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The Dose-, Energy- and Time Post-Irradiation-Dependent Radiobiological Response of MCF-7 Breast Cancer Cells to X-Rays: An *In Vitro* Study

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Abstract The successful outcome of radiotherapy in cancer patients is largely dictated by a number of physical and biological parameters, such as radiation dose, X-ray photon beam energy and the temporal evolution of cellular damage. In three separate studies (n = 3), these variables were thoroughly assessed in cancer cells and analysed using one-way ANOVA and Tukey's post hoc test; findings were deemed statistically significant at $p<0.05$. The effects of therapeutically administered photon irradiation produced by a medical linear accelerator are examined in this work, with a primary focus on oestrogen receptor-positive breast cancer cells. Photon doses of 4 and 6 (Gy) at energies of 4 and 6 (MeV) were applied to the cells. In several research, the MTT test was employed to measure cell viability at 1, 24 and 48 hours after radiation. 6 MeV photons were more effective against cancer cells than 4 MeV photons, substantial cell death according to both dosage and photon energy. Also, a time-dependent decline in cell viability was noted; 48 hours following irradiation, the lowest survival rates were noted. These results highlight the need for radiobiology research to take the temporal dimension into account; this factor may be crucial not only for understanding fundamental biological aspects but also for refining patient selection criteria and developing treatment protocols through dose fractionation schemes. The observed survival rates reflect biological effects consistent with ionizing radiation-induced cell death, potentially opening new and effective avenues for understanding the radiobiology of breast cancer.

Key Words Breast Cancer Cells, MCF-7, X-Ray Irradiation, Radiotherapy (RT), MTT Assay, Medical Linear Accelerator (LINAC)

INTRODUCTION

Breast cancer is one of the most common cancers in women worldwide and irradiation is central to breast cancer management, playing a significant role in both local control and survival [1]. The biological efficiency of radiation is high for ionizing radiations, although these efficiencies are based on complex interplay of physical (dose and intensity) [2,3] and biological factors (the capacity to undergo cellular repair and the time frame in which a cell can respond) [4]. The harmful effects of ionizing radiation at the cellular level are mediated by both (1) direct ionization of DNA and (2) indirect pathways via reactive oxygen species (ROS) that lead to single- and double-strand breaks of DNA [2]. Throughout decades of radiobiological research, it has been consistently shown that as dose increases cell survival probabilities decrease represented by accompanying dose-response or survival curves that depict the balance between lethal and sub-lethal damage repair [3]. While dose is one variable, the energy of a photon beam has long been recognized as a significant contributing factor that governs radiation - matter interactions. Megavoltage photon beams (e.g. 4-6 MeV) deliver low energy dose deposition patterns as expressed by differences in penetration depth and secondary electron spectra that influence biological response even *in vitro*, Therefore, the selection of 4 MeV and 6 MeV energy values for this experiment was done to ensure that clinically relevant photon energies of medical linear accelerator (LINAC) used to treat breast cancer are studied. It allows for the evaluation of how the radiobiology of MCF-7 cells will be affected by changes in the photon beam energy level. In addition, the two energy values fall in the clinically relevant range used to treat surface and semi-deep tumours [5-7]. For example, cancer cell lines treated with photon energies of 56 kV and 249 kV had differing amounts of DNA damage

and cytotoxicity (dependent on oxidative stress) [8,9]. More literature also focuses on the temporal evolution of damage due to radiation. While there are some effects seen within a few minutes of irradiation, such as damage to cellular membranes or chromatin elements [7]. many important, serially ordered events (e.g. apoptosis, mitotic catastrophe and delayed reproductive death) take hours-days to develop post- irradiation [10]. Indeed, while cytotoxicity assays conducted within hours after irradiation can significantly underestimate the total biological impact, more dramatic reductions in cell viability are observed at longer time windows (24-48h) [11]. While the dose-response relationship has been extensively studied, most studies focus on a single time point or do not determine and compare the effect of combined parameters dose, photon energy and time. This highlights an area of deficiency within otherwise extensively characterized knowledge of such radiobiological reactions, particularly for breast cancer cell lines (i.e. MCF-7). Accordingly, this study systematically investigates the effects of radiation parameters, specifically dose and the time elapsed post-irradiation, on MCF-7 breast cancer cells, no human participants, patient samples or animal subjects were involved in this research, while accounting for beam intensity. Multiple time points (1, 24 and 48 hours post-irradiation) were utilized, enabling a comprehensive characterization of the dynamic biological response to radiation, a detailed analysis of changes associated with clinically relevant irradiation parameters and an evaluation of the effects of 4 and 6 MeV X-ray photon beams generated by a clinical medical accelerator.

