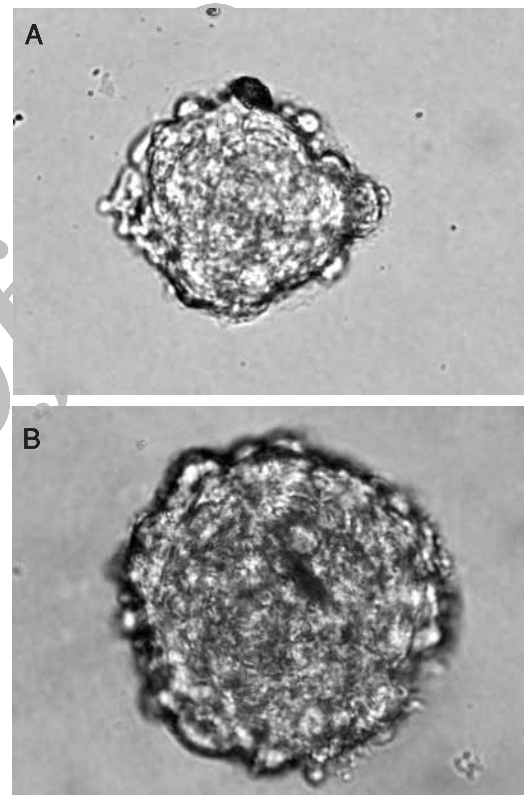

Fig 1: Phase contrast micrographs of U87MG spheroids. (A) 100µm spheroid at day 11 and (B) 300µm spheroid at day 23 after initiation of measuring the diameters.

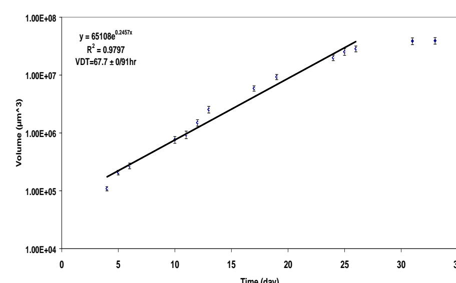

Fig 2: The growth curve of U87MG cell line in the spheroid cultures. The days 4 to 26 show the log phase of curve and used to measuring volume doubling time (67 h). Mean±SEM of 3 experiments

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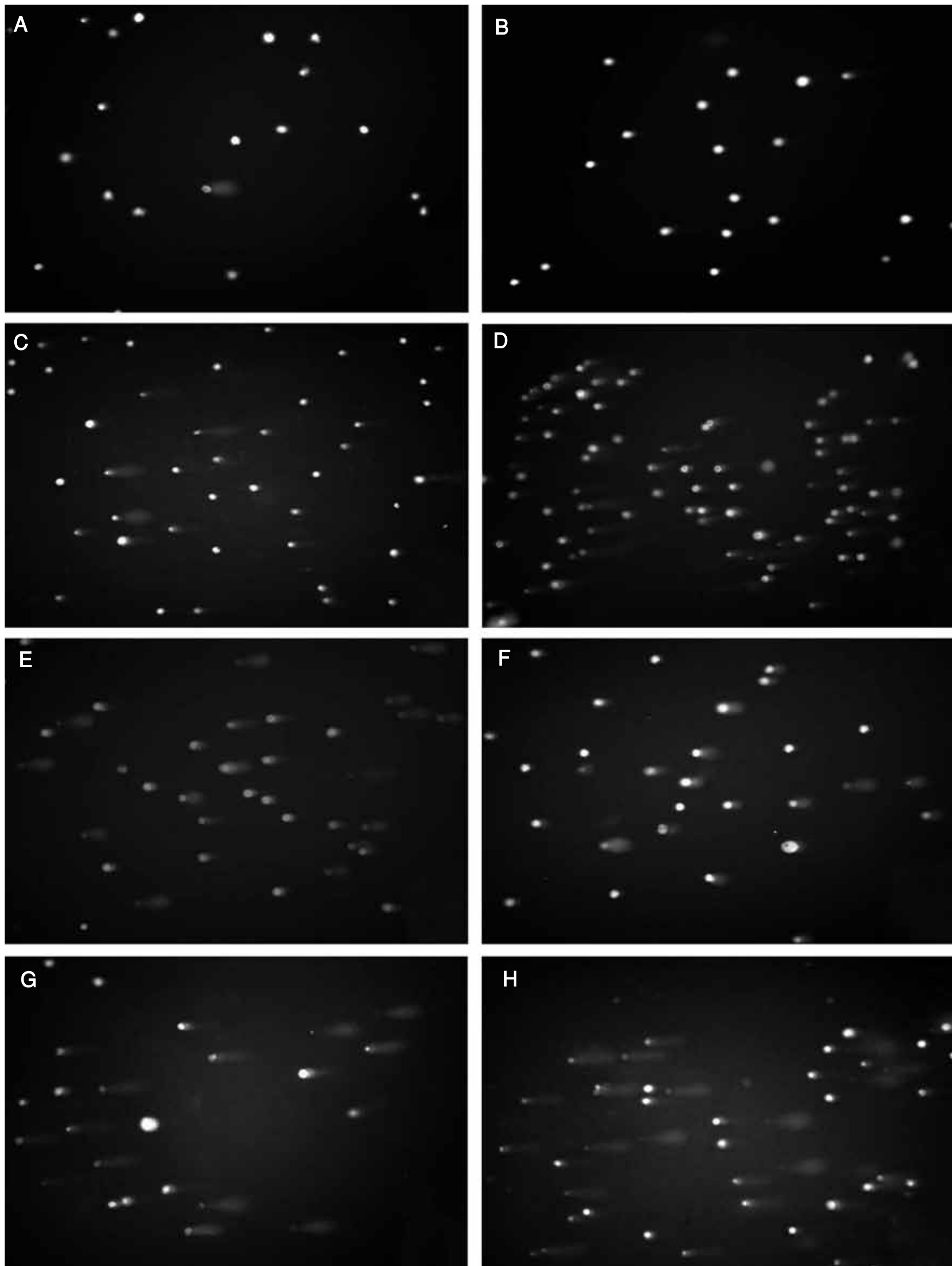

