



Influence of dose rate on radiotherapy treatment of the U87MG cell line through cell viability

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Abstract: Glioblastoma multiforme is a tumor that affects glial cells and is common in humans, being the most aggressive of gliomas. Patients, after diagnosis, have an average survival of just over a year, even when undergoing standard treatment. Given the low estimated survival of patients with this type of tumor, the objective of the study is to investigate the contribution of dose rate in radiotherapeutic treatment using a human glioblastoma cell line called U87MG, through the MTT (brometo de 3-[4,5-dimetiltiazol-2-il]-2,5-difenil tetrazólio) cell viability assay. Three dose rates were tested, including 400 cGy/min which is used clinically in patients, as well as 1.400 and 3.300 cGy/min from a clinical linear accelerator. Sterile culture plates with U87MG cells were irradiated, then, manipulated in the laboratory for the execution of the cell viability assay. The irradiated plates showed lower cell viability than the non-irradiated group, but there was no significant difference in viability among the different tested dose rates.

Keywords: Glioblastoma multiforme, Radiotherapy, cell line, U87MG, dose rates, cell viability, MTT.



Influência da taxa de dose no tratamento radioterápico da linhagem celular U87MG através da viabilidade celular

Resumo: O Glioblastoma multiforme é um tumor que afeta as glias e comum em humanos, sendo mais agressivos dos gliomas. O paciente, após o diagnóstico, possui uma média de sobrevida de pouco mais de um ano, mesmo submetido ao tratamento padrão. Tendo em vista a baixa sobrevida estimada dos pacientes com esse tipo de tumor, o objetivo do trabalho é estudar a contribuição da taxa de dose no tratamento radioterápico usando uma linhagem celular humana de glioblastoma chamada U87MG, por meio do ensaio de viabilidade celular chamado MTT (brometo de 3-[4,5-dimetiltiazol-2-il]-2,5-difenil tetrazólio). Foram estudadas três taxas de dose, dentre elas 400 cGy/min que é usada clinicamente em pacientes, bem como 1.400 e 3.300 cGy/min de um acelerador linear clínico. Placas de cultura estéril com células U87MG foram irradiadas, então, manipuladas em laboratório para execução do ensaio de viabilidade celular. As placas irradiadas mostraram menor viabilidade celular que grupo não irradiado, mas não houve diferença significativa na viabilidade entre as diferentes taxas de dose.

Palavras-chave: Glioblastoma multiforme, Radioterapia, linhagem celular, U87MG, taxas de dose, viabilidade celular, MTT.

1. INTRODUCTION

Glioblastoma multiforme (GBM) is a tumor from the glia, one of the most aggressive of the gliomas, clinically considered grade 4 and most common in humans [1]. Malignant gliomas are histologically heterogeneous and invasive tumors, of which glioblastoma can be characterized by mitotic activity and contain areas of microvascular proliferation and/or necrosis [2]. It is not possible to treat this type of tumor with surgery only, and the average survival after surgery resection and conventional therapy is about 12-15 months [3]. GBM tumors can develop, especially if it is recurring, an increased resistance.

The patient postoperative is submitted to radiotherapy (RT), because glioblastoma has an infiltrative nature and surgery leads to a neurologic loss. Around 1960s and 1970s some cases suggested that postoperative radiotherapy provides survival advantage. Despite the variation of dose on RT, in general, the reports point to an improvement in survival [4]. Therefore, is important to further investigate if varying total doses and dose rate could be more effective with least harm and exposure to radiation to the patient.

