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Original Article

Development of Spheroid in Co-Culture of Prostate Tumor with Human Fibroblast, using the Hanging Drop Technique with Plate Inversion for Analysis of GammaCell220 Irradiation in Co-60

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Abstract: The development of tumor spheroids in co-culture with human fibroblasts represents an innovative approach to modeling the prostate cancer microenvironment and assessing radiation effects. In this study, we employed the "hanging drop" technique in conjunction with plate inversion to generate three-dimensional spheroids composed of prostate tumor cells (LNCAP cell line) co-cultivated with human fibroblasts. This approach aims to create a more representative in vivo-like tumor environment, facilitating a detailed analysis of the interactions between tumor cells and fibroblasts in 3D conditions. The formed spheroids were exposed to gamma radiation using a GammaCell 220 irradiator with Co-60, with the goal of investigating the effects of radiation on both fibroblastic and tumor cells. Gamma radiation is known to induce cell death, and this study examines how such damage impacts cellular morphology within the spheroid microenvironment.

Keywords: Hanging Drop, LNCaP, GammaCell Co-60.

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Desenvolvimento de Esferoides em Co- Cultura de Tumor Prostático com Fibroblastos Humanos, Usando a Técnica de Gota Suspensa com Inversão de Placa para Análise de Irradiação GammaCell 220 com Co-60

Resumo: O desenvolvimento de esferoides tumorais em co-cultura com fibroblastos humanos é uma abordagem inovadora para modelar o microambiente do câncer prostático e avaliar os efeitos da radiação. Neste estudo, utilizamos a técnica de "hanging drop" combinada com a inversão de placas para formar esferoides tridimensionais de células tumorais prostáticas (linha LNCAP) co-cultivadas com fibroblastos humanos. Esta abordagem visa criar um modelo mais representativo do ambiente tumoral in vivo, permitindo uma análise detalhada das interações entre células tumorais e fibroblastos em condições 3D. Os esferoides formados foram expostos a radiação gama utilizando um irradiador GammaCell 220 com Co-60, com o objetivo de investigar os efeitos da radiação nas células fibroblásticas e tumorais. A radiação gama é conhecida por induzir morte celular, e este estudo analisa como esses danos influenciam a morfologia no microambiente dos esferoides.

Palavras-chave: Gota Suspensa, câncer de próstata, irradiador GammaCell 220 com Co-60.

1. INTRODUCTION

Three-dimensional (3D)cell culture systems have emerged as a transformative tool in biomedical research, offering a more accurate representation of *in vivo* cellular environments compared to traditional two-dimensional (2D) cultures [1, 2]. Unlike 2D cultures, which can impose significant constraints on cell growth and behavior, 3D cultures better mimic the complex cellular interactions and microenvironments of living tissues [3]. One widely utilized method for creating 3D cellular structures is the hanging drop technique. This approach fosters the formation of spheroids and other 3D aggregates by allowing cells to self-organize into structures that closely resemble the *in vivo* tissue architecture [4].

The hanging drop technique is particularly effective for studying cellular dynamics such as interaction, migration, differentiation, and tissue formation under conditions that closely replicate the physiological environment [5]. It is commonly used with fibroblasts, which are essential components of connective tissue involved in extracellular matrix production and tissue homeostasis. In 3D cultures, fibroblasts can produce and organize critical matrix components, including collagen, fibronectin, and elastin, thus creating a more intricate and responsive extracellular matrix that can better simulate pathological conditions [6,7].

In normal prostate tissue, fibroblasts are responsible for maintaining tissue structure and integrity, exhibiting significant changes during cancer progression [8]. In the tumor microenvironment, these fibroblasts can become activated into cancer-associated fibroblasts (CAFs), which can play a pivotal role in tumor progression by remodeling the extracellular matrix, secreting growth factors, pro-inflammatory cytokines, and enzymes that promote cancer cell invasion and metastasis [4, 9]. Understanding the behavior of CAFs and their interactions with prostate cancer cells is critical for developing effective therapeutic strategies [1, 5].

