Evaluation of glioblastoma (U87) treatment with ZnO nanoparticle and X-ray in spheroid culture model using MTT assay

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HIGHLIGHTS

- MGU87 cells (spheroid 350 μm) treated with ZNO nanoparticles for 68 h (1 VDT).
- Difference between 6 Gy and Zno groups in density of 8000 cells was significant.
- ZnO more effective than the 2, 4, and 6 Gy doses of X-Ray in the treatment of MGU87.
- That is very important because of the protection of normal tissues to radiation.
- Treated cells with ZnO and 2, 4, and 6 Gy, viability not be significantly changed.

ABSTRACT

Background: Glioblastoma is the most serious brain tumor that is often incurable with surgery. Chemotherapy and radiation have also been unable to increase viability. Metal oxides nanoparticles such as zinc oxide (ZnO) are new techniques as anticancer agents that offer new hope in the treatment of cancers. We aimed to assess viability of glioblastoma cell lines U87 with spheroid culture model with a mean diameter of 350 μm using ZnO nanoparticles with 2, 4, and 6 Gy doses of X-Ray.

Methods: The spheroids in 8 groups including control group, the group irradiating with 2, 4, and 6 Gy doses of X-Ray, the group receiving only ZNO nanoparticles, and groups receiving radiation plus nanoparticles ZnO (Zno + 2 Gy, Zno + 4 Gy, Zno + 6 Gy) were assessed. After cells treatment with nanoparticles for about 68 h (one VDT = Volume Doubling Time), and radiation to the groups should be given especial doses with an accelerator 6 MV, the results were finally assessed by MTT assay test with 8000 and 10,000 cells densities.

Results: ZnO nanoparticle was alone more effective than irradiating with 2, 4, or 6 Gy doses of X-ray 6 MV to treat the MGU87 cells. After treating cells with ZnO nanoparticle for one VDT and delivering 2, 4, and 6 Gy doses of X-ray using an accelerator 6 MV, the viability of the cells remained unchanged (p > 0.05). Also, by adding ZnO nanoparticles to cell culture medium, it was achieved a better reduction of viability in the density of 8000 cells than in the density of 10,000 cells. Most importantly, this percentage reduction of viability in the group received ZnO nanoparticles alone with the density of 8000 cells was more than compared to the density of 10,000 cells. The difference between groups and the group received radiation with 6 Gy dose of X-Ray group with the density of 8000 cells was significant (p < 0.05).

Conclusion: In treatment of glioblastoma cell lines U87, using ZnO nanoparticles with the density of 10 mM can be an alternative for 2, 4, and 6 Gy doses of X-Ray 6 MV. This result is very important for the protection of normal tissues to radiation therapy. Adding 2, 4, and 6 Gy doses of X-ray after treating ZnO nanoparticles for one VDT did may not lead to higher efficiency of ZnO nanoparticles.