METHODS

Cell Culture

The MCF-7 tumour cell lines were prearranged by the National Cell Bank of Iran (Pasteur Institute, Tehran, Iran). The cells were cultured in an incubator with standard envelopes (5% CO_2, 37˚C) in RPMI 1640 medium supplemented with 10% FBS, Gibco and antibiotics (Gibco). Before being exposed to radiation, MCF-7 cells were established in 96-well plates at a density of 1×10^4 cells per well and allowed to adhere overnight.

Radiation

X-ray photon beams with energies of 4 or 6 MeV, generated by a medical accelerator, were used to irradiate a breast cancer cell line (MCF-7). For equal irradiation of all cells and uniformity of radiation dose distribution, the culture plate with irradiated cells was positioned centrally and surrounded from all sides by water-containing cell culture plates; this was done to help in ensuring that irradiation conditions were even and the dose variation in the cells was reduced. That part is easy to miss .A full scatter environment was established with 20×20 cm^2 field size .100 cm was the source-to-surface distance .A linear accelerator type from Elekta Solutions AB in Sweden was used to irradiate cell culture plates .Total doses were 4 and 6 Gy and that actually matters .Resulting from irradiation with these photon beams. Every irradiation was done in accordance with the radiotherapy department's standard clinical operational and quality assurance protocols.

Evaluate Cell Viability

The curious thing is that cell viability is often used as an indicator of cytotoxicity and treatment efficacy in cancer research. This is because cell viability is frequently used as an endpoint in radiobiology assays. Cell viability, a point often overlooked, is defined as a cell's ability to survive, maintain its metabolic activity and proliferate after exposure to an external stressor, such as ionizing radiation. Most people ignore this fact, but ionising radiation generally damages cells through oxidative stress and DNA damage. This is the crux of the matter: it can affect mitochondrial function, thus reducing cell viability [12]. The variables that determine the extent of this reduction [13], include radiation dose, energy and cell lineage sensitivity. But why does this happen in the first place? The MTT assay is one of the most common methods for determining cell viability. In this study, an MTT solution (at a concentration of 5 mg/ml) was added to each well and the samples were incubated for 4 hours. The resulting formazan crystals were dissolved in 200 µL of DMSO. Studies [12,14] are considered good examples of this approach, as they involve the reduction of yellow tetrazolium salt to purple formazan crystals by mitochondrial dehydrogenases found in metabolically active cells. The amount of formazan produced, which is crucial, is directly proportional to the number of viable cells. Study [12] is particularly good in this respect, as the results are expressed as a percentage compared to untreated, 100% viability control cells. In this study, cell viability, the key factor, was measured by determining the optical density of the dissolved formazan product using a microplate reader at 570 nm, following a method similar to that described in Studies [15,16].

$$\text{Cell Viability (\%)} = \frac{\text{OD sample}}{\text{OD control}} \times 100 \quad (1)$$

Since radiation-induced cell death is not necessarily instantaneous, it is crucial to assess cell viability at various intervals following irradiation. Hours to days subsequent to exposure, certain cells experience delayed death as a result of processes such apoptosis or mitotic catastrophe. Consequently, a more realistic depiction of the biological consequences of radiation will be obtained by accessing viability at various time intervals [17].

Survival Curves

The survival fraction (SF) curves of MCF-7 breast cancer cells have been investigated to determine the impact of radiation dose, photon energy and post-irradiation time on cellular radiosensitivity. The survival fraction was determined by dividing the viability of irradiated cells by that of the control group. The survival fraction (SF) was calculated using experimentally determined MTT cell

viability data by normalising the viability percentage to the untreated control group. This technique gives a relative measure of post-irradiation cellular survival and is frequently employed in radiobiological investigations examining radiation-induced cell death [18]. Linear-Quadratic (LQ) radiobiological analysis was performed using the natural logarithm of survival fraction data, where ln (SF) was plotted versus radiation dosage (Gy) for different post-irradiation durations (1 h, 24 h and 48 h) at photon energies of 4 and 6 MeV .The analysis was based on the Linear-Quadratic equation [19, 20]:

$$\ln(SF)=-\propto D-\beta D^2 \quad (2)$$

Where:

SF: Survival fraction

D: Radiation dose (Gy)

α and β: Radiobiological parameters representing the linear and quadratic components of radiation-induced cell killing

Experimental Design

In order to study the effects of radiation dose and photon energy on cell viability of MCF-7 cells, the cells were subjected to different doses (4 and 6 Gy) from two different photon energies (4 and 6 MeV) to determine which radiobiological effects occurred in each case, as shown in Figure 1. All experiments were independently performed in triplicate (n = 3) format. The study was intended to be an exploratory *in vitro* radiobiological examination. In line with standard procedure in cell culture and radiobiological research, the quantity of biological replicates was determined. The study was conducted using predefined experimental groups under controlled laboratory conditions. Formal randomization and blinding procedures were not applied; however, all samples were processed, irradiated and analysed using identical experimental protocols to minimize potential sources of bias.