This study used the LNCaP cell line (FGC clone, ATCC CRL-1740), an invasive adenocarcinoma derived from a lymph node metastatic cell line which expresses androgen and estrogen receptors, irradiated by a gamma source to evaluate possible morphological changes of prostate cancer cells and human fibroblasts when co-cultured in 3D [10, 11]. By exposing the 3D spheroids to gamma radiation, the study aimed to assess how radiation-induced cell death impacts fibroblast function and its subsequent effects on tumor cell behavior [12, 13, 14].

2. MATERIALS AND METHODS

2.1. Cell lines

HF002-J (human healthy breast fibroblasts) cells were a kind gift of Dr. Monica Beatriz Mathor (CETER, IPEN/CNEN) and LNCap cells (ATCC CRL-1740) were kept and cultured at 37°C in a humid atmosphere containing 5% CO_2, maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% antibiotics (Penicillin-Streptomycin, 10.000 µg / mL – 10.000 U / mL). When reaching 60-70% confluence, the cells were detached using a 0.05% trypsin solution. Spheroids were prepared using the hanging-drop technique adapted from. Briefly, 440 µL of medium containing 3×10^4 to cells per well of a 96-well plate were plated, generating a positive meniscus. The plate was inverted and incubated as described and let to grow for 72h.

2.2. Irradiation

Cells were irradiated in PBS (Phosphate Buffered Saline) for gamma-ray irradiation at doses ranging from 0 to 2 Gy of 60-Co, in a sterile manner and at room temperature. The irradiations were carried out using a GammaCell 220 model equipment (Irradiation Unit of Canadian Atomic Energy Commission, Ltd.) located at the Radiation Technology Center of IPEN/CNEN-SP, using lead shielding corresponding to 90% radiation attenuation.

2.3. Fluorescence microscopy assays

Cells in spheroids in 96-well plates were stained with Hoechst 33342 solution (10 µg/mL) and SYTOX ® Green (1 nM) and left in the incubator for 60 minutes. The material were visualized and photographed at 4X magnification using an automated fluorescence microscope (INCell 2500 HS, Cytiva) at 24, 48, and 72 hours. Image stacks (20 to 60 slices) of each spheroid were acquired, in focal planes 4 µm apart. Cell nuclei were highlighted by blue fluorescence, labeled by Hoechst 33342, and nuclei of non-viable cells were highlighted in green, labeled by SYTOX ® Green [2].

Maximum intenstity projections of stacks were generated by Fiji/ImageJ software [6]. For better visual contrast, signal form blue channel (Hoechst) were pseudocolored in cyan, and signal from green (SYTOX) was translated to red. After backgroud correction (rolling ball radius: 10 µm), images were binarized and analyzed using the "Analyze Particles" plugin. The measured parameters of the spheroids are on Table 1:

Table 1: Measured parameters of the spheroids.

Parameter	Description
Dose	Radiation dose: 0 or 2 Gy
t (h)	Time of observations: 24, 48 or 72h
Area	Spheroid area (µm²)
Perimeter	Spheroid perimeter (µm)
BX	Lenght (µm) of the x-axis of the bounding rectangle
BY	Lenght (µm) of the y-axis of the bounding rectangle
Width	Spheroid width (µm) on x-axis of image
Height	Spheroid height (µm) on y-axis of image
Major Axis	Major axis (µm) of spheroid
Minor Axis	Minor axis (µm) of spheroid
Angle	Angle between major and Minor Axis
Circularity	0 to 1 (1 = perfect circle)
Feret	The longest distance between any two points along the selection boundary
Feret X	Starting coordinates of Feret Diameter
Feret Y	Starting coordinates of Feret Diameter
Feret Angle	The angle (0--180 degrees) of the Feret's diameter
Feret (Minimum)	Minimum caliper diameter
Aspect Ratio	Ratio between Major and Minor bounding ellipses
Roundness	Inverse of Aspect ratio

2.4. Principal Component Analysis (PCA)

To visualize which variables became more relevant in the present assay setup, data were analyzed by a PCA approach, using the GraphPad Prism 10.2.0 software. Numeric values (categorical variables) were transformed to z-score values to minimize the effects of standard deviations and to provide and absolute comparison scale. Components were chosen using the parallel analysis method. Variables were picked when associated with at least |0.95| of PC loading score. Variables were also plotted for better visualization of differences between 0 and 2Gy groups.