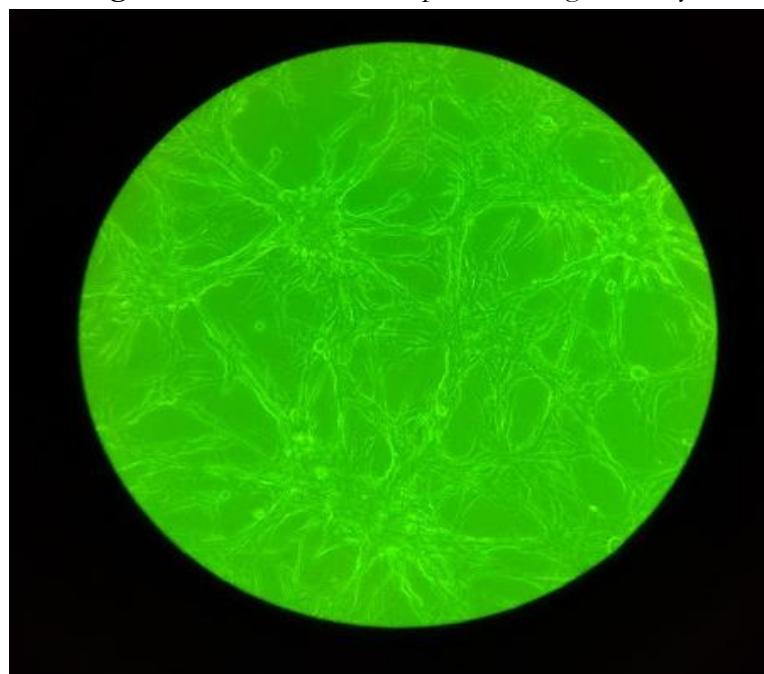
The objective of this study is to evaluate if different dose rates impact in this kind of problem in treatment. To achieve this, U87MG cells, a human glioblastoma cell line, were used for investigation in a cell model of irradiation, followed by viability quantification by the MTT assay. We analyzed three dose rates groups of samples and compare with non-irradiated group (control), and we observed reduced survival rate of irradiated samples in comparison with control group, but no significant reduction among them.

2. MATERIALS AND METHODS

2.1. Cell line

All experiments were performed using the human glioblastoma cell line U87MG. This cell line – presents a unique morphology shown in Fig. 1, where profile at high density can be observed – has been characterized as radio-resistant [5], and in this work, is maintained in DMEM culture medium, supplemented 10% fetal bovine serum in a sterile culture plate. Cells are maintained at 37°C in a humidified incubator with a 5% CO₂ atmosphere for pH buffering. Cells were manipulated using a class II, A1 laminar flow hood to guarantee sterility, located at Laboratório de Genética Molecular of Instituto Federal do Rio de Janeiro.

Figure 1: U87MG cell line profile at high density.



Source : Own authorship.

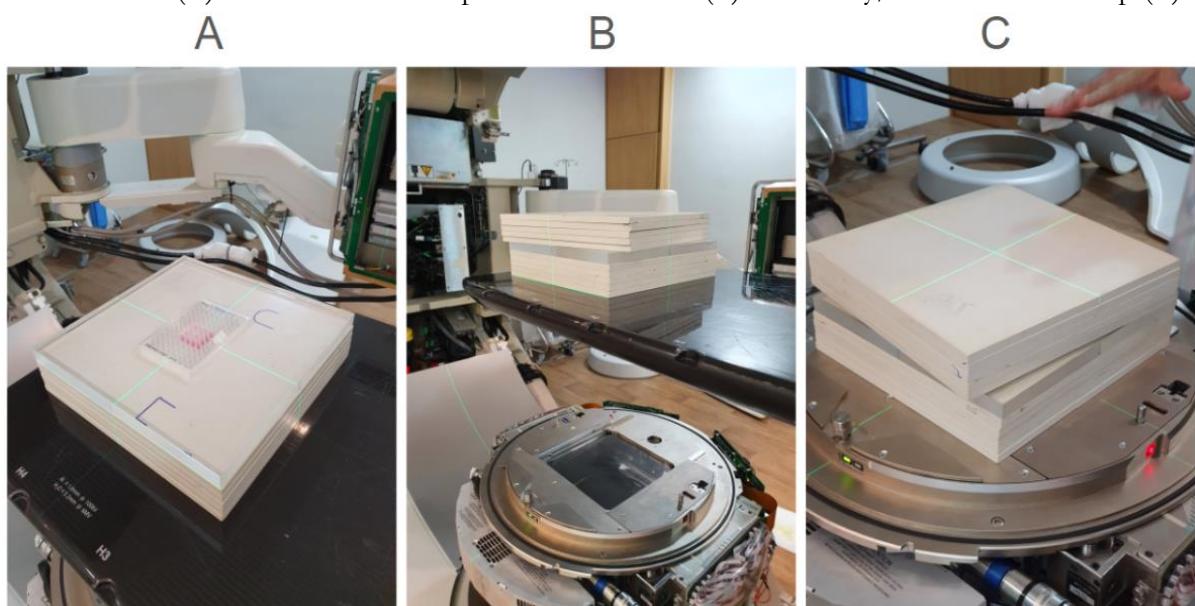
2.2. Irradiation setup

Irradiations were carried out using the clinical linear accelerator Varian TrueBeam of Hospital Quinta D'Or in Rio de Janeiro.

