Assessment of cytotoxic damage induced by irradiation combined with hyperthermia and Gemcitabine on cultured glioblastoma spheroid cells

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HIGHLIGHTS
- We studied the effect of combined therapy on glioblastoma spheroid cells.
- Gemcitabine, gamma radiation and hyperthermia were used in the combined therapy.
- This model of therapy showed synergistic effect in the treatment of glioblastoma.

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ABSTRACT
Background: Glioblastoma is a highly lethal tumor. There are several methods for the treatment of glioblastoma including surgery/chemotherapy/radiotherapy/radiosurgery or corticosteroids. However none of them could have improved treatment considerably. Therefore this study assessed the cytotoxicity of the combined treatment with Gemcitabine, radiation and hyperthermia on cultured glioblastoma spheroid cells.

Material and methods: In this study human glioblastoma cell line U87-MG, was cultured as spheroid using the liquid overlay technique. After reaching spheroid dimensions to 100 μm, cells treated by 100 nM Gemcitabine, 2 Gy gamma rays and then hyperthermia of 43 °C for an hour. Colony assay was used to determine cell induced damage and synergistic effect of Gemcitabine, irradiation and hyperthermia.

Results: The combined therapy indicated greater percentage of cellular damage, although more damages were seen when Gemcitabine, radiation and hyperthermia induced into U87-MG cell line simultaneously. Combination of those three factors in comparison to the radiation combined with hyperthermia and Gemcitabine separately showed 1.85 and 1.5 times more damages respectively.

Conclusion: This study revealed the synergistic effect for the combined therapy of Gemcitabine, radiation and hyperthermia in glioblastoma treatment.

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1. Introduction
Glioblastoma is the most common malignant brain tumor in adults that could be highly lethal (Buckner, 2003). Despite of the wide clinical studies, survival for the patient is up to 15 months (Buckner, 2003). Treatment of glioblastoma may include surgery/chemotherapy/radiotherapy/radiosurgery or corticosteroids. Surgery is the first step of treatment to remove the tumor. Since glioblastoma is a finger-like tentacles, complete removal of the tumor is very difficult, especially when the tumor is close to those parts of the brain that control vital functions such as speech, body balance and coordination. Beside there is a high propensity for recurrence of the tumor (Galanis and Buckner, 2000; Chien-Kuo and Noriyuki, 2008; Lawson et al., 2006).

Nucleoside analogues are molecules acting similar to nucleosides in DNA synthesis. Gemcitabine (GEMZAR) is used in chemotherapy as a nucleoside analog. It is an excellent radiosensitizer too. This drug is routinely used in treatment of various tumors such as breast and ovarian cancers, non-small-cell lung cancer (NSCLC), bladder and pancreatic cancers (Toschi et al., 2005). It is injected intravenously and extensively metabolized by gastrointestinal tract. Dose range is 1–1.2 g/m² of body surface according to the cancer type (Lawson et al., 2006).

Gemcitabine is a pro-drug. It is activated intracellularly by deoxycytidine kinase. After transferring into the cell,
deoxycytidine kinase adds a phosphate to the 5′ position of the ribose leading to the monophosphate (dFdCMP). The next phosphorylation results Gemcitabine diphosphate (dFdCDP) and triphosphate (dFdCTP) active metabolites targeting DNA and RNA. These are responsible for the cytotoxic effect of Gemcitabine (Huang et al., 1991; Haperen et al., 1993). Both of these metabolites inhibit required processes for DNA synthesis. DNA binding Gemcitabine triphosphate (dFdCTP) is the most likely mechanism of cell death. In other words, Gemcitabine analog triphosphate is being replaced cytidine during DNA replication and stops tumor growth since the only one additional nucleoside can be added to the defective nucleoside (dFdCTP). Therefore DNA synthesis is inhibited and finally cell death will be occurred (Haperen et al., 1993; Haperen et al., 1994).

Another target of Gemcitabine is ribonucleotide reductase (RNR) enzyme involving in DNA synthesis. Gemcitabine diphosphate (dFdCDP) binds to the active site of RNR to get inactive enzyme irreversibly (Van Moorsel et al., 2000).