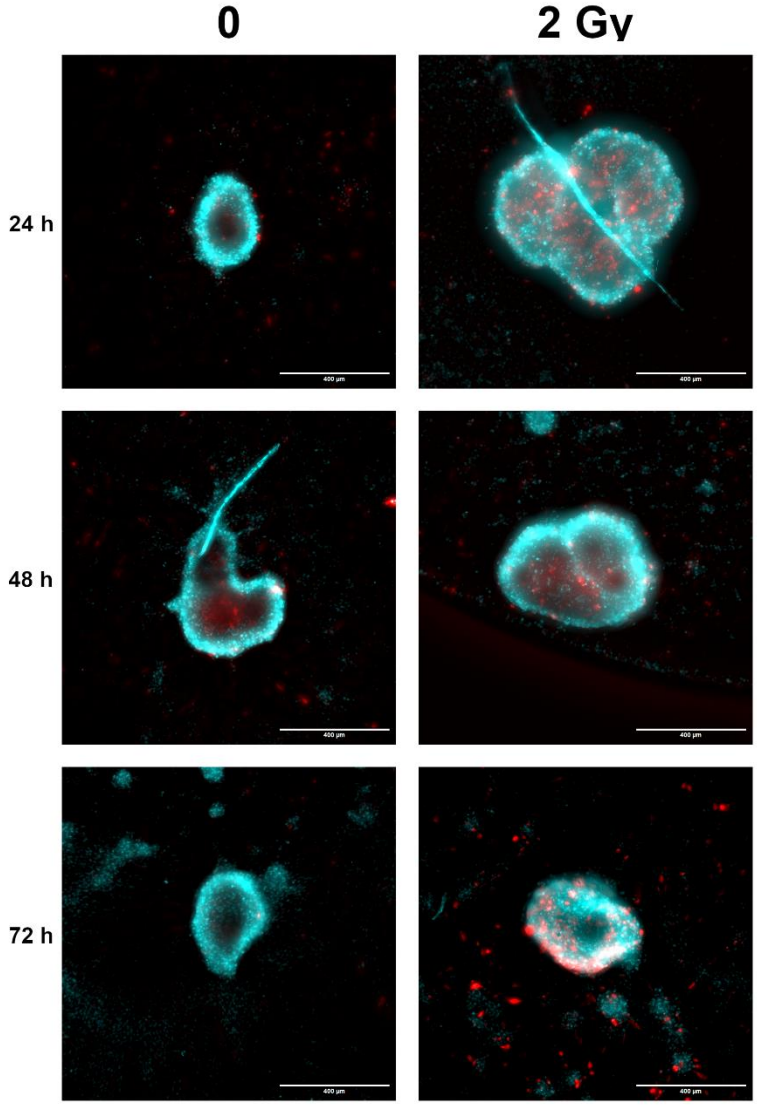
3. RESULTS AND DISCUSSIONS

This study assessed the development of spheroids in the hanging drop system after 4 days of culture, using different cell preparations. The results indicate that the spheroid preparations were found to be compact, as described elsewhere [1]. The used cell concentration was 30,000 cells / well, with a ratio of 60% LNCaP cells and 40% HF002J cells, aiming to simulate the tumor microenvironment. The analysis focused on the interactions between tumor cells and fibroblasts, which play crucial roles in various tissues, mainly in extracellular matrix production. Interactions between fibroblasts and tumors contribute to the formation of a protective envelope around the tumor, where the tumor modifies the extracellular matrix, making it stiffer to perform this protective function. However, to facilitate tumor migration, the tumor also expresses factors that promote the destabilization of the surrounding extracellular matrix, allowing infiltration into adjacent tissues.