For the cell lines irradiation, a 6 MV FFF (Flattening Filter Free) photon beam from the Varian TrueBeam linear accelerator was used with different dose rates: 400 (clinically used), 1.400 and 3.300 cGy/min. The culture plate was positioned in a central opening of a PMMA (Poly(methyl methacrylate)) slab, with adequate dimensions to accommodate the plate with cells with minimal clearance, allowing it to be placed and removed easily. The PMMA slab was sandwiched between five 1 cm thick solid water plates, with 5 plates placed on the PMMA slab and another 5 plates (Fig. 2 A and Fig. 2 B) placed under it. This apparatus was built to ensure the electronic balance of particles on the plate and the beam focused on the sample from bottom to top.

For the 400 and 1.400 cGy/min dose rates, a source-cell surface distance of 100 cm was set between the radiation source and the cell lines, with 15x15 cm² field size and 2227,4 monitor unit to achieve 20 Gy of total dose. The 3.300 cGy/min dose rate, on the other hand, was only achieved by positioning the pile of plates directly on the accelerator beam's exit window (Fig. 2 C), with 25x25 cm² field size and 901,0 monitor unit, to also obtain 20 Gy of total dose.

Figure 2: Irradiation setup for different dose rates. Culture plate in central opening of the PMMA slab (A). Pile of solid water plates with PMMA (B). 3300 cGy/min dose rate setup (C).



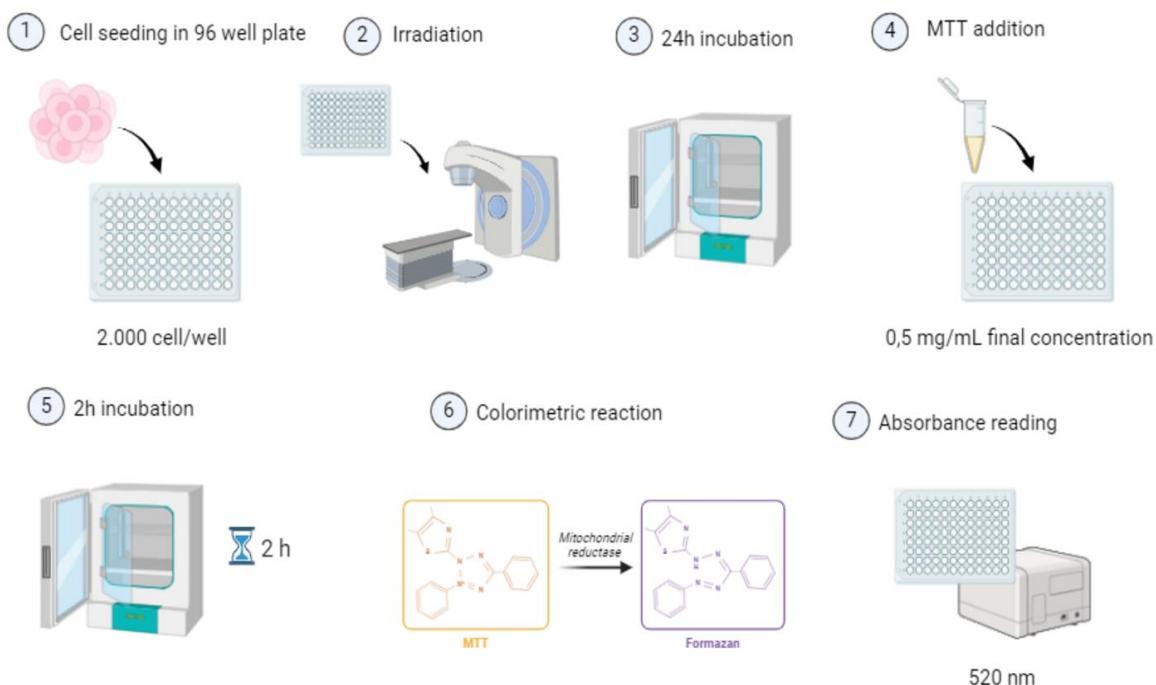
Source : Own authorship.

