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Three-dimensional cell culturing by magnetic levitation for evaluating efficacy/toxicity of photodynamic therapy

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ABSTRACT

We used three dimensional cell cultures (3D) based on the magnetic levitation method (MLM) to evaluate cytotoxicity of photodynamic therapy (PDT). First, we decorated Hep G2 and MDA-MB-321 cells with NanoShuttle by introducing it in the media and incubated overnight. Next day, we transferred the cells to a 6-well plate and placed a magnetic driver on the top of the plate to start levitation. We monitored the formation of the 3D cell culture by optical microscopy and after four days, we added the photosensitizer Photogem (PG) in the culture media in concentrations of 30, 25, 12.5, 6.25µg/ml. We incubated them for 24 hours, after that we washed the cultures with PBS and added fresh media. Samples were then illuminated for 600s using a 630nm LED-based device, generating light intensities of 30 mW/cm² in a total light fluence of 18 J/cm². Following the illumination, we added fresh media, and 30 hours later, the 3D structures were broken using a pipettor and the cells seeded in 96 well plates, 10⁴ cells per well, with a magnetic drive placed on the bottom of the plate to create cell culture dots. After 24 hours, we used a MTT assay to evaluate PDT cytotoxicity. The PDT effect, evaluated by the half maximal effective concentration (EC₅₀), in MDA-MB-231 cells (EC₅₀ =3.14 µg/ml) is more aggressive compared to the effect of PDT in Hep G2 cells (EC₅₀ = 7.48 µg/ml). It suggests that the cell culture structure and its interaction facilitated the PG uptake and consequently elevated the Photodynamic effect for MDA-MB-231.

Keywords: photodynamic therapy, Photogem; in vitro dosimetry, three dimensional cell cultures; Hep G2, MDA-MB-231, NanoShuttle.

1. INTRODUCTION

Photodynamic therapy has shown to be a powerful treatment option for many types of cancer and other neoplastic diseases, at least as a palliative and or combined with other techniques. PDT act as a combination of a photosensitizer, light with specific wavelength and molecular oxygen present into cells, to cause the oxidative damage that can kill target cells by necrosis or apoptosis¹,². There is no possible cellular resistance to large amounts of oxidative stress. In other words, if the damage is irreversible, cells shall die – and by “irreversible”, we mean no cancer recurrence after treatment³.

Dosimetry is important for PDT outcome and has been largely investigated in vivo using different approaches, since being able to determine the minimum extension of damage, to preserve main anatomical structures and to avoid unwanted effects such as thermal (and, thus, uncontrolled) damage are crucial to the success of the therapy²,³. Using liver models became interesting to observe PDT effects in healthy tissue as a pre-tumor model, because liver is usually very homogeneous concerning light propagation properties. The use of rat liver models in dosimetry showed that the threshold dose is an important parameter for PDT planning⁴. However, it is still a normal tissue and does not represent important aspects of cancer tissue, like cellular heterogeneity⁵,⁶.
The cell heterogeneity could be a possible limiting factor for PDT outcome\(^5\). When PDT outcome does not result in full elimination of lesions, partial cell killing may result in selection of the privileged ones among the heterogeneous cells in a tumor mass, with lesion recurrence. The PDT efficacy against the recurrent lesion could not change, but it does not mean those resistant cells are still there to regrow the tumor. Therefore, improving our ability to perform PDT dosimetry properly is of major importance in the development of the technique.

To further study how cells heterogeneity interferes with PDT outcome, we have applied the magnetic levitation method. By using NanoShuttle to decorate cells and to give them magnetic properties, we used the Bio-assembler to levitate them under the air media surface promoting cell–cell interaction and extracellular matrix production\(^7\). In this way two types of cells from distinct tumor tissues (liver and breast) was studied comparing the 2D and 3D models. We aim to obtain a tool to create an “in vitro” cancer tissue, which might not only reduce the use of animal models, but also allow for better-controlled conditions in a 3D arrangement to study dosimetry for PDT.

\section*{2. MATERIALS AND METHODS}

\subsection*{2.1 Cell culture}

Cells used in this study were human hepatocellular carcinoma (Hep G2) and human breast cancer MDA-MB-231 (ATCC\(^\text{®}\)) and fed with DMEM containing 10\% of FBS for Hep G2 and 5\% for MDA-MB-231. Cells were cultured in a humidified environment (37°C, 5\% CO\(_2\)) with media changes every other day.

\subsection*{2.2 Magnetic levitation}

Monolayer (2D) cell cultures of each cell type were turned into 3D cultures using the 6.well Bio-Assembler Kit (Nano3D Biosciences). Cells were grown in 2D to 75\%–80\% confluence, after that, they were treated with a nanoparticle assembly (NanoShuttle; Nano3D Biosciences) at a concentration of 50 \(\mu\)L/mL media and incubated overnight. Followed by trypsin detachment procedure, we counted the cells and resuspended in 2mL of media in 6-well plate (Corning) with 1.6x10\(^6\) cells per well. A neodymium magnet was placed on top of the plate to levitate the cells to the air–liquid interface. The magnetic field drove the cells with NS together in the center of the well under the air liquid surface stimulating extracellular matrix production\(^8\).