Furthermore Gemcitabine has several self-potentiating effects such as its enhanced incorporation into DNA by inhibition of RNR. It will lead to depletion of deoxyribonucleotides (dNTP) pools including that of dCTP (Plunkett et al., 1996). Deoxycytidine deaminase (dCDA) deaminates Gemcitabine to difluorodeoxycytidine (dFdU). Although dFdU is considered as an inactive metabolite, it can act as a radiosensitizer at concentrations which can be easily reached in plasma. At these concentrations, dFdU shows cytotoxic activity (Pauwels et al., 2006).

The combined therapy of Gemcitabine with gamma radiation could have synergistic effect for treatment of glioblastoma. It is probably due to a combination of mechanisms including deoxyadenosine triphosphate (dATP) depletion, cell cycle redistribution, reduction of apoptotic threshold, inhibition of DNA synthesis and reduction of DNA repair (Pauwels et al., 2005).

Hyperthermia or thermotherapy is another way to treat cancer through damaging or destroying cancer cells. Therefore these cells will be more sensitive to the ionizing radiations and certain anticancer drugs. Hyperthermia has been used for many years and has led promising results in cancer treatment (Engin, 1994; Hulshof et al., 2004).

Many studies have shown hyperthermia at temperature range of 41–43 °C could cause tumor regression without complete cure. But the combined therapy with ionizing could lead better treatment (Hulshof et al., 2004).

Three-dimensional multicellular spheroids culture is a comprehensive examination of tumor response to the treatment modalities. In contrast to monolayer culture, three-dimensional culture meets specific biochemical and morphological reliability similar to the corresponding in vivo tissue. Spheroids behavior in many aspects is similar to the in vivo-tumor such as proliferation or nutrient gradients/pH/antigen/the influence of growth factor/cell interaction with extracellular matrix and oxygen gradient in the environment (Leoni et al., 1998).

According to above explanations we decided to study the combined therapy of drug, irradiation and hyperthermia for glioblastoma using colony assay.

2. Materials and methods

2.1. Cell line

Human glioblastoma cell line U87MG was bought from the Institute of Pasteur in Iran. Cells were cultured in a minimum essential medium (MEM; Gibco) including 10% fetal bovine serum (FBS; Gibco), penicillin (100 U/ml), streptomycin (0.25 µg/ml) (Sigma) and 20 U/ml of fungizone (Gibco).

2.2. Spheroid culture

Spheroids were cultured using the liquid overlay technique. 5 × 10⁵ cells were seeded in 100 mm T-25 flask (NEST) coated with a thin layer of 1% agar with 10 ml of MEM supplemented with 10% FBS. The plates were incubated at 37 °C in a humidified atmosphere and 5% CO₂. Every three days, half of the culture medium was replaced with the fresh culture medium for cell nutrition.

2.3. Growth curve

3 days after culturing spheroid the related growth curve was obtained. One spheroid cell was seeded in each well of multi-well plates coated with a thin layer of 1% agar including 1 ml MEM. The multi-well was incubated at 37 °C in a humidified atmosphere and 5% CO₂. For 30 days, every 3 days two vertical diameters of spheroid were measured by a microscope and then spheroids volume (V) was calculated as follows:

\[ V = a \cdot b^2 \cdot e^{kt} \]

where (a) and (b) are the small and large diameters of spheroid respectively.

In the linear area or logarithmic phase of the curve, the spheroid volume was calculated as follows:

\[ V = V_0 \cdot e^{kt} \]

where \( V_0 \) is the initial spheroids volume, \( V \) is given by spheroids volume after time \( t \), and \( k \) indicates the gradient of the logarithmic phase of the curve.