Statistical Analysis

The results are reported in a mean±SD, all experiments were independently performed in triplicate (n = 3) format. OriginPro 2024 (OriginLab Corporation, Northampton, MA, USA) software was used to conduct statistical analysis. The Shapiro-Wilk test was implemented to determine normality and Levene's test was utilised to determine homogeneity of variance. Tukey's HSD post-hoc test was used after one-way ANOVA to examine group differences. Eta squared (η^2) was used to calculate the effect size. Analysis of variance and Tukey's test were used for statistical analysis ($p<0.05$).

RESULTS AND DISCUSSION

The impact of ionizing radiation on cell viability was assessed at various photon energies (4 and 6 MeV), radiation doses (4 and 6 Gy) and post-irradiation durations (1 h, 24 h and 48 h). Figure 1 which shows a multipaneled comparison of such under different experimental conditions.

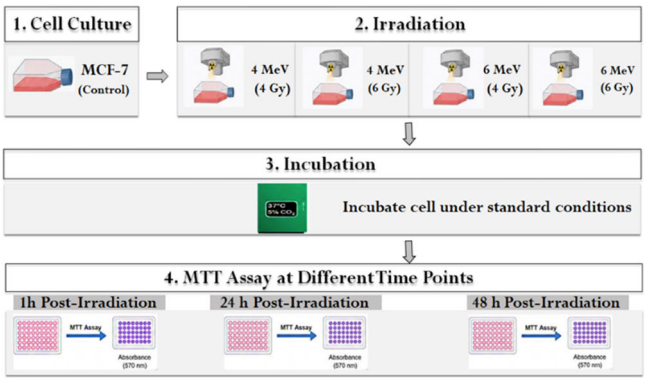

Figure 1: Schematic representation of the experimental design used to assess MCF-7 cell viability.

Results in Figure 2a show a time-dependent decrease of cell viability after 4 Gy and 4 MeV at 24h/48h post-irradiation. One-way ANOVA revealed a very statistically significant difference between groups ($p<0.0001$). Although we observed a decrease in AGEs at 24 and 48 hours, Tukey's test revealed that the control group as well as the one-hour group were similar (Figure 2b). The transmission of ionizing radiation fraction (6 Gy) associated with X-ray fractions (4 MeV): similar results as obtained in presence 6 Gy but stronger reducing of cell viability effect right after irradiation, particularly at 24 and 48 hours post-irradiation. The statistical indicated that there were significant differences among groups ($p<0.0001$). By contrast, irradiation at 4 Gy and 6 MeV resulted in a marked loss of cell viability in the first hour after irradiation (Figure 2c). It was subsequently shown to gradually increase over time with the lowest viability seen 48 hours after the irradiation process. In the same manner, irradiating with 6 Gy and 6 MeV (Figure 2d) treated cells where a time-dependent decrease of cell viability was observed. The cytotoxic effect, however, was much stronger, especially at the 48 h post-irradiation point when cell viability was minimal sequentially over time. The results obtained in the current research are consistent with those obtained by Hargrave *et al.* [21], proving that the effects of radiation on cellular level do not stop at the moment of radiation itself, but continue developing over the subsequent hours and days due to ongoing damage to DNA and other cellular processes induced by radiation. In the current study, a gradual decrease in cell viability for MCF-7 cells was demonstrated at 1, 24 and 48 hours post-irradiation, which means that the effect of radiation becomes more severe over time. It can be explained by the fact that radiation induces direct and indirect DNA damage in the form of reactive oxygen species (ROS) and it accumulates even after the end of exposure. Also, damaged cells need time to enter apoptosis or become non-proliferating due to inability of repairing their DNA. Therefore, the effect of radiation occurs with a delay, leading to decreased cell viability and higher mortality rates over the period of post-irradiation incubation. This time-dependent bio reductive loss of cell viability indicates that radiation

