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Establishment and validation of a method for cell irradiation in 96-well and 6-well plates using a linear accelerator

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Abstract To establish and validate a method for cell irradiation in 96-well and 6-well plates using a linear accelerator, three irradiation methods (*G0B0F40, G0B1.5F40,* and *G180B1.5F40*) were designed to irradiate cell culture plasticware simulated with RW3 slab phantom and polystyrene. The difference between the actual physical measured dose and the preset dose was compared among the three methods under the preparatory conditions of 2, 4, 6, 8, and 10 Gy. MDA-MB-231 cells were analyzed by using a cell proliferation assay and a clonogenic assay to verify the difference between the three cell irradiation methods on cell radiosensitivity. For each preset dose, the difference between the actual measured dose and the preset dose was the lowest for *Method G0B1.5F40*, the second lowest for *Method G180B1.5F40*, and the maximum for *Method G0B0F40*. The ranges of the differences were − 0.28 to 0.02%, − 2.17 to − 1.80%, and − 4.92 to − 4.55%, and 0.31 to − 0.12%, − 3.42 to − 2.86%, and − 7.31 to − 6.92%, respectively, for 96-well and 6-well plates. The cell culture experiments proved that *Method G0B1.5F40* was an accurate, effective, simple, and practical irradiation method. The most accurate and effective cell irradiation method should always be used, as it will reduce dose differences and instability factors and provide improved accuracy and comparability for laboratories researching cellular radiosensitivity.

Keywords Linear accelerator · Radiotherapy · Cell irradiation methods · Cell culture plate · Dose

1 Introduction

Over the last 20 years, the global incidence of malignant tumors has increased steadily [1]. The current clinical experience has revealed significant differences in radiosensitivity among different types of tumors [2, 3], but also differences in radiosensitivity among different individuals with the same tumor type [4–7]. This means that basic research on tumor radiotherapy is very important, as it provides an experimental basis and theoretical support for the personalized treatment of patients to increase the precision of specific radiotherapy treatments. For the radiosensitivity of tumor cells, the radiation source is used to irradiate cells, so that the cells receive a certain dose, so as to observe its radiosensitivity. Irradiation conditions are different for each research unit. In recent years, irradiation with a 6 MV X-ray linear accelerator has been conducted in most hospitals by using different irradiation methods. Yang et al. [8]. used 6 MV

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X-ray irradiation treatment on MDA-MB-231 cells during the logarithmic growth phase; the source-to-skin distance (SSD) was noted to be 100 cm, but the build-up and back-scattering materials were not stated.

Some studies have described the irradiation conditions and methods; however, the description was not sufficiently detailed. A study by Jin et al. [9]. described cells that were irradiated with 6 MV X-ray radiation with an SSD of 100 cm and an irradiation field of 25 × 25 cm^2. The build-up and back-scattering materials were not explained. In a study by Chen et al. [10], a vertical beam irradiation method with 6 MV X-ray radiation, gantry angle of 180°, and 1.5 cm of build-up materials were adopted whereas Zachari et al. [11]. irradiated cells with 6 MV X-ray radiation and a 10 × 10 cm^2 irradiation field. In summary, many studies on the radiosensitivity of various tumors have been reported; however, the specific irradiation conditions and methods differ between studies, which mean that many irradiation experiments cannot be reproduced between different research centers. Meanwhile, it has become ever more important to establish a convenient and feasible cell irradiation method able to deliver an accurate dose.

The question of whether the cell receives the preset dose is of importance. Without other factors, and only considering the source-cell distance (SCD), build-up and back-scattering effect, cavity effect, and the convenience and feasibility of cell culture, three cell irradiation methods were designed for the most frequently used 96-well and 6-well plates by physical measurement. The human breast cancer cell line, MDA-MB-231, was used to verify the differences in the three cell irradiation methods on cell radiosensitivity by using cell proliferation and clonogenic assays. The aim of the present study was to establish and validate an accurate and convenient cell irradiation method, thereby providing an experimental basis and theoretical support for the study of radiosensitivity of tumor cells.