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1. Introduction

Glioblastoma constitutes more than 50% of all tumors of the nervous system. The World Health Organization categorized Glioblastoma as the components of four degree malignancies. Enam (2000) Glioma, originated from malignant transformation of glial cells (astrocytes, ependymal and oligodendrocytes), which eventually gets grade of glioblastoma multiform. Mahaley and Mettlin (1990) In vitro the cells set in a single layer in contact with culture medium and are properly oxygenated. In the spheroid
model, the superficial cells can be exposed to medium culture and thus the cells at depths greater than 100 μm remained oxygen deficient. Leek et al. (2005) Currently surgery, chemotherapy and radiotherapy are used for the treatment of glioma. Surgical treatment of the tumor is complete or partial surgical removal of the tumor (Galani and Buckner, 2000). The first decision to treat patients with glioma is surgery in combination with radiotherapy (Jemal et al., 2003). Unfortunately, the optimal surgery for malignant brain tumors is rarely feasible because most have recurring feature. In chemotherapy, many tumors are resistant to chemical agents (Galani and Buckner, 2000). High-dose radiotherapy for glioma may be also failed because of intolerance of normal brain tissues (Schultheiss et al., 1995). Surgery combined with radiotherapy is of utmost importance in the treatment of most cancers. Due to the anatomical location of the brain, critical brain areas and limitation of radiation dose, advantages of this therapy for brain tumors are limited (Barker et al., 1996). Making the disappointing results led to focusing on targeted methods to reduce the dose of ionizing radiation. The basis of these targeted methods is the use of molecules that can be selectively entered the cancerous cells, cause damage to cells, or cause increased vulnerability of cancer cells to radiation (Sullivan et al., 1994). Radiation therapy combined with metal nanoparticles is a new approach for the treatment of cancers (Zhang et al., 2008). Various types of metal nanoparticles and metal oxide nanoparticles are available that among them, metal oxide nanoparticles are widely used as versatile materials. In this group, ZnO nanoparticles have an especial role because of their role in biological and chemical sensors (Baxter and Aydil, 2005; Sawai et al., 1996). The applications of ZnO nanoparticle depend on their characteristics such as size, density, shape, crystal structure, and artificial method of making it (Vaseem et al., 2010). It is expected that selecting cancerous cells is more advanced even from engineering design to minimize damage in cancer cells (Hanley et al., 2009). The studies could show that treating the glioma cells lines LN22, LN18, LN2308, A172, and U87 using ZnO nanoparticle lead to cytotoxicity in these cells that is inactive in healthy cells, thus ZnO nanoparticle have selective effects on cells (Ostrovsky et al., 2009). One of the main advantages of these particles is their inherent and preferential cellular toxicity in cancerous cells in vitro (Hanley et al., 2008; Wang et al., 2009).

The effects of ZnO nanoparticle on tumor cells line U251 could demonstrate its cytotoxicity so that the cytotoxic effect depend directly on the size and density of nanoparticle (Chue and Valiyaveettil, 2009). In tumor cells, the use of nanoparticles with a high atomic number increases damage when used in conjunction with X-ray radiation to the tumor. The findings could confirm that in treating tumor cells, nanoparticles can increase the radiation-related damage. This damaging is dependent on the size and density of nanoparticles that in the sizes of 2–400 nM, the particle size is inversely related to the degree of damage (Hossain and Su, 2012). Pre-treatment of DU145 cancer cells with FeO nanoparticles and irradiated with 2 Gy dose of X-ray with 6 mega-volts led to 1/2 dose increase factor that is equal to increase in cells sensitivity by 24% (Khoei et al., 2014). Also, using FeO nanoparticles on CT26 cells, it was shown that the cells receiving FeO nanoparticles and 10 Gy dose of X-ray indicated tumor regression by 100% after 5–35 days and also those cell lines receiving FeO nanoparticles and 40 Gy dose of X-ray showed regression by 100% (Gi-Hwan Choi et al., 2012). In 2005, in the Monte Carlo simulation model, it has been shown that dose increase factor in tumors with 7 mg of gold nanoparticle concentration and X-ray 4–6 MV ranged from 1% to 7% (Cho, 2005).

In current study, we aimed to assess the effects of a combined regimen with ZnO nanoparticles and 2, 4, and 6 Gy doses of X-ray with 6 mega-volts on viability of glioblastoma cell lines U87 using MTT assay. In this study, we assessed the results using two different 8000 and 10,000 cellular densities.

2. Materials and methods

The study was a practical laboratory research on population of 350 μm of glioblastoma spheroids. MGI87 cell lines of glioblastoma were prepared from cell bank of the Pasteur Institute. For preparing 100 ml of cellular culture medium, 0.96 gr of minimum essential medium (MEM; Gibco/Invitrogen, USA) containing l-Glutamine, Penicillin 1 mL (GmbH/PAA, Austria), and 0.22 g of NaHCO3 (Sigma/Aldrich, Germany) were dissolved in 80 mL of deionized water and regulated at pH of 6.9 to 7.1. The solution volume was reached to 90 ml and was filtered by a filter of 0.22 mm (Orange). Then, 10 mL of Fetal Bovine Serum (FBS; Gmbh/PAA, Austria) was added to solution.