Thus, the three-dimensional models used in this study resemble *in vivo* tumor morphology, as the hanging drop model allows for the formation of spheroids with regions similar to those found *in vivo*, such as necrotic, quiescent, and proliferative zones. Initially, it was possible to identify, in Figure 1, dead cells within the tumor, stained with SYTOX Green and pseudocolored in red, which likely correspond to the necrotic zone due to tumor hypoxia

in non-irradiated spheroids, but may represent cell death upon irradiation in 2Gy-irradiated models. However, it was not possible to clearly identify the quiescent and proliferative zones.

Figure 1. Maximum fluorescence projections of image stacks of LNCap/HF002J spheroids. Cyan: Hoechst 33342 (nuclei). Red: SYTOX Green® (nuclei from dead cells). Magnification: 4X. Bar: 400 µm.



Source: Prepared by the author.

Gamma radiation can induce a cascade of biological effects within cells, often culminating in alterations to cellular morphology and size. One prominent consequence of gamma irradiation is the induction of DNA damage, which can trigger cell cycle arrest and subsequent cellular responses aimed at repairing the damaged genetic material (Bharat et al., 2024; Johnson & Carrington, 1992). This DNA damage response can lead to an increase in

cell size as cells halt their progression through the cell cycle, providing them with additional time to repair DNA lesions before replication or division (Panganiban et al., 2013). Furthermore, the activation of the p53 signaling pathway, a critical regulator of cellular responses to stress and DNA damage, plays a significant role in mediating these changes (A. Marcellus et al., 2024). Specifically, p53 activation can promote the expression of genes involved in cell cycle arrest, DNA repair, and apoptosis, further contributing to the observed increase in cell size. Irradiated cells frequently exhibit an elevated production of reactive oxygen species, which can inflict oxidative damage on cellular components, including lipids, proteins, and DNA (Ruan et al., 2023). Oxidative stress can disrupt cellular homeostasis and contribute to an increase in cell size (Ruan et al., 2023). Moreover, gamma irradiation can disrupt cellular membrane integrity, leading to changes in cell volume and morphology. Thus, the area of spheroids, as a measurement proportional to spheroid size, can be used to detect if spheroids were affected by radiation doses, even in cases such as presented when cell death cannot be easily detected.

High-Content Screening (HCS) has emerged as a powerful methodology for investigating spheroid morphology due to its capacity to automate image acquisition and analysis, thereby enabling the rapid and quantitative assessment of three-dimensional (3D) cell cultures (Mittler et al., 2017). Spheroids, as self-assembled three-dimensional cellular aggregates, offer a more physiologically relevant model compared to traditional two-dimensional monolayer cultures, as they better mimic the in vivo microenvironment, including cell-cell and cell-matrix interactions, nutrient gradients, and drug penetration profiles (Sart et al., 2017). The utilization of HCS platforms allows for the automated acquisition of high-resolution images of spheroids, coupled with sophisticated image analysis algorithms to extract a multitude of morphological parameters (Horman et al., 2013). These parameters can include spheroid size, circularity, solidity, surface roughness, and internal texture, providing a comprehensive characterization of spheroid shape and structure (Zanoni et al., 2019). Furthermore, HCS enables the assessment of spheroid viability through the