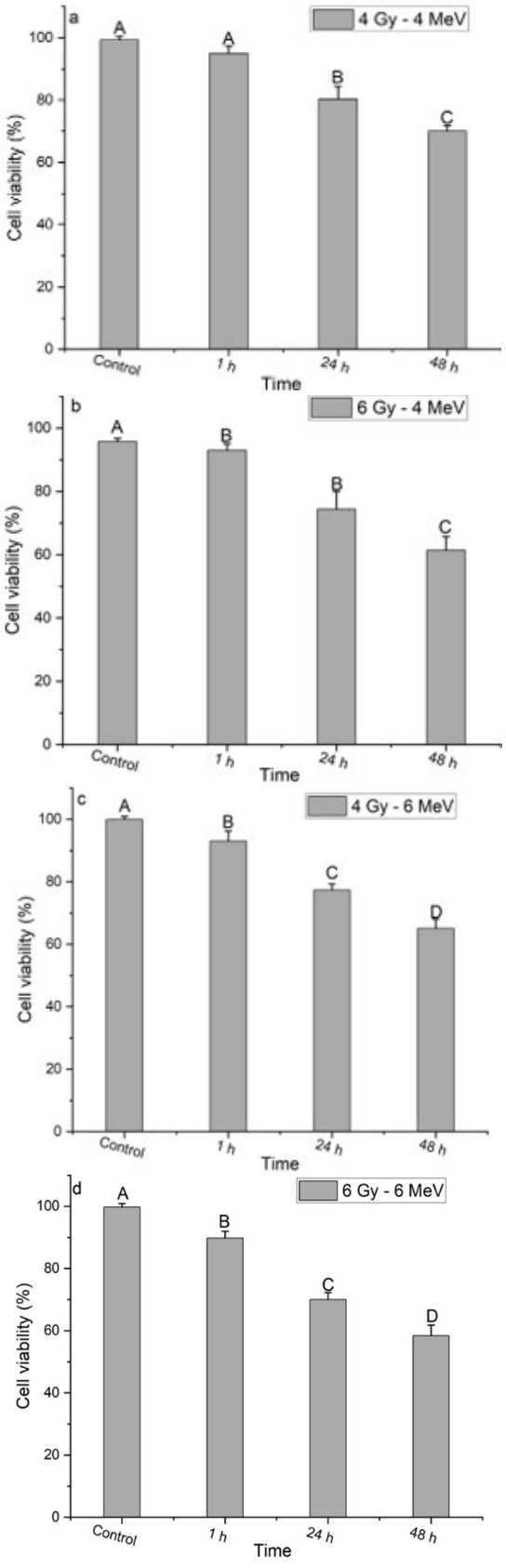

Figure 2 (a,b,c,d): Effects of post-irradiation time on cell viability at different radiation doses (4 and 6 Gy) under photon energies of 4 and 6 MeV. Data are expressed as mean±SD (n = 3). Different letters indicate significant differences ($p < 0.05$).

damage is not solely instantaneous but also cumulative. Although in the first days after irradiation cells are able to be viable and even proliferative despite DNA damage, progressively increased cumulative cellular damage leads to delayed cell death mechanisms like apoptosis and mitotic catastrophe, which compromise proliferation ability in long term assays [7].

Due to the time-dependent effect, however, a clear dose-dependent response was also noted; namely that with an increase from 4 Gy to 6 Gy at all-time points after irradiation the reduction in cell viability was greater (see Figure 3). That is probably due to the fact that at high doses oxidative damage and incidence of DNA double-strand breaks occur more frequently. This is consistent with reference [22] which proves the capability of radiation in killing cells if cellular repair mechanisms are exceeded and irreversible cell death occurs [23]. The findings from the current study corroborate those reported by Alzibdeh *et al.* [20] where an increase in the radiation dose causes more cell damage and decreases cell survival, which is due to the increase in the ionizing effect and interactions induced by the radiation dose in the cell. This is attributed to the fact that when the radiation dose is increased, more energy deposition occurs in the cells, thereby increasing the formation of ROS and causing more DNA damage including double-strand breaks (DSBs). In situations where the damage surpasses the cell's ability to repair, then the processes of cell death get triggered or the cells become unable to divide and proliferate; hence a decrease in cell viability and an increase in cell death rates occur as the radiation dose is increased.

