

Radiosensitization of Glioma Cells by Temozolomide (TMZ): A Colony Formation Assay

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ABSTRACT

Background: Glioblastoma is one of the most radioresistant cancers. It is suggested that combination of radiotherapy with other cancer treatment modalities may increase control of tumor. Temozolomide (TMZ) is one of the most known drugs for glioblastoma. It has shown that TMZ via induction of mutation and cell death can kill glioma cells.

Objective: In the current study, we aimed to show possible radiosensitization effect of TMZ for glioma cells. In addition, results compared to response of normal fibroblast cells to TMZ and irradiation.

Material and Methods: This is in vitro study for evaluation of the effect of TMZ and irradiation on high grade glioma cells and normal fibroblasts. The human fibroblast and glioma cells were cultured as monolayer. The cells were treated with 2000 μ M TMZ, which was equal drug dose for IC50%. In addition, irradiation was done with 5Gy gamma rays. The formation of colony was observed following irradiation, treatment with TMZ, and combination both of them.

Results: The formation of colony for both glioma and fibroblast cells showed a reduction following irradiation or treatment with TMZ. Irradiation showed more toxicity compared to TMZ for glioma cells, but not fibroblast cells. Combination of TMZ and irradiation showed a significant reduction in the colony formation compared to irradiation or TMZ treatment alone.

Conclusion: This study showed that TMZ increases sensitivity of both glioma and fibroblast cells to ionizing radiation.

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Keywords

Temozolomide; Radiation; Glioblastoma; Fibroblast Cells

Introduction

Glioblastoma multiforme (GBM) is one of the most resistant human tumors that can originate from astrocytes within brain or spinal cord [1]. Patients with GBM has poor survival [2]. Control of growth of glioblastoma is very difficult and usually complete treatment isn't possible [3]. Radiotherapy and chemotherapy are the most common modalities for reduction of cancer cells survival [4]. This can reduce tumor growth, thus increase survival of patients [5]. Radiotherapy is able to kill cells via direct interaction with genomic content

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of cells, and also through radiolysis of water molecules. Free radical production plays a main role for killing cells following exposure to radiation [6]. Because of severe toxicity of radiation for adjacent normal tissues, tumors can receive only a limited dose of radiation. Thus, it is necessary that patients receive another adjuvant for more suppression of tumor growth [7].

Temozolomide (TMZ) is an alkylating agent chemotherapy drug, approved by the food and drug administration (FDA) for some brain tumors, including glioblastoma [8]. It can be administrated orally because of its stability within acid environment [9]. Furthermore, because of small size and lipophilic properties of TMZ, it can pass from blood-brain barrier (BBB) easily [10]. It is suggested that anti-cancer activity of TMZ is different from ionizing radiation. It seems that TMZ can affect DNA directly by post-translational modifications. Moreover, TMZ can cause methylation of histone H3 protein and purine bases in DNA, leading to suppression of glioma cells proliferation [11].

In current study, we aimed to determine radiosensitization effect of TMZ in glioma cells. We evaluated the effect of TMZ combination with ionizing radiation on colony formation of glioma cells. Furthermore, we evaluated the toxic effect of this combination of the normal fibroblast cells.

Material and Methods

This is in vitro study for evaluation of the effect of TMZ and irradiation on high grade glioma cells and normal fibroblasts.

Cell Lines

U87MG human glioma cell line (ATCC® HTB-14™) was provided by the cell bank of the Pastor Institute of Iran and fibroblast cell line of primary culture from the eyelid skin biopsy of a 45-year-old woman. Cells were maintained in Dulbecco's modified eagle's medium (DMEM, GIBCO), containing 10%

fetal bovine serum (FBS, GIBCO), 100 U/ml penicillin (Sigma), 100 U/ml streptomycin (Sigma), and 2 mM L-glutamine.

Monolayer Cell Culture

Both cell lines were cultured as monolayer at a cell density of 10^4 cell/cm² in T-25 flasks (Nunc). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂. Cultures were propagated and cells were harvested by trypsinizing cultures with 1mM EDTA/ 0.25% Trypsin (w/v) (SIGMA) in Phosphate Buffer Saline (PBS).