2 Materials and methods

2.1 Radiotherapy equipment

The radiotherapy equipment included a linear accelerator (Synergy VMAT, Elekta), a dosimeter (UNIDOS E), a plane-parallel ionization chamber (34001, PTW, Germany), and a RW3 slab phantom (T29672, PTW, Germany, that is solid water) with thicknesses of 0.1, 0.2, 0.5, and 1 cm and dimensions of 30 × 30 cm^2.

2.2 Simulated “cell culture plate” system

The cell culture plate system was made up of ware cover, cavity, culture medium, and ware bottom. As the density of the solid water, which is often adopted as the clinical homogeneous phantom that is close to that of the cell culture plate, the system was built up and simulated by the solid water. The cavity was built of four pieces of polystyrene with a density merely equal to that of air that was placed on the solid water. The well wall was ignored, as shown in Fig. 1. The specific parameters of the cell culture plate simulated by solid water are shown in Table 1.

2.3 Irradiation methods

The 6 MV X-ray was used, the depth of the maximum dose is 1.5 cm below the water, and $D_{20/10}$ = 0.585. The thickness of the irradiated material required is 1.5 cm for the 6 MV X-ray beam. The simulated “cell culture plate” system was irradiated at five different preset doses: 2, 4, 6, 8, and 10 Gy, by the three methods. Each measurement was repeated five times.

(i) *Method G0B0F40* The 6 cm solid water (including the thickness of ware bottom and the gap between the bed and the bottom of the cell culture plate) was placed below the plane-parallel ionization chamber. This solid water is used as a back-scattering material. There were no additional build-up materials, as shown in Fig. 2a.

(ii) *Method G0B1.5F40* The 6 cm solid water (including the thickness of ware bottom and the gap between the bed and the bottom of the cell culture plate) was placed below the plane-parallel ionization chamber. This solid water is used as a back-scattering material. Solid water of 1.5 cm thickness was then placed over the plane-parallel ionization chamber (including the thickness of ware cover and the height of culture medium). A certain thickness of solid water was placed on the above simulated “cell culture plate” system, in order to construct a 1.5 cm thickness of build-up materials, as shown in Fig. 2b.

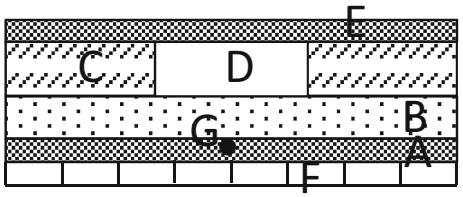

Fig. 1 A cross section of the simulated “cell culture plate” system. *A* Ware bottom; *B* culture medium; *C* the phantom of building cavity; *D* cavity; *E* ware cover; *F* the gap between the bed and the bottom of the cell culture plate. (*A*, *B*, and *E* are all built of solid water). In the real cell irradiation experiment, *F* is escapable and filled by using solid water. Thus, the gap is filled by using solid water in the simulation. *C* is built of polystyrene, in which the density is nearly equal to that of air, in order to build the cavity. *G* is used to put the plane-parallel ionization chamber

Table 1 Specific parameters of cell culture plastic ware

	Ware height (cm)	Culture medium height (cm)	Cavity height (cm)
96-well plate	1.1	0.6	0.5
6-well plate	1.7	0.3	1.4