Effect of photon energy, radiation dose and time period after irradiation: a combined analysis (Figure 4). Results indicate an overall decreased number of viable cells at the highest dosage (6 Gy), energy (6 MeV) and longest time point post-irradiation (48 h). This suggests that during the time period, biological response of cells and physical properties of radiation may act synergistically. Statistical analysis found differences between the experimental groups to be highly significant ($p<0.0001$). These findings are consistent with well-established tenets of radiobiology that suggest that high exposure levels will deplete cellular repair mechanisms and that the effects of irradiation is cumulative over time[13]. This might lend credence to the idea of hypofractionation, which holds that because radiation causes continuing DNA damage and delayed biological reactions, a therapeutic impact can be obtained with fewer treatments. This result has been examined, verified, the data in previous theoretical and practical studies also confirm the role of ionizing radiation as a powerful source of progressive cellular damage [24-27].

Tests of Shapiro-Wilk proved that none of the groups deviated from the assumption of normality ($p>0.05$). One-way ANOVA analysis suggested that there were significant differences between the experimental groups ($F(17,36) = 92.83$, $p<0.0001$), as is illustrated in Table 1. From the data obtained, it can be seen that there was a progressive reduction in the viability of MCF-7 cells with increase in radiation doses

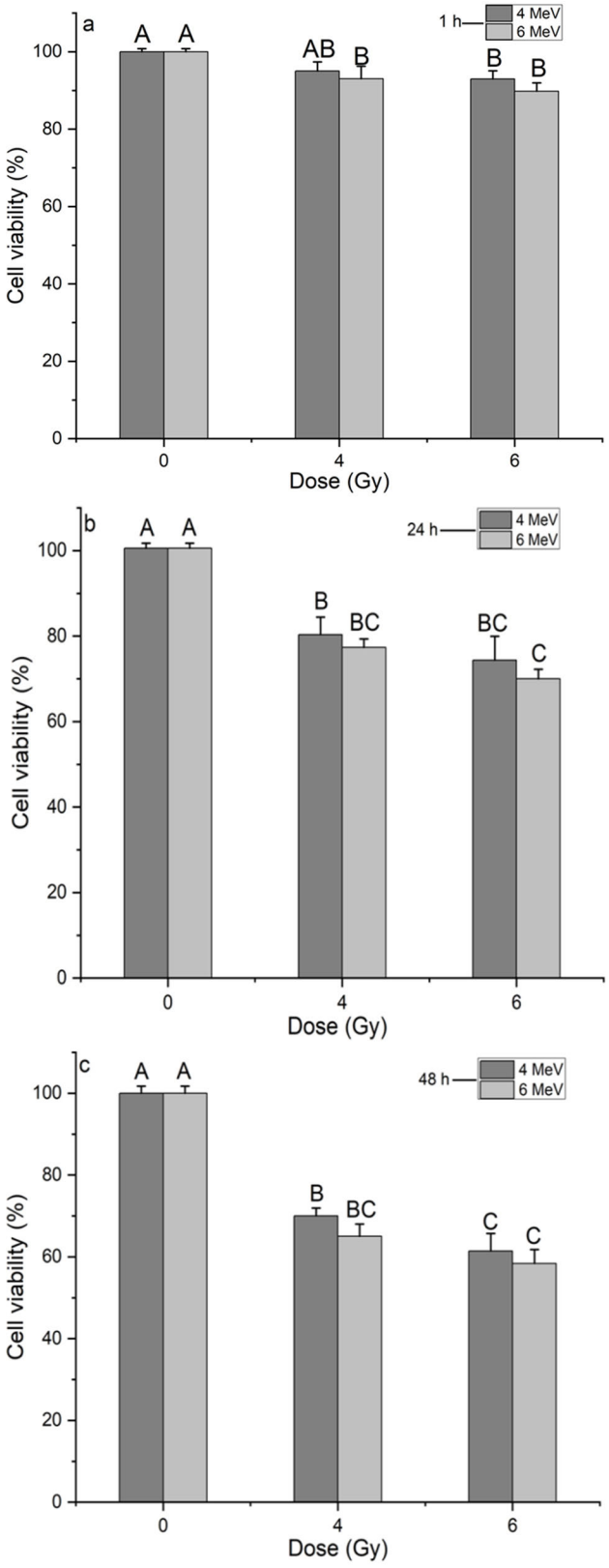

Figure 3 (a,b,c): Effect of radiation dose (4 and 6 Gy) at given photon energies (4 and 6 MeV) on cell viability, measured at 1, 24, and 48 h post-irradiation. Data are expressed as mean±SD (n = 3). Different letters indicate statistically significant differences according to Tukey's HSD test ($p < 0.05$).

