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Cytotoxic effects of hyperthermia, chemotherapy (Navelbine) and radiation on glioma spheroids

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HIGHLIGHTS

- Effects of hyperthermia, chemotherapy and radiation were evaluated on the glioma cells.
- Results suggest significant synergistic effect on the cells at low concentration.
- The frequency of DNA damages was approximately 20 to 26 times more than cell death.

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ABSTRACT

Introduction: Glioblastoma multiform (GBM) is the most common malignant primary brain tumor in humans. Current conventional treatment could not effectively increase the survival rate of patients who suffered from GBM. In clinical view, some experiments which can mimic in vivo conditions to evaluate anticancer agent effects on tumors are very useful. We can fairly reach to this goal using 3-dimentional spheroids rather than monolayer culture. The aim of this study was to determine the cytotoxicity caused by Navelbine, hyperthermia and radiation on glioma spheroids.
Methods: The spheroid cells were separately or simultaneously treated with the doses of 7.5 μg/ml Navelbine for 50.3 h, irradiated with 2 Gy and incubated at 43 °C for 1 h. Comet and colony formation assays were applied to assess the DNA damages and survival rate of the cells.
Results: In both comet and colony formation assays, cell damage in all treated groups was significantly higher in comparison with control group ($P < 0.05$). Moreover, the synergistic effect of combined groups such as: hyperthermia and radiotherapy, Navelbine and radiotherapy and combination of Navelbine-hyperthermia-radiotherapy was observed.
Conclusions: Combined agents with low concentration can cause a synergistic effect on treatment procedure and increased damages in multicellular spheroids.

1. Introduction

Glioblastoma multiform (GBM) or grade 4 astrocytoma is the most malignant astrocytoma and includes more than 50% of gliomas (Bleeker et al., 2012). Because most chemical drugs cannot pass through the blood-brain barrier and on the other hand high dose radiotherapy can cause damages in normal brain tissues, glioma cells do not respond to the conventional treatment (Lawson et al., 2007). In this situation, median survival of patients who undergone comprehensive surgery, radiotherapy and chemotherapy can reach to 14.6 months (Mirimanoff et al., 2006) and only 27% and 10% survive for two and five years respectively (Stupp et al., 2009). To resolve this problem, new treatment approaches are in progress.

Radiotherapy followed by surgery is the most prevalent treatment for GBM but it can only increase the survival up to 11% for 2 years (Stupp et al., 2009) hence, complementary treatment methods are welcome.

Hyperthermia, raising tumor temperature more than 37.5–38.3 °C (Axelrod and Diringer, 2008), is used to degrade the intracellular proteins, shrink cellular components or enhancing chemical reactions to induce cell death finally (Hynynen and Lulu, 1990). Although hyperthermia has been widely used as a method of treating cancer for centuries but based on current information, there is a general agreement that the local hyperthermia cannot play a key role in controlling the tumors (Hall and Giaccia, 2006).

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In fact, hyperthermia is a complementary treatment that destroys those cancer cells that untreated by irradiation or it can increase the effects of anticancer drugs (Ozben, 2006).

Navelbine is a chemotherapeutic agent that used to treat non small cell lung cancer (Group ELCVIS, 1999) and breast metastatic carcinoma (Romero et al., 1994). Previous studies have shown Navelbine can be used to treat recurrent head and neck cancers (Degardin, 1998) and neuroblastoma spheroids in vitro (Bilir, 2010). Also in an interesting report, a young girl with recurrent GBM, showed great improvement after Navelbine treatment (Biassoni et al., 2006). The use of Navelbine has shown much promising results (Elderly Lung Cancer Navelbine Italian Study Group, 1999), but neutropenia and leukemia induction are considered to be dose-limiting factors for Navelbine (Mathe and Reizenstein., 1985). In this in vitro study we used of appropriate doses of Navelbine (Markasz, 2007), Radiotherapy and Hyperthermia that can well tolerated in clinical experiments.

2. Material and methods

2.1. Cell line

In this study we used U87MG human glioma cell line, which was prepared from the cell bank of the Pasteur Institute of Iran.