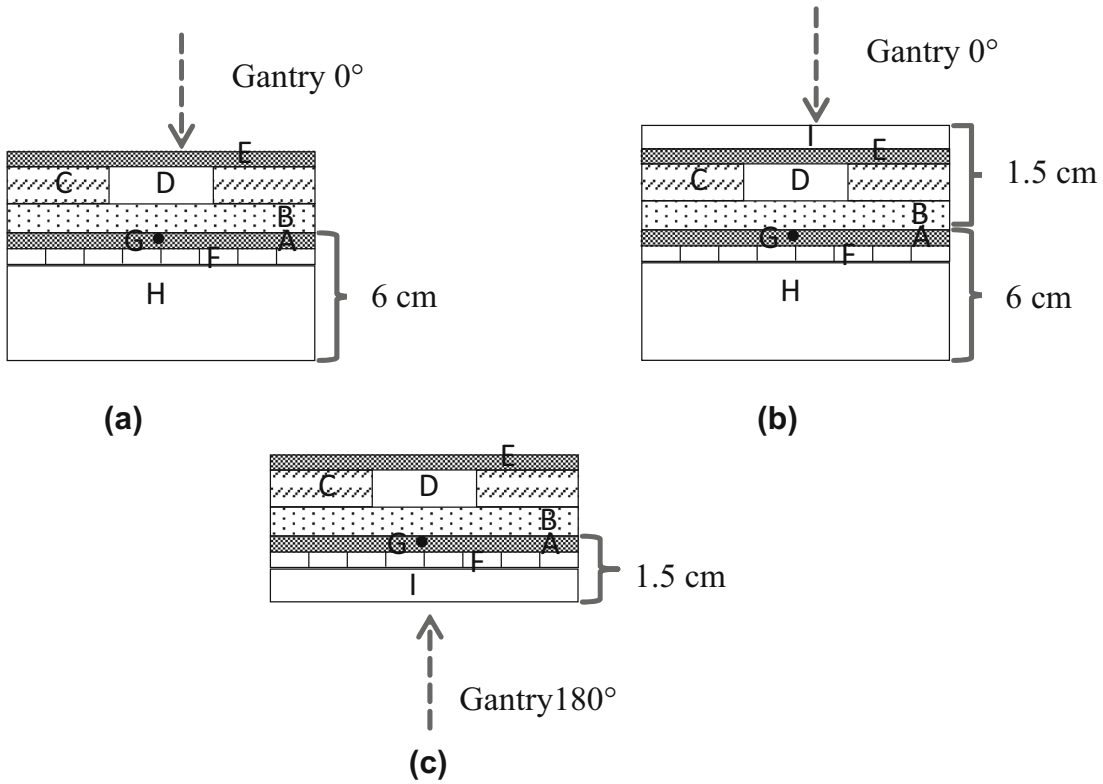

Fig. 2 Three irradiation methods for the simulated "cell culture plate" system. **a** *G0B0F40* (a certain thickness of solid water (*H*) is placed on the below simulated "cell culture plate" system, to construct a 6 cm thickness of the back-scattering materials). **b** *G0B1.5F40* (a certain thickness of solid water (*I*) is placed on the above simulated "cell culture plate" system, to construct a 1.5 cm thickness of the back-scattering materials; a certain thickness of solid water (*H*) is placed on the below simulated "cell culture plate" system, to construct a 6 cm thickness of back-scattering materials). **c** *G180B1.5F40* (a certain thickness of solid water (*I*) is placed on the below simulated "cell culture plate" system, to construct a 1.5 cm thickness of the back-scattering materials). *Note*: *H* stands for the additional back-scattering materials; I stands for the additional adding build-up materials; For *G*, *B*, *F* with the name of Method *G0B0F40*, *G0B1.5F40*, *G180B1.5F40*, *G* stands for gantry degree; *B* stands for the thickness of build-up materials; and *F* stands for field size

(iii) *Method G180B1.5F40* Solid water of 1.5 cm thickness (including the thickness of ware bottom and the gap between the bed and the bottom of the cell culture plate) was placed below the plane-parallel ionization chamber. A certain thickness of solid water was placed below the simulated "cell culture plate" system, in order to construct a 1.5 cm thickness of the build-up materials, as shown in Fig. 2c.

2.4 Cell culture

The breast cancer cell line, MDA-MB-231, was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and stored in the Central Laboratory of Shanghai Tenth People's Hospital. The MDA-MB-231 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C in an incubator with 5% CO_2 and 95% humidity.