Drug Treatment with TMZ

In order to evaluate the radiosensitizing effect of TMZ on glioma and primary fibroblast cells in the presence of irradiation (IR); 2000 μM of TMZ were added to the plates. Based on our previous study, this is the minimum concentration of TMZ in which cell growth is reduced by 50% (IC50) [12]. The plates contained monolayer cells with 2×10^3 cell/cm² density. Mentioned concentration was acquired with a solution of 2.5 mg TMZ powder in 1 ml of 0.2% DMSO in ambient temperature and kept at -20 °C. Samples were maintained under drug treatment for a cell doubling time (27 hours).

Irradiation Treatment

Glioma and primary fibroblast cells were treated with TMZ 27 hours before being exposed to ionizing radiation. The concentration of the chemical was 2000 μM, in MEM containing 10% FBS. After the treatment time, the medium containing drugs was removed and the cultures were washed 3 times with PBS and the cells were immediately irradiated using ⁶⁰Co source (Theratron 780, Canada) at a dose rate of 116.76 cGy/min for 5 Gy. For radiation treatment, culture flasks were put under collimator of equipment at 80cm distance of the head at ambient temperature, and the field size and the period of irradiation were 20×20 cm² and 4.31min, respectively.

Colony formation assay

After drug treatment and irradiation, single cells were plated for colony formation tests. In day 10 after plating, cultures were washed 3 times with PBS, fixed with 2% formaldehyde in PBS for 15 min and stained with 0.5% crystal violet; in addition, colonies were counted by invert microscope (Olympus).

In order to evaluate the ability of cells to form colonies, different concentrations of individual cells (50, 150, 250, 500 cells) from spheroid seeded into 60mm were plated with 10 ml of DMEM supplemented with 10% FBS. Plating efficiency (PE) and surviving fraction (SF) were calculated, using the following equations:

$$PE (\%) = \frac{\text{the number of colonies}}{\text{the number of seeded cells}} \times 100 \quad (1)$$

$$SF = \frac{\text{the number of colonies}}{\text{the number of seeded cells} \times \%PE} \quad (2)$$

Experimental Design

In order to evaluate the effects of TMZ and IR on U87MG and skin fibroblast cell lines, growth curve was drawn in semi-logarithmic scale with cell numbers versus time during 12 days. For the combined treatment based on IC50 of TMZ, a concentration of approximately 2000 $\mu\text{M}/\text{ml}$ was used. Therefore, the concentration of TMZ in the last step was con-

sidered as 2000 μM in order to achieve the desirable effect of less TMZ dose in combination with irradiation. The groups were treated with radiation and TMZ were divided as follows:

1. Control: received neither drug nor radiation
2. Control solvent: received neither drug nor radiation, but received 0.2% DMSO
3. TMZ: treated with 2000 μM dose of TMZ
4. Co: treated with 5 Gy of Co-60 gamma-rays
5. TMZ+IR: treated with TMZ (2000 μM) in combination with 5 Gy of Co-60 gamma-rays.

Statistical Analysis

Results were analyzed using SPSS version 16.0 and Excel 2010 software and data were obtained with triplicate experiments. The significance level of the data was compared by one-way ANOVA and Kruskal-Wallis non-parametric tests. A value of $p \leq 0.05$ was considered statistically significant.

Results

Figure 1a and b show the U87MG cell line and human skin Fibroblast cells in the monolayer culture model. The growth curve of U87MG cells was drawn in semi-logarithmic scale with number of cells versus time during 12 days. By calculating the slope of the curve

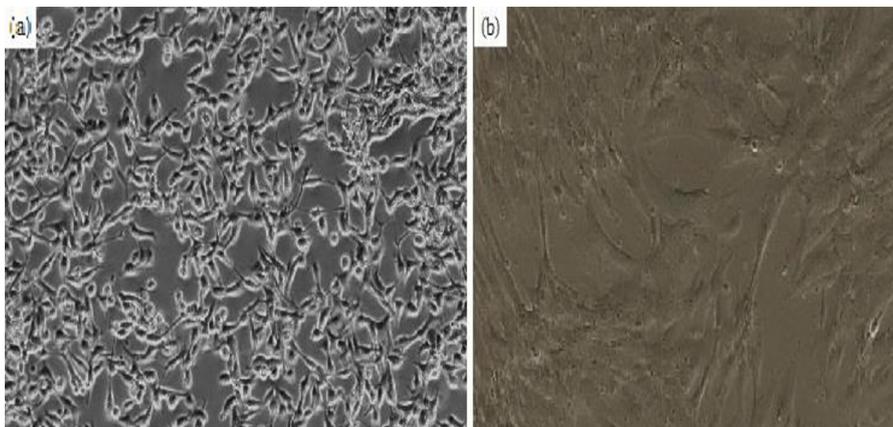


Figure 1: (a) U87MG cell line and (b) skin Fibroblast cells in the monolayer culture with 10X magnification.

in logarithmic region, the doubling time (DT) of U87MG cell growth was obtained which was equal to 27.36 ± 0.89 hours.