3. ZNO nanoparticle

According to molecular weight of ZnO nanoparticle for preparation of 10 mM of solution, the powder was measured and placed in a micro-tube with measuring phosphate-buffered saline (PBS; MPBiomedicals/Germany). ZnO was sonicated by a sonicator before adding it into culture medium.

3.1. Monolayer culture

Glioblastoma cell lines U87 are adhesive cells that were cultured with the density of 25 × 10³ cells/cm² in T-25 tissue culture flasks (NEST) and maintained in maximum 6 passages. Cell culture was done in MEM culture medium containing PBS 10%. After the cells were filled container floor, the flask was removed from the incubator, supernatant medium was poured out, the floor of the plate was washed using PBS, and then EDTA/trypsin solution was added. The flask was placed at incubator 37 °C.

4. Spheroid culture

The spheroids were cultured by liquid overlay technique. First, a solution 1% of agar (Sigma-Aldrich, Germany) was prepared. After heating and cooling solution, 8 ml of the solution was poured and agar gel was prepared. Equivalent to half a million viable cells were isolated and cultured at 12 ml of culture medium consisted of PBS 10%. Carefully once every three days, half of the spheroid was replaced with fresh medium.

4.1. Growth curve

For drawing the spheroid growth curve, the cells in a Petri 100 ml were cultured as spheroids. After three days of culture and the formation of spheroids, cells were transferred from Petri 100 ml to a MEM culture medium containing PBS 10%. The volume of each spheroid was calculated using the following formula: 

\[ V = \frac{4}{3} \pi \frac{a^3}{b^2} \]

that (a) and (b) variables were large and small diameters. Then, the curve of volume per time was drawn on semi-logarithmic scale. In the linear region of the curve or logarithmic phase, spheroids follow the following link: 

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

where \( V_0 \) is initial spheroid volume, \( V \) is spheroid volume, \( t \) represents the time, and \( k \) represents the slope of the linear plot. The time for Spheroid volume doubling was also calculated by the following formula:

\[ VDT = \ln \frac{2}{k}. \]
4.2. Drug treatment with ZnO nanoparticle

For drug treatment with ZnO, first tumor cells were cultured in culture medium to achieve spheroids with the diameter of 350 μm in medium. Then, nanoparticle in PBS was sonicated by sonicator and was then added to the culture medium with concentration of 10 mmol. For performing this test, drug treatment was done for all spheroids candidates for receiving nanoparticle for one VDT (68 h) and the cells were maintained in the incubator. After one VDT, the cell groups which considered for irradiation received 2, 4, and 6 Gy doses of X-ray. Then, spheroids were tripisinated and converted to single cells mechanically and then the level of cellular damage was determined and analyzed by MTT method.

4.3. MTT assay

In this analytical method that was primarily developed by Mosmann in 1983, the density of cells can be determined based on cell color in small volumes (Mosmann, 1983). After converting spheroids to single cells, two different densities of single cells including 8000 and 10,000 cells in different plates were selected for MTT assay. After a period of 24 h, dissolved MTT in PBS with final density of 0.5 mg/ml was added and after incubation for 4 h, MTT solution was extracted and DMSO was then added and was shaken for 20 min. Finally, absorption of the samples was read by regulating 570-nanometer filter as the main wavelength and also 630-nanometer filter as the referenced wavelength. The absorption rate of the wells without cells was diminished from absorption of the wells with cells to obtain pure cellular absorption. According to the following equation, there is a direct association between pure absorption and rate of viable cells:

\[
\frac{\text{Mean absorption of sample}}{\text{Mean absorption of referenced}} \times 100 = \text{percentage of viable cells}
\]

For irradiation of the cells, a 6 megavoltage accelerator was used and 2, 4, and 6 Gy doses of X-ray were irradiated. All required data were defined in Iso Gray software. For assessing the level of cellular damage, spheroids with the mean diameter of 350 μm was divided in 8 groups including the control group, the group irradiating with 2, 4, and 6 Gy doses of X-Ray, the group receiving only ZnO nanoparticle, and groups receiving radiation plus nanoparticle ZnO (ZnO + 2 Gy,ZnO + 4 Gy,ZnO + 6 Gy). The quantitative results were finally compared with T test or ANOVA test using SPSS software version 20.0 (Chicago, IL, USA).