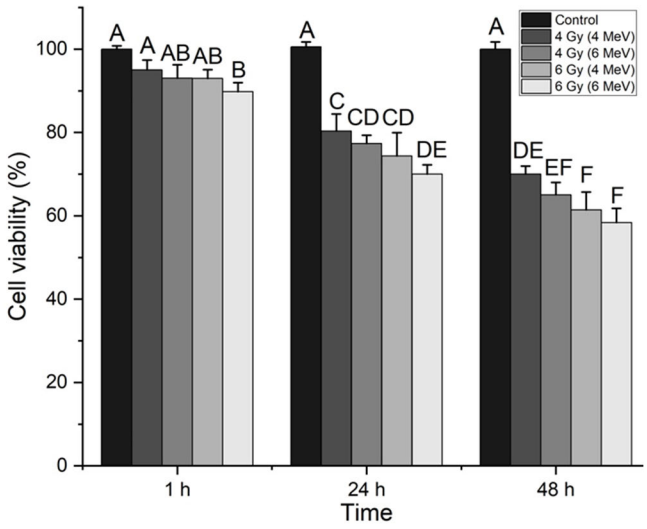

Figure 4: Combined impact on MCF-7 cell viability of radiation doses (4 and 6 Gy), photon energy (4 and 6 MeV), and post-irradiation intervals (1, 24, and 48 h). The data can be seen as mean±SD (n = 3). According to Tukey's HSD post-hoc test ($p<0.05$), distinct letters denote statistically significant differences, while groups that share at least one letter do not vary substantially.

Table 1: Effect of radiation dose, photon energy, and post-irradiation incubation time on the viability of MCF-7 breast cancer cells compared with the control group.

Treatment	Mean±SD	Significance vs Control	p value
Control	100±0.79	—	—
4 Gy (4 MeV) – 1 h	95.01±2.35	ns	0.7217
6 Gy (4 MeV) – 1 h	92.97±2.10	ns	0.1917
4 Gy (6 MeV) – 1 h	93.05±3.23	ns	0.2048
6 Gy (6 MeV) – 1 h	89.80±2.14	**	0.00587
4 Gy (4 MeV) – 24 h	80.31±4.13	****	<0.0001
6 Gy (4 MeV) – 24 h	74.36±5.59	****	<0.0001
4 Gy (6 MeV) – 24 h	77.37±1.95	****	<0.0001
6 Gy (6 MeV) – 24 h	70.03±2.23	****	<0.0001
4 Gy (4 MeV) – 48 h	70.02±4.31	****	<0.0001
6 Gy (4 MeV) – 48 h	61.41±4.31	****	<0.0001
4 Gy (6 MeV) – 48 h	65.06±2.95	****	<0.0001
6 Gy (6 MeV) – 48 h	58.40±3.38	****	<0.0001

ns: Non-significant at $p > 0.05$, ** at $p \leq 0.01$, **** Highly significant at $p \leq 0.0001$

(4 to 6 Gy), photon energies (4 to 6 MeV) and post-irradiation time (1 to 48 hours). The minimum viability value was obtained at doses of 6 Gy and 6 MeV at 48 hours (58.40±3.38%), implying that these values were the most potent for causing cell death. The effect size of $\eta^2 = 0.978$ shows a highly significant effect of the treatment. In order to establish statistically significant differences between the groups, Tukey's HSD post hoc test was applied. These findings demonstrate that radiation dosage, photon energy and incubation time work together to reduce cancer cell viability, as shown in the statistical analysis data. The results from the present experiment match with what is demonstrated recently in the scientific literature concerning the effect of ionizing radiation on breast cancer cells [28], which indicated a significant suppress in the viability of the