2.5 Cell verification test

2.5.1 Cell proliferation assay

Cell proliferation was assessed by using the Cell Counting Kit (CCK)-8 assay (Shanghai Yisheng Biotechnology Co., Ltd, Qingdao, China) and a microplate reader (BioTek Company, VT, USA). Briefly, the hybrids and

Table 2 Actual measured doses of two different cell culture plastic wares for *Method G0B0F40, G0B1.5F40* and *G180B1.5F40*

Method	Preset dose (Gy)				
	2	4	6	8	10
(a) 96-well plate					
G0B0F40					
Measure dose (Gy)	1.9090 ± 0.0010	3.8092 ± 0.0008	5.7188 ± 0.0008	7.6274 ± 0.0023	9.5078 ± 0.0114
Difference (%)	− 4.55	− 4.77	− 4.69	− 4.66	− 4.92
G0B1.5F40					
Measure dose (Gy)	1.9990 ± 0.0007	3.9890 ± 0.0007	5.9940 ± 0.0016	8.0012 ± 0.0013	9.9960 ± 0.0019
Difference (%)	− 0.05	− 0.28	− 0.10	0.02	− 0.04
G180B1.5F40					
Measure dose (Gy)	1.9628 ± 0.0004	3.9132 ± 0.0024	5.8848 ± 0.0023	7.8556 ± 0.0047	9.8202 ± 0.0034
Difference (%)	− 1.86	− 2.17	− 1.92	− 1.81	− 1.80
(b) 6-well plate					
G0B0F40					
Measure dose (Gy)	1.8572 ± 0.0011	3.7076 ± 0.0015	5.5754 ± 0.0029	7.4468 ± 0.0018	9.3062 ± 0.0036
Difference (%)	− 7.14	− 7.31	− 7.08	− 6.92	− 6.94
G0B1.5F40					
Measure dose (Gy)	2.0062 ± 0.0004	4.0000 ± 0.0016	6.0020 ± 0.0012	8.0058 ± 0.0033	9.9876 ± 0.0086
Difference (%)	0.31	0.00	0.03	0.07	− 0.12
G180B1.5F40					
Measure dose (Gy)	1.9316 ± 0.0021	3.8694 ± 0.0024	5.8184 ± 0.0039	7.7708 ± 0.0047	9.7138 ± 0.0052
Difference (%)	− 3.42	− 3.27	− 3.03	− 2.87	− 2.86

All the measure dose are presented as mean ± SD. Deviation is presented as $\frac{\text{measure mean dose} - \text{preset dose}}{\text{preset dose}} \times 100\%$

their parental counterparts were plated at 1200 cells/well in 96-well plates. After incubation for the indicated time, CCK-8 was added at 200 µl/well, and irradiation with 6 MV X-ray of 4 Gy was performed, with a source-axis distance (SAD) of 100 cm by using three different physical methods. Given the length of the paper, only the data for 4 Gy were provided and discussed; the other data will be discussed more extensively in another paper. The optical density was measured at 450 nm by using a microplate reader (BioTek Company, VT, USA).

2.5.2 Clonogenic assay

MDA-MB-231 cells in the logarithmic growth phase were harvested and 500 cells/well were reseeded in 6-well cell culture plates. After incubation for 24 h, the cells were irradiated with 6 MV X-ray of 4 Gy, a source-axis distance (SAD) of 100 cm, by using the three different physical methods. Similarly, only the data for 4 Gy were provided and discussed in this experiment. After 9 days, medium-free cells were fixed with absolute ethyl alcohol for 10 min, stained with 0.5% crystal violet for 15 min, and then washed three times with PBS. The colonies containing > 50 cells were counted. The plating efficiency (PE) and surviving fraction (SF) were calculated from the following formulae:

$$\text{Plating efficiency (PE)}\,(\%) = (\text{Numbers of colonies formed (non-irradiated)}/\text{Numbers of cells plated}) \times 100$$

$$\text{Surviving fraction (SF)}\,(\%) = \text{Numbers of colonies formed}/(\text{Numbers of cells plated (irradiated)} \times \text{Plating efficiency (non-irradiated)}) \times 100.$$

3 Results

3.1 Simulated "cell culture plate" system irradiation

The actual measured doses in the "cell culture plate" system (96-well and 6-well plates) using the methods *G0B0F40*, *G0B1.5F40*, and *G180B1.5F40* are shown in Table 2. For the 96-well plate, at each preset dose the range of the differences between the measured dose and preset dose was between − 0.28 and 0.02% with *Method G0B1.5F40*, between − 2.17 and − 1.80% with *Method G180B1.5F40*, and the maximum difference was between − 4.92 and − 4.55% with *Method G0B0F40.* For the 6-well plates, at each preset dose, the range of the