Spheroids volume doubling time (VDT) is calculated according to the gradient of the logarithmic phase of the curve as follows:

\[ \text{VDT = Ln}_2/k \]

2.4. Cell preparation

The glioma cell line was grown in a liquid media in the spheroid form with diameter of 100 µm and then classified to 8 groups as follow:

- A: a control group
- B: a group irradiated with 2 Gy gamma-ray
- C: a group treated with Gemcitabine
- D: a group treated with hyperthermia
- E: a group treated with Gemcitabine + gamma-ray
- F: a group treated with gamma-ray + hyperthermia
- G: a group treated with Gemcitabine + hyperthermia
- H: a group treated with Gemcitabine + gamma-ray + hyperthermia

2.5. Drug treatment

In order to study Gemcitabine effect on glioma cells in presence of radiation and hyperthermia, 7.5 µl of Gemcitabine solution per 10 ml of MEM was added to the flasks containing spheroid in related groups (C,E,G, and H) to get 100 Nano molar concentration. The samples were treated with Gemcitabine for 24 h, then washed with PBS to remove the drug (Haveman et al., 2006; Gregoire et al., 1999).

2.6. Irradiation

Spheroids were irradiated by 2 Gy gamma ray of 60Co (Teriton 76) with dose rate of 89.23 cGy/min from posterior. 24 h after treatment with Gemcitabine. The field size was 20 × 30 cm².
2.7. Hyperthermia

To apply hyperthermia, flasks containing spheroids (D,F,G, and H) were placed in water bath (Memmert) immediately after irradiation at a constant temperature of 43 °C for an hour.

At the end of treatment, the cytogenetic damages were evaluated by the colony assay.

2.8. Colony assay

Spheroids were converted to single cells to assess colony formation ability. These cells were counted and their viability determined. Then 5000 cells from each sample were cultured into 60 ml pellet containing 5 ml MEM with FBS10%.

After 10 days incubation (the best time for colony formation), samples were fixed and stained by crystal violet 0.5% and finally colonies were counted.

Culture efficiency was calculated as follows:

(Number of colony counted/the number of cultured cells) × 100 = culture efficiency

In this study after spheroids conversion to single cells and before colony formation the cell viability was being measured. It was more than 90%.

2.9. Statistical analysis

Statistical analysis was done by one-way Anova.

3. Results

In order to assess the growth process of U87-MG cells in monolayer culture model, cells were cultured in a monolayer form and growth curve drawn on the base of the cell numbers during 10 days (Fig. 1). According to this curve the cell population doubling time was measured, 27.95 h.

Then cells were cultured as spheroids in liquid overlay method. After getting spheroid growth curve, volume doubling time (VDT) for spheroids was measured, 66.79 h (Fig. 2).

To assess integration effect of the Gemcitabine, radiation and hyperthermia, spheroids were treated with Gemcitabine for 24 h and then irradiated by gamma radiation and exposed to hyperthermia at 43 °C for an hour. To evaluate the cytotoxic damage of this integration, the colony formation assay was used. Fig. 3 shows culture efficiency percent (PE%) in all samples after different treatments.

Table 1 reveals the combined therapy of Gemcitabine, irradiation and hyperthermia significantly reduces colonies number in comparison that of control group (p < 0.05). Treatment with Gemcitabine or hyperthermia alone made slight reduction in culture efficiency and survival fraction, whereas this reduction was greater for treatments with R+H as well as G+R. The highest losses of PE% and SF% were happened for samples treated by Gemcitabine + radiation + hyperthermia (G+R+H) along with the loss of cellular repair mechanism.

4. Discussion

Nucleoside analogues are molecule acting similar to nucleosides in DNA synthesis and prevent cell proliferation. Gemcitabine (GEMZAR) is a pyrimidine nucleoside analogue in which the hydrogen atom on the carbon 2 of deoxycytidine is replaced by
Gemcitabine is used as a chemotherapy drug in pancreatic cancer treatment. It is applied for treatment of different solid tumors including NSCLC, small cell lung cancer, head and neck squamous cell cancer and cancers of the bladder, breast, ovary, cervix and pancreas (Gregoire et al., 1999).