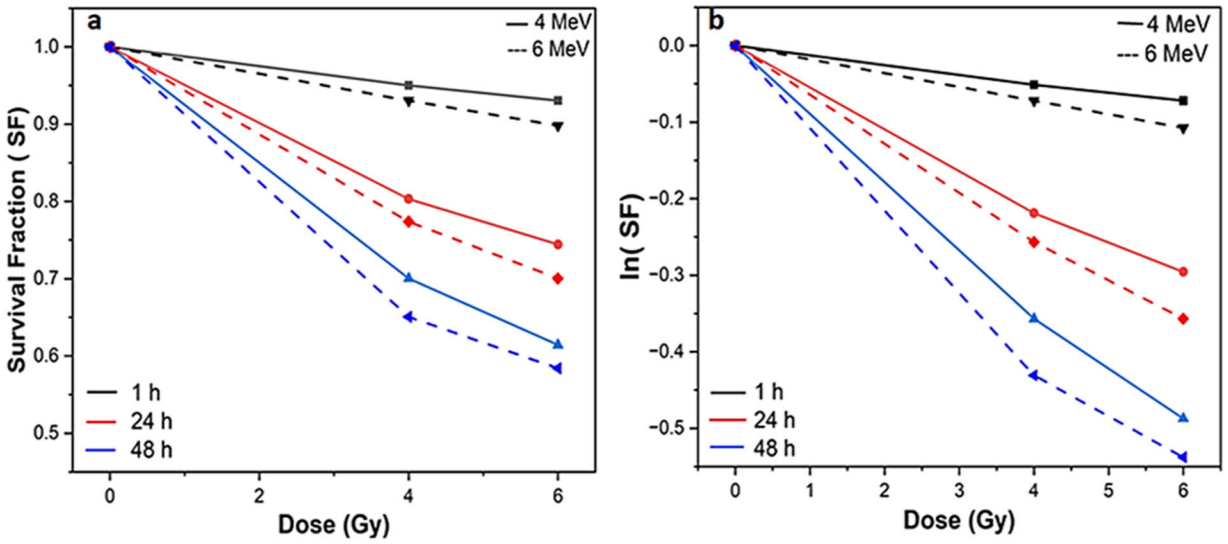

Figure 5 (a&b): A combined radiobiological survival analysis of MCF-7 cells subjected to various photon energies, radiation dosages, and post-irradiation durations. (a) Survival Fraction (SF) curves versus radiation dosage at 4 and 6 MeV photon energies. (b) A linear-quadratic plot of cell survival expressed as ln(SF) versus radiation dosage under various irradiation conditions.

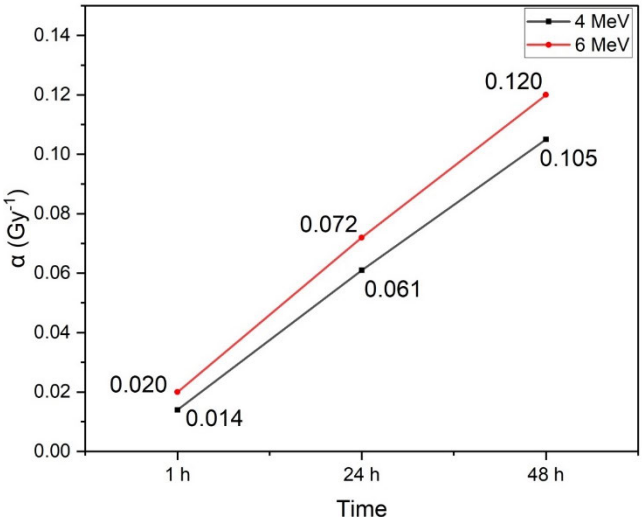

Figure 6: Variation of the α parameter with post-irradiation time for MCF-7 cells exposed to 4 and 6 MeV photon energies.

MCF-7 cells as a result of an increased radiation dosage, increased photon energy and longer periods after irradiation. The above conclusion can be substantiated by the fact that the increased doses of the X-ray rays cause increased damage to DNA molecules (double-stranded breakage) and ROS production, thus increasing the possibility of failure of repair mechanisms resulting in cell apoptosis or mitotic death. The findings of this present study reveal that the effect of radiation on cells is influenced not only by the amount of radiation but also by radiation energy and the time passed after the exposure because the reactions in cells and accumulating of damages in molecules occur during hours and even days after the irradiation. In medical practice, the possibility of obtaining better cell killing due to better irradiation conditions could help to make the effectiveness of radiotherapy better in the future. It might become possible to reduce the number of procedures or the total dose of radiation for certain patients which would prevent unnecessary irradiation of healthy tissues. However, this assumption necessitates additional research.