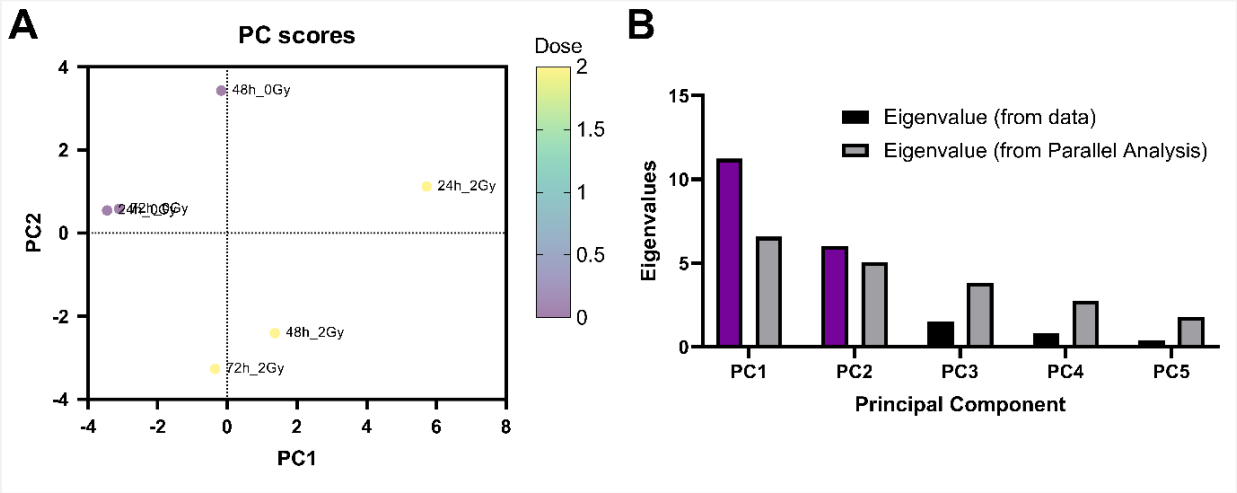
utilization of fluorescent dyes that indicate metabolic activity or cell death (Ivanov et al., 2014). The ability to monitor these parameters over time or in response to various stimuli, such as drug treatments or genetic manipulations, makes HCS an invaluable tool for drug discovery, toxicology studies, and fundamental cell biology research (Bassi et al., 2021; Mehta et al., 2012). The integration of HCS with spheroid models not only enhances the throughput of experiments but also improves the reproducibility and objectivity of data analysis, leading to more reliable and informative results.

The application of HCS to spheroid morphology studies generates a high-dimensional dataset consisting of numerous morphological parameters, which can be challenging to interpret and analyze effectively. Principal Component Analysis provides a robust dimensionality reduction technique to simplify the data and extract the most relevant information (Sgouros et al., 2003). PCA transforms the original variables into a new set of uncorrelated variables called principal components, which are ordered by the amount of variance they explain in the data (Eilenberger et al., 2019). By focusing on the principal components that capture the majority of the variance, researchers can reduce the complexity of the dataset while retaining the most important information about spheroid morphology. PCA can identify the key morphological features that distinguish different experimental groups or treatment conditions, allowing for a more targeted and efficient analysis of the data (Jensen & Teng, 2020). For instance, PCA can reveal whether changes in spheroid size, circularity, or texture are the primary drivers of the observed differences between control and treated spheroids. Furthermore, PCA can be used to identify correlated variables, which may indicate underlying biological processes or pathways that are influencing spheroid morphology (Ivosev et al., 2008).

The PCA analysis could retain only 2 main components that contained 86.35% of total variance, and individual variances of PC1 and PC2 of 56.21% and 30.15%. The PC scores of each experimental group (24h / 0 Gy; 48h /0 Gy; 72h / 0 Gy; 24h / 2 Gy; 48h / 2 Gy; 72h

/ 2 Gy) are shown in Figure 2, as PC eigenvalues obtained after parallel analysis, justifying the choice of the components.