The groups that need to be treated with TMZ were subjected to it for one doubling time, i.e. 27 hours, to initiate apoptosis process. Then all the groups were transferred to the radiotherapy department for irradiation. Finally, after drug and irradiation treatment, the number of colonies was counted for the evaluation of radiosensitization effects of TMZ. The average of maximum and minimum numbers of forming colonies were 112, 29 and 96, 33 for U87MG and Fibroblast cells, respectively.

Based on the results of colony formation of different numbers of pre-cultured cells, the optimum cell number to create colonies from single cells of U87MG was ~ 150 per 60 mm dishes. The results are shown clearly in Figure 2.

To ensure that any observed reduction in the survival fraction of the monolayer culture cells is not related to the immediate death of the cells based on staining them with Trypan-Blue dye, the percentage of alive cells were immediately determined after the exposure. According to Figure 3, the percentage of alive cells in all treated groups were more than 95%.

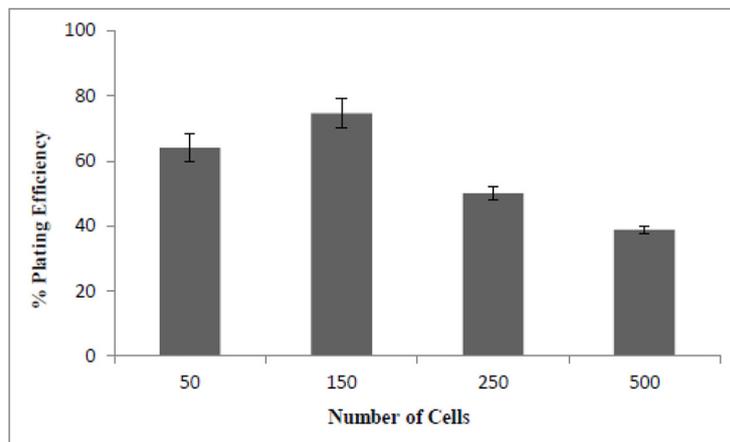


Figure 2: Plating efficiency using U87MG cells of monolayer culture in Dulbecco's modified eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) in different concentrations. Mean \pm Standard Error of the Mean (SEM) of 3 experiments.

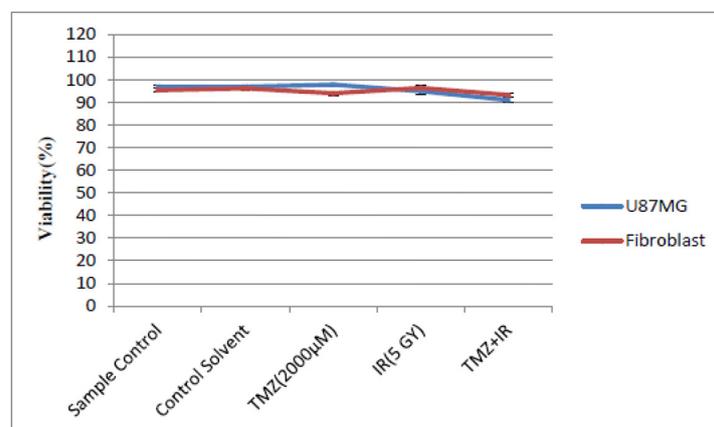


Figure 3: The percentage of living cells in different treated groups of U87MG and Fibroblast cells. Mean \pm Standard Error of the Mean (SEM) of 3 experiments.

Eventually, after exposing of samples by ⁶⁰Co in combination with TMZ, the ability of cells to form colonies was assessed using colony assay. Tables 1 and 2 show the number of colonies and PE percentage of cells in control and experimental groups under TMZ and IR treatment in both cell types.