Fig 3: SCGE (Comet Assay) of U87MG cells of 100µm spheroids after treatment for 67 h with 1µM IUdR alone or simultaneous treatment with 6mM MX. microphotographies are representative samples of following slides: A) control, B) MX, C) IUdR, D) IUdR+ MX, E) Gamma, F) MX+Gamma, G) IUdR+Gamma, H) IUdR MX+Gamma

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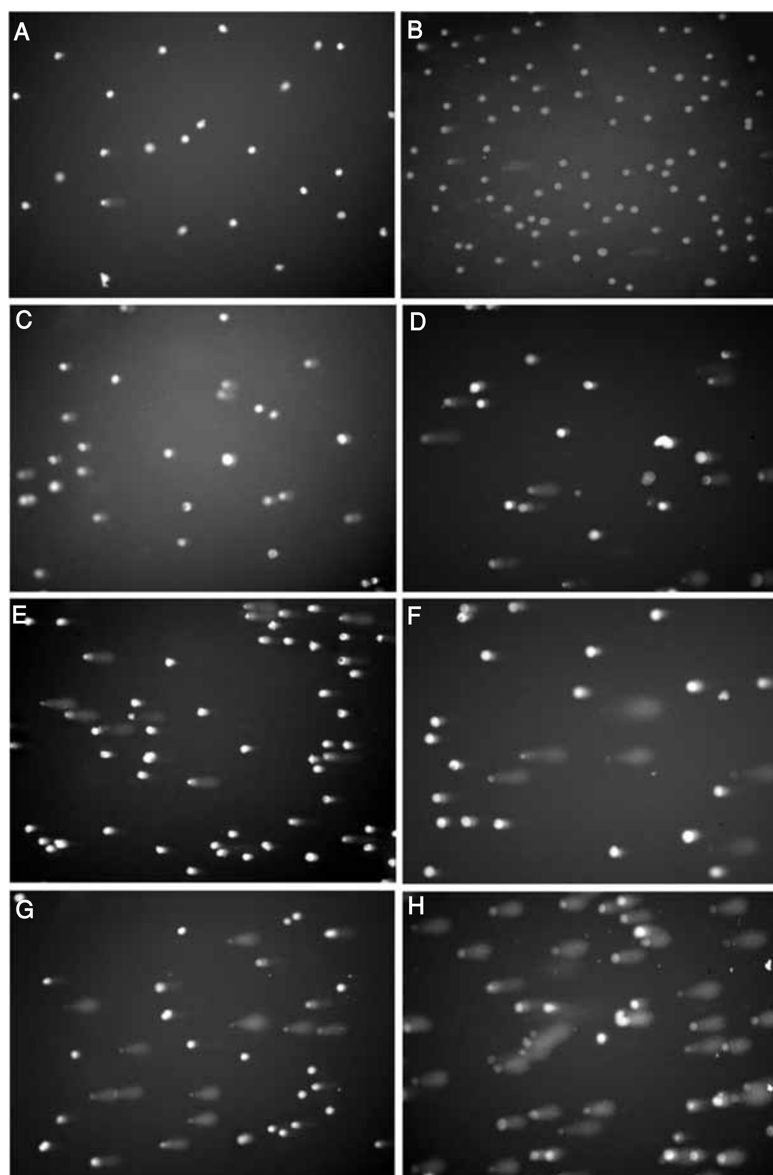