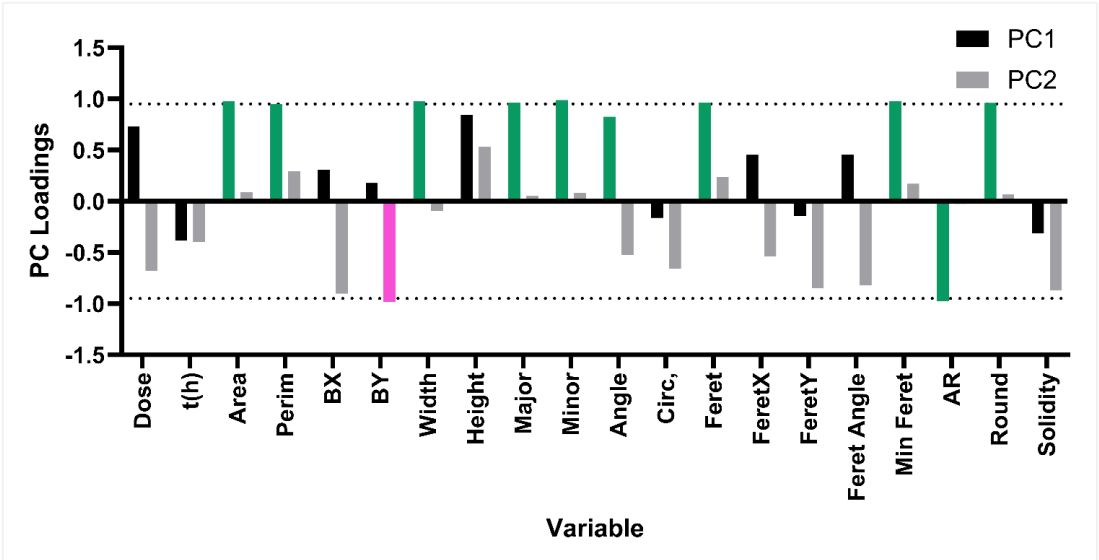
Figure 2: A: PCA Scores of experimental groups. B: Eigenvalues of obtained components.



The 0 and 2Gy could be segregated accordingly, what can show the usefulness of the proposed analysis to discriminate the effects of gamma radiation on spheroid morphology.

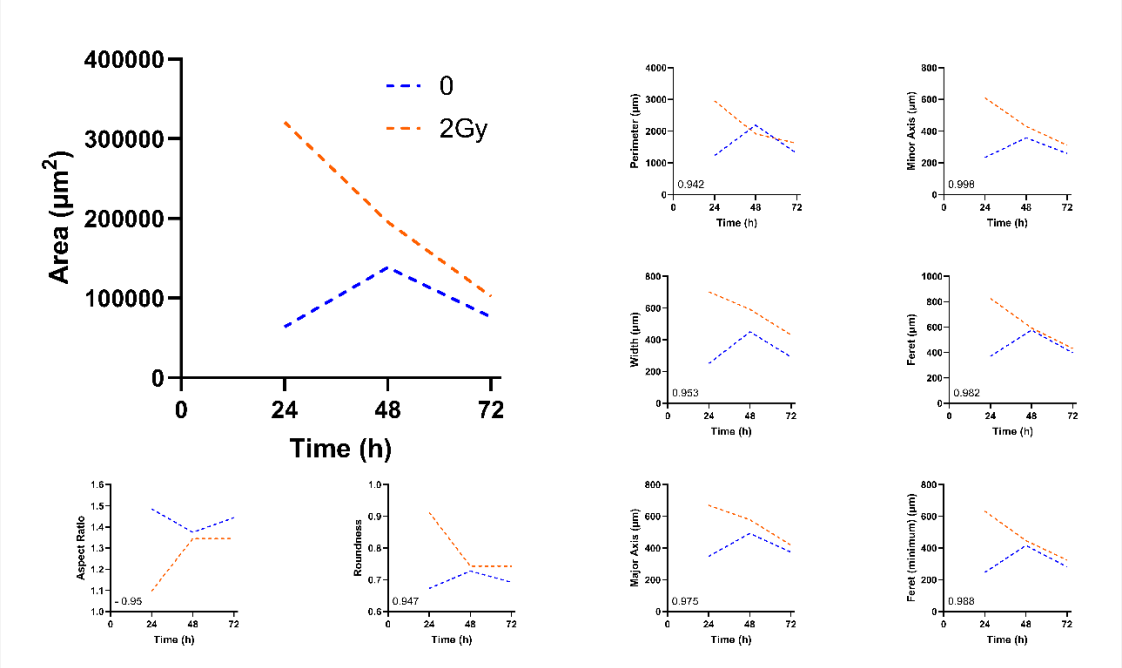
Variables in PC1 and PC2 were chosen whether its PC loadings were higher than 0.95, or lower than -0.95. Selected variables on PC1 (green) and PC2 (magenta) are shown Figure 3. Besides the "Area" parameter, were chosen "Perimeter", "BY", "Width", "Major Axis", "Minor Axis", "Feret", "Feret Minimum", "Aspect Ratio" and "Roundness".