2.3. MTT

To quantify changes in cell metabolism and cell viability, we performed an MTT assay. The MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) is a yellow-colored salt that pass through cellular membranes and when reduced by mitochondrial hydrolases, it is converted into an insoluble violet-blue molecule called formazan, which is then dissolved in an organic solvent, such as DMSO (Dimethyl sulfoxide), for quantification by absorbance in a spectrophotometer to indirectly measure the metabolic activity of the cell [6].

To establish an acceptable dose rate to observe cell viability, cells were plated into 96-well plates at the concentration of 2.000 cells/well. The following conditions were designed and executed in triplicate: control, 400 cGy/min, 1.400 cGy/min and 3.300 cGy/min. Cells were plated into 16 center wells of a sterile 96-well plate to ensure that all cells would receive a homogeneous dose from the beam. The protocol is summarized in Fig. 3.

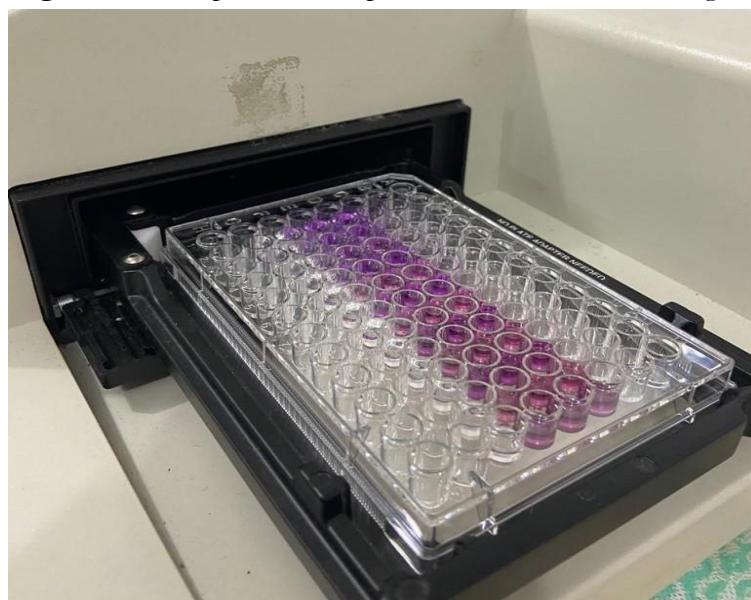
Figure 3: Schematic protocol to perform MTT assay for cell viability.



Source: Reprinted from “MTT Assay Citotoxicity evaluation”, by BioRender.com (2020). Retrieved from <https://app.biorender.com/biorender-templates>.

After irradiation, in which each plate received the correspondent dose rate reaching 20 Gy of total dose, all plates were incubated in a CO₂ incubator for 48 hours. Next, we added the MIT reagent at a final concentration of 0,5 mg/mL, followed by incubation for 2 hours at 37°C, where the colorimetric reaction occurs through mitochondrial reduction converting MTT salt into Formazan, and finally, the medium was discarded. To conclude, we added DMSO to dissolve the formazan, as seen in Fig. 4. Absorbance at 520 nm was acquired using SpectraMax Plus microplate reader (Molecular Devices).

Figure 4: Example of MTT plate with DMSO dissolving formazan.



Source : Own authorship.