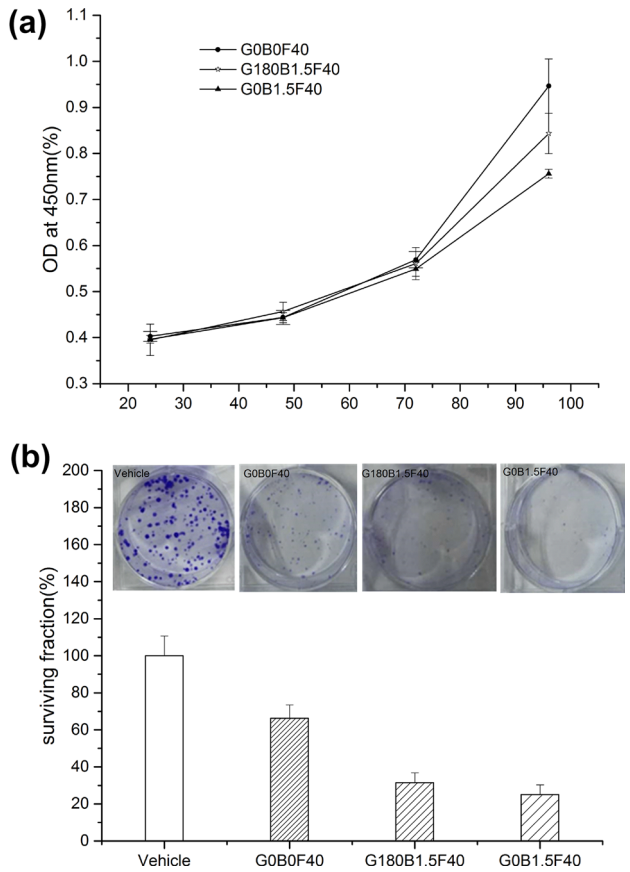

Fig. 3 (Color online) **a** the cell growth curves for the irradiated MDA-MB-231 cells with *Method G0B0F40*, *Method G0B1.5F40*, and *Method G180B1.5F40* ($n = 6$ per group, values represent the mean ± SD); **b** the comparison of the surviving fraction of the MDA-MB-231 cells after the three methods. All data are presented as the mean ± SD from four replicate wells

differences between the measured dose and the preset dose was between 0.31 and − 0.12% with *Method G0B1.5F40*, between − 3.42 and − 2.86% with *Method G180B1.5F40*, and the maximum difference was between − 7.31 and − 6.92% with *Method G0B0F40*. In addition, the dose differences in the 96-well plates were generally lower than that of the 6-well plate for *Method G0B0F40* and *Method G180B1.5F40*. Overall, for each preset dose, the actual measured dose was closest to the preset dose with *Method G0B1.5F40* and lower than the preset dose with *Method G180B1.5F40*. Under the conditions of *Method G0B0F40*, the measured actual dose was lowest, with the maximum deviation from the preset dose. Therefore, the above results show that *Method G0B1.5F40* was chosen as the optimal method for cell irradiation.

3.2 Comparison of cell verification test

3.2.1 Comparison of proliferation of cells

MDA-MB-231 cells were irradiated with 4 Gy, and the cell growth curves were measured by CCK-8 assay after irradiation using *Method G0B0F40*, *G0B1.5F40*, and *G180B1.5F40*, as shown in Fig. 3a. There was no significant difference in the survival rate of MDA-MB-231 cells at 24 h after attachment between the three methods. The cells entered the logarithmic growth phase at 48 h after attachment. The difference appeared at 72 h after attachment, and it became larger as time progressed. The OD value with *Method G0B0F40* was highest, followed by *Method G180B1.5F40,* and the lowest for *Method G0B1.5F40*, which indicated that the actual irradiated dose of cells was lower than that with the other two methods. *Method G0B1.5F40* had the lowest OD value and the lowest cell survival rate; the actual irradiated dose of cells in this method was higher than that of *Method G0B0F40* and *Method G180B1.5F40*. It was also confirmed that the actual irradiated dose of cells for *Method G0B1.5F40* was the highest overall, followed by *Method G180B1.5F40* and then *Method G0B0F40*, in which the actual irradiated dose of cells was the lowest.