As shown in Figure 4, the number of colonies counted in treated and irradiated samples was less than the control group. In addition, there were no statistical differences of data obtained for the %PE between the control and control solvent groups (P=0.2 and 0.4), meaning 0.2% DMSO does not cause any cytotoxic

Table 1: A summary of data obtained for U87MG cells in treated samples. Data shows mean values from triplicate experiments. SD is the standard deviation of mean values.

Groups	Mean No of colonies	SD	Mean PE%	SD
Control	112.33	6.03	74.88	4.02
Control solvent	105.33	8.50	70.22	5.67
TMZ	78.33	4.04	52.22	2.69
IR	57.33	3.81	38.22	2.34
TMZ+IR	29	3.00	19.33	2.00

SD: Standard deviation, PE: Plating efficiency, TMZ: Temozolomide, IR: Irradiation

Table 2: A summary of data obtained for primary fibroblast cells in treated samples. Data shows mean values from triplicate experiments. SD is the standard deviation of mean values.

Groups	Mean No of colonies	SD	Mean PE%	SD
Control	96.33	5.03	64.22	3.36
Control solvent	95.66	3.06	63.77	2.04
TMZ	70	5.57	46.66	3.71
IR	60.33	7.09	40.22	4.73
TMZ+IR	33.66	4.16	22.66	2.77

SD: Standard deviation, PE: Plating efficiency, TMZ: Temozolomide, IR: Irradiation

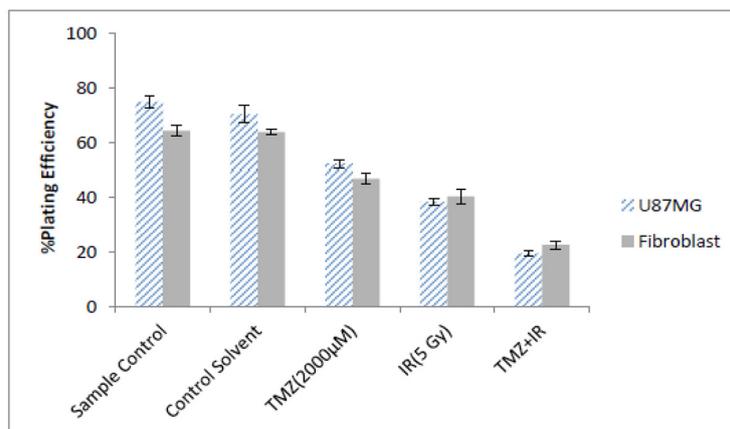


Figure 4: Plating efficiency using U87MG and fibroblast cells of monolayer culture in Dulbecco’s modified eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in different treated groups. Mean ± Standard Error of the Mean (SEM) of 3 experiments.

effect. Moreover, the difference between IR alone and TMZ alone for fibroblast cells was not statistically significant ($P=0.5$). However, for glioma cells, results showed a significant difference ($p<0.05$).

Treated group with TMZ+IR in both cell lines was compared to TMZ alone which showed a significant difference in U87MG and fibroblast ($P=0.002$ and 0.001 respectively). Beside, treated group with TMZ+IR in U87MG and fibroblast cells presented a significant difference compared to IR alone ($P=0.009$ and 0.03 respectively).

There was also a big difference, in TMZ+IR in comparison with the control as well as other groups ($p<0.002$ and <0.03 for U87MG and fibroblast respectively). In order to acquire the cytotoxic effect of TMZ on the cells in the presence of gamma radiation, the percentage difference of mean %PE in TMZ+IR relative to the mean %PE in IR exposed group was evaluated. This discrepancy was 50% and 44% for U87MG and fibroblast, respectively.

Furthermore, according to Figure 5 that shows the survival fraction in both cell lines, the lowest survival fraction was observed in the combination of TMZ and IR that was 25% for U87MG and 34% for fibroblast cells. In addition, there was no significance difference between the results obtained for U87MG cells

and primary fibroblast ($P\geq 0.05$). All the results were in agreement with our results published before [12].