5. Results

The spheroids were cultured by liquid ovary and the mean doubling time of spheroid volumes was obtained 67.90 ± 0.57 h (Fig. 1).

In second phase, damaging spheroids with the diameter of 350 μm following drug treatment with ZnO nanoparticle (10 mmol) and radiation with the different dosages of 6 MV X-ray in 8000 was assessed.

In the figure of 2, the percentage of cellular viability in the group irradiating with 2, 4, and 6 Gy doses of X-ray and in the group that only received nanoparticle is shown. Compared to the control group, the decrease in cellular viability in the groups received 2, 4, and 6 Gy doses of X-ray was 19.0%, 29.5%, and 36.5%, respectively with a significant difference (p < 0.05). However, the rate of viability in the group received only ZnO nanoparticle was 47.5% that accompanied with a 52.2% decrease in the cells survival. Thus, the use of ZnO nanoparticle alone could be accompanied with more effectiveness on compared with irradiating with 6 Gy dose of X-Ray. According to the points in Fig. 2, the decrease of viability in cells received both ZnO nanoparticle and different doses of X-Ray was significantly higher than the control group. In this regard, receiving nanoparticle and each 2, 4, and 6 Gy doses of X-Ray resulted in decreasing viability by 44.5%, 42.5%, and 41.5% that were all higher than in control group (p < 0.05). The percentage of viability in those MGU87 cells received ZnO nanoparticle and in the cells received both ZnO nanoparticle and different doses of X-ray was statistically similar. According to the Fig. 2 indicating results related to cells with 8000 density revealed significant difference compared to the control group.

Fig. 3 indicates the results related to treatment with ZnO nanoparticles (10 mmol) and radiation with the different dosages of 6 MV X-ray in 10,000 cellular densities. The percentage of cellular viability in the group irradiating with 2, 4, and 6 Gy doses of X-ray was 81.73%, 71.47%, and 63.32%, respectively with 18.67%, 28.5%, and 36.5% decrease in viability, respectively showing a significant difference (p < 0.05). As a matter of fact it was found there was significant difference between treatment with ZnO nanoparticle alone and treatment with 2 and 4 Gy doses of X-ray. However it was not found significant difference in regard of 6 Gy. Comparing the results related to treatment MGU87 cells with ZnO nanoparticle alone and combined nanoparticle with the different dosages of 6 MV X-ray in 10,000 cellular densities showed no significant difference. However, according to the Fig. 3 control group had significant difference with other groups.
As shown in Fig. 4, the percentage of cellular viability decrease in the control group and the groups received 2, 4, and 6 Gy doses of X-ray in two 8000 and 10,000 cellular densities was not different. The viability decrease in MGU87 cells in the group of cells with two 8000 and 10,000 densities received nanoparticle significantly decreased when compared with the control group. In inter-group comparing, the highest difference was found between control group and the group received ZnO nanoparticle alone and this difference was higher in the 8000 density than in the 10,000 density (with 5% difference in percentage).

6. Discussion

In the present study, we assessed the effects of ZnO nanoparticles alone or in combination with different 2, 4, and 6 Gy doses of X-ray in two 8000 and 10,000 cellular densities for treating U87 cell lines of glioblastoma. In the density of 8000 cells, all study groups were different to the control group so that there were significant differences in cellular viability between those cells receiving ZnO nanoparticle alone, radiation alone, or ZnO nanoparticle combined with 2, 4, and 6 Gy doses of X-Ray in both cell densities in comparison with the control group.