3.2.2 Comparison of the clonogenic capacities of cells

As can be seen from Fig. 3b, obvious differences in the surviving fraction were found for cells after the three different irradiation methods. For methods *G0B0F40, G180B1.5F40*, and *G0B1.5F40*, SF(%) was 66.31 ± 7.10, 31.52 ± 5.27, and 25.12 ± 5.21, respectively. In addition, the surviving fraction of the cells for *Method G0B1.5F40* was significantly lower than that of *Method G0B0F40* and slightly lower than that of *Method G180B1.5F40.* This showed that the actual irradiated dose of cells was highest with *Method G0B1.5F40* and closest to the preset dose. When using this method, the radiosensitivity applied to the cell was accurate.

4 Discussion

It is not clear if the actual irradiated dose for the cells reaches the preset dose, as the results from different irradiation methods are contradictory. Although many research articles have reported data on cellular radiosensitivity, the published descriptions of the irradiation methods tend to be imprecise [12, 13]. In this study, the results showed that no matter what type of cell culture plate was used, the best irradiation method, in which the irradiated dose was closest to the preset dose, occurred after our *Method G0B1.5F40*

above. For method *G0B1.5F40*, there was a 1.5 cm thickness of build-up materials (including the thickness of the ware cover and the height of culture medium) on the upside of the cell, the irradiated dose of cells is at the point of maximum dose, and the 6 cm thickness of the back-scattering materials (including the thickness of ware bottom and the gap between the bed and the bottom of the cell culture plate) at the back of the cell increases back-scattering. In this case, a millimeter of difference in the height of the culture medium has little impact on the experiment. For *Method G0B0F40*, although the 6 cm thickness of solid water (including the thickness of the ware bottom and the gap between the bed and the bottom of the cell culture plate) can increase back-scattering, there is insufficient build-up of materials (for 6 MV, the thickness of the build-up materials is 1.5 cm) on the upside. In this case, the actual irradiated dose of cells falls within the range of the high energy X-ray dose build-up region, and the actual irradiated dose is low. The millimeter of difference in the height of the culture medium in the actual experiment leads to a larger difference in the actual irradiated dose of cells. In *Method G180B1.5F40*, although there is a 1.5 cm thickness of build-up materials (including the thickness of ware bottom and the gap between the bed and the bottom of the cell culture plate), there was no material in the emission direction of the X-ray to increasing back-scattering, which results in a lower actual irradiated dose of cells than the preset dose. This occurs because the actual cell irradiation is not feasible. Therefore, it is important to add a 1.5 cm thickness of build-up materials (including the thickness of ware cover and the height of the culture medium) into the area above the cells after irradiation of the cells with 6 MV X-ray. In addition, a certain thickness of back-scattering material should be added to the area under the tissue culture plastic ware. In a study by Zheng et al. [14], who used a 6 MV X-ray linear accelerator, it was suggested that at least 5 cm thickness of solid water should be added as the back-scattering material. Therefore, in this paper, a 6 cm thickness of solid water was used as the back-scattering material. The thickness of back-scattering material influences the cell dose, but this is not discussed in this paper.

The dose differences of the 96-well plates were generally less than that of the 6-well plate for methods *G0B0F40* and *G180B1.5F40*. For *Method G0B0F40,* the thickness of the build-up materials was 0.6 cm in the 96-well plate and 0.3 cm in the 6-well plate. Thus, the measured dose was closer to the preset dose: The dose difference was smaller for the 96-well plate, but not for the 6-well plate. For *Method G180B1.5F40,* the thickness of back-scattering material was 0.6 cm in the 96-well plate and 0.3 cm in the 6-well plate. In a 5 cm thickness of back-scattering material range [13], the thickness of the back-scattering material is thicker, there are more back-scattering photons, and the back-scattering effect of the dose was larger. Thus, the measured dose was closer to the preset dose, and the dose difference was smaller for the 96-well plate, but not for the 6-well plate. This also demonstrated that it was important to add the appropriate thickness of the build-up and back-scattering materials for cell irradiation according to the energy of the linear accelerator.