When Gemcitabine gets into the cell, it will be phosphorylated to active metabolite Gemcitabine diphosphate and triphosphate by nucleoside kinase. Diphosphate molecule is able to inhibit ribonucleotide reductase.

Our study showed the combination of Gemcitabine and radiotherapy could be promising combined therapy. Experimental evidence shows that Gemcitabine is a potent radiosensitizer in vitro and in vivo (Pauwels et al., 2005).

Damage to DNA with ionizing radiation occurs through two routes: direct and indirect effects. In direct effect ionizing radiation could make ionization of DNA directly. Indirect effect happens through ionization of that portion of the solvent shell that is tightly bound to the DNA. Thereafter holes and electrons created in the DNA solvation shell were transferred to the DNA. These events could lead some lesions such as single strand break (SSB) and double strand break (DSB). Since DNA is the repository of genetic information in each living cell, its integrity and stability are essential to life. On the other hand DNA lesions could cause mutation and cell death. Finally ionizing radiation with cell death and inhibition of some genes responsible for vasculogenesis such as vascular endothelial growth factor (VFGF) could cause tumor regression. It explains why radiotherapy is used for cancer treatment.

Hyperthermia is another cancer treatment method. Scientists have used integration of that with other methods especially radiation therapy for many years and reported promising results (Engin, 1994; Leoni et al., 1998). Pei-yu et al. indicated apoptotic cell death would be one of the mechanisms of hyperthermia therapy for malignant glioma (Pei-yu et al., 2000). Also hyperthermia imposes toxicity in cancer cells and induces apoptosis. It sensitizes cells to ionizing radiation. As a matter of fact hyperthermia increases blood flow rate delivering more oxygen to tissue. It causes oxygen effect that is known as a radiosensitizer. Heating increases DNA damages induced by irradiation and also protein injuries. Heat has an important role in prevention of DNA repair (Engin, 1994; Hulshof et al., 2004).

Cells in S-phase of the cell cycle are more sensitive to hyperthermia (Buckner, 2003). Gemcitabine induces an S-phase arrest. Pawel et al. revealed Gemcitabine role in cells accumulation in S phase [24]. Therefore combination of this accumulation with S-phase cell sensitivity to hyperthermia could create a synergistic effect to kill cancer cells.

Our aim was to apply colony assay to determine cytotoxic damage induced by the combination of radiation + hyperthermia after treatment with Gemcitabine in spheroid culture of human glioblastoma U87-MG cell line.

Our study revealed treatment with Gemcitabine, irradiation or hyperthermia separately reduces cell viability significantly in comparison to that of the control group. Even though the combined therapy including G+R, R+H and G+R+H showed the greater percentage of killing in glioblastoma cells significantly than that of the control group. Of course the greatest reduction in glioblastoma cells was caused by G+R+H due to loss of the colony formation ability.

It was found the combination of Gemcitabine and irradiation respectively 1.46 and 4 times more than the radiation and Gemcitabine alone had cytotoxic effect on glioblastoma cells.

Our study showed hyperthermia could sensitize glioblastoma cells respectively 1.2 and 1.4 times to gamma irradiation and Gemcitabine alone. Meanwhile integration of Gemcitabine, radiation and hyperthermia simultaneously could reduce colony formation respectively 1.85 and 1.5 times less than that of using radiation plus hyperthermia and Gemcitabine plus radiation.

Gemcitabine as a DNA synthesis inhibitor, inhibits the repair of genomic damage induced by irradiation and as a cytotoxic product decreases the number of tumor colonies. Tumor shrinkage induced by Gemcitabine may improve tumor oxygenation. It could reduce the effect of tumor hypoxia and finally could gain radiation response. In addition Gemcitabine incorporation into the DNA could cause an apoptotic response (Gregoire et al., 1999).

On the other side hyperthermia accelerates chemical reactions leading to cell death and preventing radiation-induced damage repair.

5. Conclusion

On the whole this study showed the combined therapy of Gemcitabine, radiation and hyperthermia could cause synergistic effect in treatment of glioblastoma.

Declaration of interests

There is no conflict of interest.

Acknowledgment

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