ZnO nanoparticle has natural OH groups that stick to its surface and play a key role in superficial charge behavior (Qu and Morais, 1999; Qu and Morais, 2001). Metal nanoparticles may increase the effectiveness of radiotherapy by screening or by absorption of ionizing radiation leading decrease of total radiation dosage or its related side effects. Since glioblastoma is resistant to radiation, has no distant metastasis, and has a rapid growing, the use of ZnO nanoparticle to treat these malignant cells can be an especial issue (Petras Juzenas et al., 2008; McGinn et al., 1996). Nanoparticles can circulate in the body, target especial tissues and organs, penetrate cellular membrane, entering into the mitochondria, and stimulating cellular apoptotic responses (Andre Nel et al., 2006). The decrease in cellular viability by using with 2, 4, and 6 Gy doses of X-ray were 19.5%, 29.5%, and 36.5%, respectively that were significantly different to the control group ($p < 0.05$). Comparing viability between the groups receiving different radiation doses showed only significant difference between the groups irradiated by 2 or 6 Gy doses of X-Ray. The percent of the viability of MGU87 cells that received ZnO nanoparticle alone was lower than that in the control group with a 47.8% viability percentage. The recent studies have showed that the use of elements with high atomic numbers such as gold nanoparticles accompanied with irradiation can increase cancer cellular damage and even higher than using X-ray alone (Garnica-Garza, 2012). These elemental nanoparticles can increase the absorbed dose to the tumor as a radio-sensitizer. This effect is dependent on nanoparticles structure, size, diameter, and shape of nanoparticles as well as to the type of radiant energy (Hossain and Su, 2012). In our study, it was shown that the viability percentage in MGU87 cells treated with ZNO nanoparticle alone was not different to other cells received this nanoparticle and different 2, 4, and 6 Gy doses of X-ray. In fact, irradiating cells previously receiving ZnO nanoparticle may not led to a synergistic effect that is consistent with a study performed in 2012 that showed lower effect of using X-ray with the source of LNAC and FeO nanoparticles in comparison with monochromatic radiation (7% versus 50% to 70%) (Kleinauskas et al., 2012; Pradhan et al., 2009). We also showed no difference in cellular viability between the cells receiving different doses of X-ray without ZnO nanoparticle. Similar to the density of 8000 cells, the difference between cellular viability in the density of 10,000 cells was only revealed between the groups received 2 and 6 Gy doses of X-Ray.

We also showed that after treating the cells with ZnO nanoparticles for one VDT time, the percentage of cellular viability by irradiating cells with dosages of 2, 4 and 6 Gy found no significant change ($p > 0.05$).

Also, the difference between cellular viability in the groups receiving $\text{ZnO}_x + 2 \text{Gy}$, $\text{ZnO}_x + 4 \text{Gy}$, and $\text{ZnO}_x + 6 \text{Gy}$ in comparison with the group received only ZnO nanoparticles was not significant. Moreover, inter-group difference for $\text{ZnO}_x + 2 \text{Gy}$, $\text{ZnO}_x + 4 \text{Gy}$, and $\text{ZnO}_x + 6 \text{Gy}$ also remained insignificant ($p > 0.05$).

Comparing two 8000 and 10,000 cellular densities showed that in all groups except for the group received only ZnO nanoparticle; a slight non-significant difference was revealed between them in cellular viability. However, none of the similar groups in two densities had any difference in viability. But, similar groups in two densities which received ZnO nanoparticle alone, better viability was observed in the density of 8000 than in the density of 10,000. In fact, the decrease in viability in the group receiving ZnO nanoparticle with the density of 8000 cells was higher than in another group which received ZnO nanoparticle alone with the density of 10,000 cells ($p < 0.05$). Beside in the density of 8000 cells there was significant difference for viability between the groups receiving ZnO nanoparticle alone and 6 Gy ($p < 0.05$).
However this significant difference was not found in the case of 10,000 cells ($p > 0.05$). As a matter of fact further damage could not be done by 6 Gy.

7. Conclusion

In conclusion, ZnO nanoparticles is more effective than the use of the one of 2, 4, and 6 Gy doses of X-Ray in the treatment of MCGU87 tumor cells that is very important because of the protection of normal tissues to radiation. In this regard, by treating cells with ZnO nanoparticles for one VDT time using an accelerator 6 MV and by adding 2, 4, and 6 Gy doses of X-Ray, the cellular viability may not be significantly changed.

Conflict of Interest

There is no conflict of interest.

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