Discussion

TMZ is known as one of the most famous drugs for temporary reduction of tumor bulk [13]. It has been reported that TMZ via induction of mutation induces death in glioma cancer cells [13]. Furthermore, it has known that TMZ is able to induce hypermethylation in the promotor of DNA mismatch repair (MMR) genes, thus causes accumulation of unrepaired DNA damage and finally cell death [14]. For first time, in 2005, a clinical trial study showed that TMZ in combination with radiotherapy could increase survival of patients with glioblastoma significantly. Besides, this study showed that the toxicity for combination of TMZ with radiotherapy is minimal [15]. This lead to approval of TMZ by FDA for patients with newly diagnostic glioblastoma [16]. TMZ also has shown promising results for patients with high-grade glioblastoma cancer [17, 18]. In recent years, a clinical trial study showed that TMZ is more useful for patients with methylated O6-methylguanine-DNA methyltransferase (MGMT) [4].

In the current study, we evaluated sensitization effect of TMZ in combination with radia-

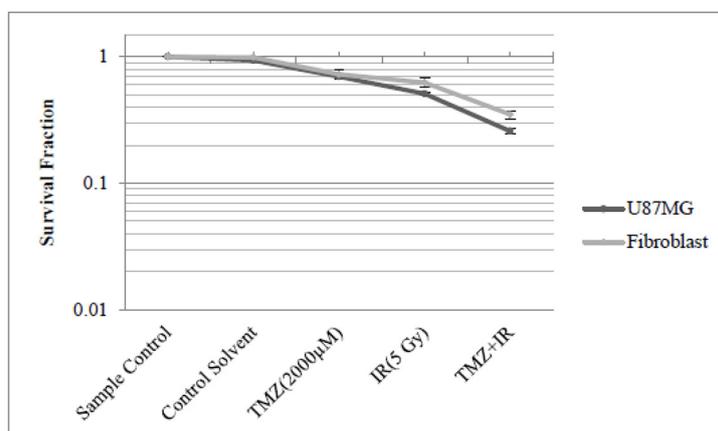


Figure 5: Survival fraction using U87MG and fibroblast cells of monolayer culture in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in different treated groups. Mean \pm Standard Error of the Mean (SEM) of 3 experiments.

tion for malignant glioma and normal fibroblast cells. At the first level, our results showed that appropriated concentration of glioma cells for colony formation is 150 per 60 mm dishes. Lower or higher concentrations showed less efficiency. For evaluating the toxicity of TMZ on both normal fibroblast and glioma cells, we measured colony formation following treatment with TMZ. Our results showed that TMZ has a significant toxicity and causes the reduction of colony formation by more than 25% for both fibroblast and glioma cells. Results of PE% showed no difference between glioma and fibroblast cells following treatment with TMZ as alone. Irradiation of glioma cells with a single dose of 5 Gy showed more efficiency compared to TMZ. Irradiation could reduce colony formation near to half of glioma cells. However, this reduction for fibroblast cells was not significant. Combination of TMZ with irradiation showed a remarkable reduction of colony formation and PE%. Results for both fibroblast and glioma cells presented a significant reduction compared to irradiation alone without treatment with TMZ. These results showed a significant radiosensitization effect of TMZ for glioma and fibroblast cells. Results of this study showed that TMZ might suppression of colony formation sensitize glioma cancer cells to radiation treatment.

Conclusion

In the current study, we show the effect of TMZ as a radiosensitizer using the evaluation of colony formation assay. Furthermore, the effect of both ionizing radiation and TMZ was alone determined. Results demonstrated a significant reduction of colony numbers following irradiation or treatment with TMZ. Irradiation showed more toxicity compared to TMZ alone for glioma cells, but not for fibroblast cells. Furthermore, the combination form of TMZ and irradiation was more toxic compared to treatment or irradiation alone. In conclusion, TMZ showed significant radiosensitization effects for glioma and fibroblast cells

via reduction of colony formation.

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Authors' Contribution

Somayyeh Babaloui, Ramin Jaberi and Hossein Mozdarani investigated and conceived the idea. The original draft of the paper was written by Somayyeh Babaloui and Masoud Najafi. Samira Borhani gathered the images and the related literature and also helped with writing of the related works. The method implementation was carried out by Samira Borhani. Results and Analysis was carried out by Somayyeh Babaloui, Masoud Najafi and Samira Borhani. The Project administration was performed by Mahdi Aghili and supervised by Ramin Jaberi. All the authors reviewed, modified, and approved the final version of the manuscript.

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Conflict of Interest

None

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