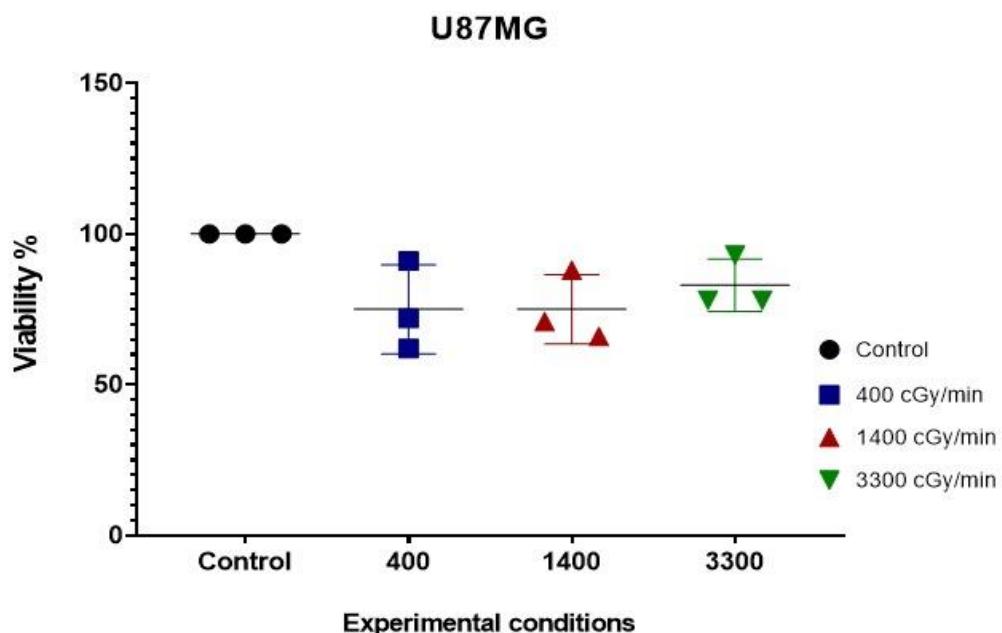
2.4. Statistical analysis

For the data analysis, the Shapiro-Wilk test ($p < 0,05$) was used to evaluate normality and Bartlett's test was used to confirm equal variances. Both tests assured, respectively, normality and homogeneity to run the One-Way ANOVA (Analysis of variance) ($p < 0,05$) with the software called Prism (Graphpad Prism version 8.0.1) by Dotmatics. Following that, a Tukey test was held to evaluate mean values.

3. RESULTS AND DISCUSSIONS

The protocol was followed and tested experimentally. Using MTT assay, we analyzed a group of triplicates with a spectrophotometer, and it was possible to observe that the irradiated cells presented a discrete viability decrease when compared to the control group, but there was no significant variation among them, as seen in Fig. 5.

Figure 5: MTT done to 400 cGy/min, 1.400 cGy/min and 3.300 cGy/min in comparison with control group. Graph created with the software Prism by Dotmatics.



Source : Own authorship.

Table 1 shows the comparison between the results of cell viability by MTT. The first column shows which samples are being compared. Second column points the value to the difference of samples to the mean. Third column is the 95.00% confidence interval of difference to the conditions and fourth column is the Adjusted P value, that is most important result of this table, because it gives the statistical significance. Although control vs. 400 and control vs. 1.400 P value are above of 0,05, is nearly. Therefore, the P value should be less than 0,05 with more samples analyzed.

Table 1: Analysis of cell viability. Tukey's post-test ($p < 0,05$).

Tukey's multiple comparison test	Mean diff	95.00% CI of diff.	Adjusted P Value
Control vs. 400	25,00	-19,522 to 51,95	0,0694
Control vs. 1.400	25,00	-19,522 to 51,95	0,0694
Control vs. 3.300	17,00	-9,952 to 43,95	0,2573
400 vs. 1.400	0,00	-26,95 to 26,95	>0,9999
400 vs. 3.300	-8,00	-34,95 to 18,96	0,7798
1400 vs. 3.300	-8,00	-34,95 to 18,96	0,7799

Additionally, we could observe that, in the tested conditions, U87MG is radioresistant, not showing significant changes in viability when compared with the control group and that is in accordance with literature [5].

4. CONCLUSIONS

The results obtained by the MTT assay indicate that the viability of irradiated cell cultures was reduced compared to the control group, although the difference was not statistically significant among them. This is in accordance with literature, which suggests that the U87MG cell culture exhibits radioresistance. Further studies are necessary to characterize the effects of different dose rates to U87MG survival, specially, the application of additional methods to quantify cell death. As a next step, we plan to investigate cell survival/death at 400 cGy/min while testing different total doses (between 2 to 20 Gy) in order to evaluate if different total dose may be more effective to reach significant tumor cell death.

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CONFLICT OF INTEREST

All authors declare that they have no conflicts of interest.

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