As the cells are protected from contamination, the culture medium cannot be filled generally; a certain gap should be left between the upper surface of the culture medium and the culture plastic ware cover, and it is inescapable in the process of cell culture. This gap will cause the cavity effect of X-ray irradiation, which has been reported in several studies [15, 16]. A study by Xiao et al. [17]. has suggested that the cavity effect was lower when the air cavity was located in the deeper region within the tissues than when it is located in the shallower region within tissues. For the methods *G0B1.5F40* and *G180B1.5F40*, there is a 1.5 cm thickness of build-up materials, so the cavity effect was lower than that of *G0B0F40*. A study by Wang et al. [18]. suggested that when the area of the radiation field was greater than that of sagittal interface of the nasopharynx cavity, the nasopharyngeal cavity has very little influence on the dose distribution of the coaxial penetrating field, and it can therefore be neglected. In this study, a 40 × 40 cm^2 radiation field was adopted for the three methods [19] and the area of the radiation field was greater than that of cavity, so the cavity effect can be ignored and the actual measured dose would be closer to the preset dose; that is, the cavity effect can be ignored for method *G0B1.5F40*. Thus, *Method G0B1.5F40* represents the optimal method for cell irradiation.

In summary, when the cell will be irradiated, we must pay attention to the influence of the build-up effect and cavity effect in the future.

According to the experience of proliferation and the colony formation assays for MDA-MB-231 cells from literature reports [20–22], the cell proliferation assay and clonogenic assay of MDA-MB-231 cells were performed to verify the difference of the three methods in this paper. For the cell proliferation assay, we found no significant difference in the survival rate of cells at 24 h after cell attachment for the three irradiation methods. This mainly resulted from the fact that cell apoptosis was not clear at this point and the obvious effect of radiotherapy on cell viability was small. After 48 h, the radiotherapy started to exert an effect on the cells. The dose was higher, and the cell survival rate was lower at 72 h after cell attachment. The cell survival rate was highest in *Method G0B0F40*, because the actual irradiated dose of cells was lowest, followed by *Method* G*180B1.5F40*, and then *Method G0B1.5F40*. Compared with the other two methods, the actual measured dose in *Method G0B1.5F40* was closest to

the preset dose and the dose was highest, which resulted in the lowest cell survival rate. The cell growth curves demonstrated that there were significant differences for the three cell irradiation methods after 72 h. For the cell clonogenic assay, we can see that SF was highest for *Method G0B0F40*, second highest for *Method G180B1.5F40*, and lowest for *Method G0B1.5F40* after 9 days. This is the dose of *Method G0B1.5F40* that was higher than that of other methods, and its dose was closest to the preset dose. This confirmed that method *G0B1.5F40* was the optimal method for cell irradiation. From the above two experiments, which showed no significant dose difference in the cell experiments after 48 h for the three methods, but the dose difference was obvious in cell experiments (e.g., the clonogenic assay) after 48 h for the three methods. Each cell experiment lasts for a different time; for example, the cell clonogenic assay usually takes longer than 9 days. Thus, method *G0B1.5F40* should be chosen at the time of cell experiment. Therefore, an accurate cell irradiation method must be established before conducting a cell radiosensitivity test. An irradiation dose that is too high or too low will affect the experimental results, which will be detrimental to the comparison of the relevant experimental results of authors from different research laboratories.

In this study, three different irradiation methods were used to measure the physical doses of the cell for the simulated "cell culture plate" system. It was demonstrated through the growth curve and colony-forming efficiency of MDA-MB-231 cells that *Method G0B1.5F40* was the best cell irradiation method. In the cell culture process, the SCD, build-up and back-scattering effect, cavity effect, and the feasibility and convenience of the operation should be fully considered with the linear accelerator. The most accurate and effective cell irradiation method should always be used, which will reduce the dose differences and instability factors and provide a greater accuracy and comparability for laboratories conducting research into cellular radiosensitivity.

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