Figure 3: Variables in PC1 and PC2 that met the criteria.



In Figure 4 are shown the area of 0 and 2 Gy irradiated spheroids across time of experiments, as the dynamics of the other chosen variables from PC1.

Figure 4: Variables of PC1 component and its dynamics across experiment time.



The behavior of spheroid area over time exhibits a complex interplay of factors, particularly when considering the influence of irradiation. In untreated spheroids, an initial increase in area, followed by a subsequent decrease, can be attributed to the dynamic balance between cell proliferation, extracellular matrix production, and the development of central necrosis (Sgouros et al., 2003). Initially, cells on the outer layers of the spheroid experience exponential growth, leading to an overall expansion in volume and, consequently, area (Petry & Salzig, 2022). This proliferation is fueled by readily available nutrients and oxygen from the surrounding culture medium (Chaicharoenaudomrung et al., 2019). As the spheroid grows, however, diffusion limitations become more pronounced, resulting in a decreasing gradient of oxygen and nutrients from the periphery to the core (Bassi et al., 2021). This gradient creates a hypoxic environment in the center of the spheroid, triggering cell cycle arrest and ultimately cell death via apoptosis or necrosis (Zanoni et al., 2019). The balance between cell proliferation and cell death will initially favor growth, leading to an initial

increase in spheroid area. As the spheroid volume increases, the core becomes more hypoxic and the rate of cell death begins to overtake the rate of proliferation on the outer edges, leading to a decrease in spheroid volume, and area (Olea et al., 1992; Sart et al., 2017). The compaction of the spheroid, resulting from cell death and the breakdown of the extracellular matrix in the core, further contributes to the reduction in area.

In contrast, irradiated spheroids typically exhibit a monotonic decrease in area over time. The introduction of ionizing radiation induces DNA damage, leading to cell cycle arrest, mitotic catastrophe, and ultimately cell death (Chiari et al., 2023). Tissues with rapid cell turnover, such as the epithelium, manifest radiation injury earlier than cells that divide slowly (Johnson & Carrington, 1992). Radiation-induced cell death surpasses cell proliferation in irradiated spheroids, even in the initial hours following exposure. The extent of DNA damage inflicted by irradiation overwhelms the DNA repair mechanisms of the cells, leading to genomic instability and ultimately triggering programmed cell death pathways (Panganiban et al., 2013). Furthermore, radiation can disrupt the extracellular matrix and vasculature, further impeding nutrient delivery and waste removal (Wani et al., 2019). The disruption of cell-cell adhesion molecules by radiation can also lead to cell detachment and spheroid disintegration, contributing to the overall decrease in area (Koch & Funk, 2001).

The analysis of the area of spheroids, three-dimensional cellular aggregates, is crucial for evaluating cell death and fate due to its direct correlation with cellular behavior, drug penetration, and overall spheroid integrity (Eilenberger et al., 2019). Spheroids mimic the in vivo tumor microenvironment, offering a more realistic platform for studying cellular responses compared to traditional two-dimensional (2D) cell cultures (Zanoni et al., 2019). The area of a spheroid provides a quantitative measure of its size and compactness, reflecting the balance between cell proliferation, death, and adhesion (Horman et al., 2013). By monitoring changes in spheroid area over time, researchers can gain insights into the efficacy of therapeutic interventions and the underlying mechanisms of cell death (Sart et al., 2017). Changes in spheroid size and morphology can be indicative of treatment response, with a

reduction in area suggesting drug effectiveness and induction of cell death, while an increase in area might indicate proliferation or resistance.

4. CONCLUSIONS

Based on the results obtained, it was possible to develop an initial prototype of spheroids from human fibroblast cells that can resemble tissues *in vivo* due to their cellular interactions, thus providing a new tool for the study of drugs and treatments. Radiation treatments can be mimicked using the approach, and measuring responses through assessment of spheroids' area.

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CONFLICT OF INTEREST

The authors do not disclose any conflicts of interest.

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