***Fig 4:** SCGE (Comet Assay) of U87MG cells of 300μm spheroids after treatment for 67 h with 1μM IUdR alone or simultaneous treatment with 6mM MX. microphotographies are representative samples of following slides: A) control, B) MX, C) IUdR, D) IUdR+ MX, E) Gamma, F) MX+Gamma, G) IUdR+Gamma, H) IUdR+MX+Gamma*

Fig 5 shows the effect of IUdR/MX pretreatment on DNA damages following IR in 100 and 300 micrometer spheroids. According to the figure, MX alone is not effective in changing IR-related cytotoxicity in both diameters. In contrast IUdR/MX treatment, significantly ($p<0.001$) increased the tail moments before and after IR compared with the control group in both spheroid diameters. On the other hand, except control and MX treated groups, the DNA damages in 100 micrometer spheroids were more significantly increased than 300 micrometer spheroids.

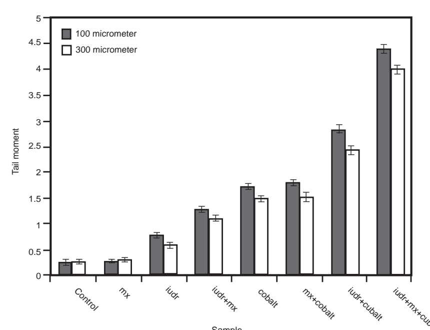

***Fig 5:** Comparative chart of comet tail moment in U87MG cell line of 100 and 300μm spheroids after treatment with 1μM IUdR alone or simultaneous treatment with 1μM IUdR and 6mM MX. Spheroids were treated and processed as described in "Material and Methods." Comet tail moment is calculated as the product of tail length and fraction of DNA in the tail. Data are expressed as fold increase compared with tail moment in untreated control cells; Mean±SEM of 3 experiments.*

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Tab 1 shows the Comparison of DNA damages (tail moments) in 100 and 300μm spheroids treated with IUdR/MX/IR in comparison with IUdR/IR. The combined treatment with IUdR/MX for 67 hours before IR induced tail moments to a greater extent (42% increase for 100μm and 34% increase for 300μm spheroids) than IUdR pretreatment alone.

Table 1: Percentages of increasing damages in U87MG spheroids from combination IUdR and Methoxyamine with and without IR in 100μm and 300μm diameters.

Spheroid diameter	Percentage of increasing damages after pretreatment with IUdR+MX	Percentage of increasing damages after pretreatment with IUdR+MX+IR
100μm	100μm	42.34%
300μm	19.47%	33.8%

Discussion

IUdR is known as radiosensitizer that increases IR-induced DNA DSBs (6-12). It is reported that MX potentiated IUdR-induced radiosensitization in human tumor cells (14, 15). IUdR with MX radiosensitization studies, entirely, are restricted to laboratory researches on monolayer culture cells from human colon cancer and it remain unclear whether these results could be extended into tumor in vivo. on the other hand, in glioma cells which are one of the most important choices for IUdR indications, there is a debate whether MX induces IUdR radiosensitization.

Spheroid cultures are interesting and valuable in vitro model systems, which allow many properties of in vivo tumor systems to be studied quantitatively (34-35). In the present study, we have studied the IUdR radiosensitization combined with MX in spheroid cultures of human glioblastoma cell line U87MG. To better clarify the role of hypoxic and G_0 cells in large spheroids (36), we performed this experiment with two different sizes of spheroids (100μm and 300μm in diameter). Using comet assay (Fig 5), IUdR pretreatment significantly sensitized cells to IR, and the radiosensitization was further increased when cells were pretreated with IUdR/MX compared with control in both spheroid diameters. These results showed for the first time that, in human glioma cells, the use of MX significantly increases the DNA damages induced from IUdR radiosensitization.

It is not completely understood that why MX alone is not effective in changing IR-related cytotoxicity in these cell lines. A study proposed that MX may inhibit short-patch BER by blocking APE activity, therefore enhancing long-patch BER to remove MX-AP (14). Comparisons of tail moments in spheroids with 100 and 300μm diameters showed that, except for MX treated and control groups, cell damages in larger spheroids, 300μm, are lesser than smaller one, 100μm. This may be due to the existence of G_0 cells and also hypoxic cells in 300μm spheroids which IUdR was not incorporated into them and therefore MX couldn't perform its inhibition activity. To solve this problem and increase the efficacy of this method. Agents can be used to inhibit the proteins which make cells hypoxic and carry them to G_0 phase, this can also increase the efficacy of modality treatment.

Conclusion

Pretreatment of cells with IUdR and MX before irradiation enhances tumor radiosensitization and may improve therapeutic index for IR.

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