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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.

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/m^2 of body surface according to the cancer type (Lawson et al., 2006).

Gemcitabine is a pro-drug. It is activated intracellular by deoxycytidine kinase. After transferring into the cell,

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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 difluorodeoxyuridine (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. 5×10^5 cells were seeded in 100 mm T-25 flasks (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% CO_2. 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% CO_2. 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:

$$V = a \cdot b^2 \cdot \pi/6$$

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:

$$V = V_0 \times e^{kt}$$

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:

$$VDT = Ln2/k$$

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 ^{60}CO (Teriton 76) with dose rate of 89.23 cGy/min from posterior, 24 h after treatment with Gemcitabine. The field size was 20×30 cm^2