Survival fraction (SF) values were estimated from experimentally determined MTT cell viability percentages using established radiobiological formulae. The appropriate ln(SF) values were then obtained for radiobiological examination in Figure 5 that shows the survival of MCF-7 cells decreased progressively. Lower 6-MeV versus 4-MeV survival values suggest destructive effectiveness and therefore, increased radiation-induced cell death at 6-MeV. Similarly, the loss of viability was persistent at 24 and 48 hours post-irradiation pointing to a combination of chronic radiation injury as well as some delayed cellular response. Tissue reaction to radiation is a function of dose and time from exposure (taking into account the energy of photons). Furthermore, increasing in the photon energy not only makes reactions to this kind of irradiation stronger, but also induces non-instantaneous one after irradiation injuries whose effects can most appropriately be demonstrated by cell defects [13,29] . Similarly, ionizing radiation (IR), on both theoretical and practical levels appears to induce cellular damage in a more stepwise fashion as well [25,27]. The current findings are consistent with numerous radiobiological studies that use the survival fraction (SF) and ln(SF) curves as main markers for evaluating cancer cells' radiosensitivity. A drop in the SF value and a steeper slope of the ln(SF) curves provide direct evidence of increased radiation-induced damage and lower cell proliferative ability following radiation exposure [29]. Butterworth *et al.* found that ln(SF) curves can be helpful for characterising the radiation response of cancer cells and that increasing the slope of these curves is related with greater irreversible damage [18].

We showed that α was gradually increased at 2 to 6 h after irradiation suggesting floating radiosensitivity and with more lethality cellular damage (Figure 6). Furthermore, already at an energy of 6 MeV α was higher than at 4 MeV, indicating a difference cell killing by this high-energy radiation. Typically, a higher α means larger irreparable DNA damage and fewer surviving cells 1 day after irradiation, which agrees with survival curves and trend analysis here. These data are corroborated by the work done by Cui *et al.* [30] on the basis of which it was established that the growing value of the α-parameter indicates higher radiation damage of lethal cell effects resulting in the reduced cell viability and radiosensitivity. In this respect, the growing value of the α-parameter, especially at 6 MeV, testifies to the higher efficiency of radiation in inducing irreparable cellular damage. These findings are in accordance with earlier studies showing that the parameters of LQ models such as α are directly associated with radiosensitivity as well as lethal damage to cells resulting from irradiation. Thus, the increasing trend in the values of the α parameter with time, especially with 6 MeV of energy, shows that there is an increase in the linear part of radiation damage, which results in more irreparable DNA damages to the cells [31,32]. Although the LQ model traditionally relies on both the α and β parameters, the present study focused on calculating the α parameter due to the limited range of radiation doses examined and the study's exploratory nature. Under these conditions, estimating the β value would have introduced significant uncertainty and compromised the reliability of the data fit; this is because the β parameter is derived from the curvature of the cell survival curve, a process requiring a wide range of doses [33], whereas the current study utilized a limited number of dose points. Consequently, the α parameter was used as an indicator of cellular response to radiation across the various experimental groups. Future research covering a broader range of radiation doses and additional biological endpoints will enable more precise determination of both the α and β parameters.

This work provides a detailed assessment of the radiobiological response breast cancer cells (MCF-7) to therapeutically relevant X-ray photon beams produced by a medical linear accelerator (LINAC). Unlike many prior research that concentrated on a single radiation parameter, the current study explored effects of photon energy (4 and 6 MV), radiation dosage Gy) and post-irradiation incubation period (1, 24 and 48 hours) under the same experimental settings. The results demonstrated that cell viability was influenced by all three factors, highlighting the importance of considering their combined contribution when evaluating the biological and dynamic responses induced by clinical radiation. These findings provide additional empirical evidence regarding the behaviour of breast cancer cells under irradiation conditions that mirror clinical practice; furthermore, they may contribute to optimizing future radiotherapy strategies, potentially reducing the number of treatment sessions for breast cancer patients undergoing X-ray therapy.

CONCLUSIONS

This work aims to summarize the results of MCF-7 breast cancer cells differential radiobiological response as a function of radiation dose, photon energy and post-irradiation time. Maximum radiation-induced cytotoxicity with delayed cellular damage for the cell survival fraction (SF) due to irradiation and post-irradiation incubation in greater doses and for longer periods, is reduced. The cumulative effects of radiation over time are also reflected in the minimum survival rates following irradiation, which were obtained at 6 Gy/48 h. The absence of crossfire and long-range DNA double-strand break rejoining at 6 MeV photons translated into smaller cell survival fractions than at 4 MeV, indicating greater effectiveness with respect to a photon energy. These plots show a measure of the death after ionising radiation exposure and differences in the biological response based on dose are apparent. These data indicate that the spectral properties of photon energy as well as dose and time-dependent effect post exposure to radiation, are major determinants for radio-sensitivity and final cell death. The radiation dose range, photon energy and post-irradiation time employing ionising radiation from a linear accelerator as inducing cytotoxic mechanisms on cancer cells progressively or as a method implementable for in-depth study of radiobiological behaviour of mammalian breast carcinoma cellular cocktails under conditions of rhythmic exposure to ionising radiation.

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