2.2. Medium

To prepare 100 ml of medium, 0.96 g Minimum Essential Media (MEM) powder (with L-Glutamine) (Merck), 1 ml penicillin/streptomycin (PAA) and 0.22 g NaHCO3 (Sigma) were dissolved in 80 ml deionized water (double distilled water) and 200 μl Fungizone (Merck) added to the final solution. PH of solution was set in the range of 7–7.1. The volume of the solution was brought to 90 ml with deionized water and filtered at the end followed by addition of 10 ml heat inactivated fetal bovine serum.

2.3. Cell culture

Monolayer cells were detached from the flask by Trypsin-EDTA and counted and then 3×10^5 living cells separated and cultured in each T-25 flasks covered by a thin layer of %1 agar containing of 8 ml of medium. Flasks were incubated at 37 °C with 5% CO2 and saturated humidity. Half of the medium was replaced with fresh medium twice a week.

2.4. The growth curve and volume doubling time

Spheroid volume doubling time (VDT) was calculated to determine the duration of drug treatment. After two days of culture and the formation of spheroids, they were transferred to a multi-well plate (24 well) in which each well was covered with a thin layer of %1 agar containing 1 ml of medium. Spheroid transfer was done in a manner that one spheroid seeded in each well. Plates were incubated at 37 °C with 5% CO2 and saturated humidity. Every three days, half of the medium was replaced with fresh one and every other day two cross diameter of three spheroids were measured randomly using a lens calibrated optical microscope. Spheroid volume was calculated according to the following equation:

$$V = a \times b^2 \times \pi/6 \quad (1)$$

where a is the small and b the large diameter of spheroid. Volume curve was plotted according to semi-logarithmic scale versus time. Spheroid volume represented by the following equation for linear curves or logarithmic phase:

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

V_0 is the initial volume; V is the volume of the spheroid after time (t), and k the slope for the linear plot. Finally spheroid volume doubling time (VDT) was obtained using the following equation:

$$\mathrm{VDT} = \boldsymbol{Ln}\ 2/\boldsymbol{k} \quad (3)$$

Tumor cords with a radius more than 160 μm (with a diameter more than 320 μm) are made from 3 layers of necrotic, hypoxic and stromal tissue (Hall and Giaccia, 2006). On 18th day after seeding, spheroids diameter were about 300 μm. After a VDT drug treatment their diameter became more than 320 (on 20th day of seeding). Thus, we expected that spheroids had been made from 3 layers of necrotic, hypoxic and stromal tissue on this day. We think this structure is useful because it makes spheroids more similar to the tumors.

2.5. Drug treatment

0.75 ml of Navelbine (0.1 mg/ml) was added to each 9.25 ml of medium proportionally to reach the final concentration of 7.5 μg/ml to T-25 flasks containing spheroids with a diameter of 300 μm. The spheroids were then treated at one volume doubling time to ensure formation of arrested metaphase in cells.

2.6. Hyperthermia

The water bath was used for heat treatment. After 20 days of seeding that spheroids are in 350 μm in diameter, the hyperthermia group was treated by incubating cells initially at 43 °C for 30 min followed by 10 min cooling at room temperature and heated again for extra 30 min. The group which should be treated with both hyperthermia and radiation was irradiated in the duration interval between heat treatment.

2.7. Irradiation

Cells were irradiated (MU = 185) with dose of 2 Gy using a linear accelerator 6 MV photon beam. After 20 days of seeding, the irradiation groups were transferred into T-25 flasks and flasks were then sandwiched between 3 cm slabs (PMMA Density 1.2 g/cm^3). Radiation dose was delivered to the center of the slabs via a single port.

2.8. Converting spheroids to single cells

For colony formation or comet assay, spheroids should be converted to single cells. Therefore, each of the control and treated groups transferred to falcon tube and centrifuged for 7 min at 3000 rpm. After removing the medium, samples were centrifuged in 1 ml phosphate buffered saline again. Buffer samples were removed and spheroids were incubated in 300 μl solution of trypsin/EDTA for 5 min. Cell-cell connections of spheroids were loose under the influence of trypsin/EDTA and converted to single cells. At last, 700 μl of medium containing 10% FBS was added to the each sample and counting and viability was determined.

2.9. Comet assay

In order to assess the DNA damage in the cells, alkaline comet assay was performed using Singh et al. (1988) protocol. Tail moments were photographed using camera on the fluorescent microscope with wide green filter. Photographs were converted to bmp format and analyzed by comet score software (Collins, 2004).