\subsection*{2.3 Optical microscopy}

We monitored the 3D structure assembling using an inverted transmitted light microscope (Leitz Labovert) coupled to a digital camera taking pictures every day until the PDT application.

\subsection*{2.4 Photosensitizer and laser source}

The photosensitizer used was Photogem\(^\text{®}\) a hematoporphyrin derivative made in the Russia Federation (Moscow, Russia) in concentrations of 50, 25, 12.5, 6.25 \(\mu\)g/ml. The light source employed was a LED-based device made in a support laboratory (LAT- innovation projects in biophotonics) operating at 630 nm. The irradiance was 30mW/cm\(^2\) and the fluence was 18 J/cm\(^2\) (Figure 1).
2.5 Patterning Dots

To replenish the culture media the magnetic drive was placed on the bottom of the plate to settle down cells, using a 1 ml pipettor the media was changed every other day. After 30 hours of the PDT procedure, we broke the 3-D structures using a 1 ml pipette, re-suspended them in fresh media and diluted them to adjust the concentration 10^5 cells per ml of media.

Next, we positioned the 96-well magnetic drive (Nano3D Biosciences) for Dots on the bottom of the 96-well low attachment plate, seeded 1e5 cells per well plus 200 µl of media and incubated it for 1 hour. After that, we removed the 96-well magnetic drive and incubated the samples during 24 hours. We executed the patterning to create six Dots for each experimental group to guarantee a higher statistical significance in the MTT assay.

2.6 MTT assay

After 24 hours of incubation, we washed the cell culture dots with PBS. Then, to evaluate cell viability, 10 µL of MTT solution (5 mg/mL MTT [Sigma] in saline stored at -20°C) was added into each well, and after shaking, the plate was incubated for 4 hours in a humidified environment (37°C, 5% CO2). Formazan crystals were dissolved with 100 µL of 0.04 N HCl-isopropyl alcohol (acid isopropanol) in a mixer for 15 minutes. The optical density (OD) of triplicated samples was measured with an absorbance microplate reader (SpectraMax Plus384, Molecular Devices, produced in the US.) at 540 nm. The EC50 (half-maximal effective concentration), for Photogem was calculated for both cell lines using a sigmoidal dose response fitting.

3. RESULTS

After levitation under the surface air-liquid, Hep G2 cells start to produce extracellular matrix and form a 3D structure that presents different morphology when compared to 2D culture, as we can see in Figure 1 (A) and (B). Looking more closely in Figure 2 (C), we see the Hep G2 cells in groups containing a few to dozens cells. There are cellular division, interaction cell-cell and interaction cell-plastic happening all the time. It is a flat surface and besides Hep G2 could grow and form a few layers, the cells suffer the action of the gravity what reduces the possibility of interaction cell-cell to the proximity and causes changes in the cell morphology. Using the magnetic force to compensate gravity gives to cells the possibility of motion and exclusively interact cell-cell.
Different cell types forms different amounts of extracellular matrix, and this could changes how permeable is the whole structure. As we can see in Figure 3 (A) the MDA, darker regions suggest that the culture different densities, the presence of the nanoparticles turns the cell darker but do not change absorption properties of the photosensitizer used in this experiment. In the Figure 3 (B) Hep G2 cells after 96 hours of levitation seems to be covered, you cannot differentiate one cell from another, they are forming a denser structure, which represents a barrier for Photogem distribution and uptake.

The result of MTT assay gave us the dose response curves illustrated in the Figure 4. These result evidenced that MDA-MB-231 cell culture is more sensitive than Hep G2 to PDT effect using Photogem at different concentrations from 6.25 to 50µg/ml, irradiated at fluency of 18 J.cm⁻². The EC₅₀ calculated using a sigmoidal fitting was 7.48 µg/ml for Hep G2 and 3.14 µg/ml for MDA.
4. DISCUSSION

We used the magnetic levitation method in an attempt to create an in vitro model, mimetic to cancer tissue, to evaluate the PDT effects in cellular heterogeneity in a solid cellular mass. The cells were driven together using nanoparticles bounded to them combined with the magnetic force.

We cannot affirm that metabolism of cells has changed in 3D, but the way they interact has. Comparison of 3D cell cultures of Hep G2 and MDA-MB-231 suggests that Hep G2 levitating cell culture present more dense structure than MDA-MB-231 levitating cell culture. We could expect that it can make MDA-MB-231 more sensitive to PDT than Hep G2 in this condition.

Each cell line shows different sensibility to different types of drugs since that one's shows more sensitive than others do. The cytotoxicity value found (EC50) can change depends on cells studied. Important targets of PDT are membranes, having protection by being in the middle of the culture, where the gradient of Photogem molecules and molecular oxygen, and the light fluence are possibly lower. This gradient could make some cancer cells survive and regroups a tumor mass. In this case, multiple sessions would be necessary. The EC50 of Photogem for Hep G2 was two folds higher than the EC50 for MDA; this fact might have a strong correlation with the structural differences between these two cell lines. These results emphasize the real importance to study the specific tissue targets of the photosensitizers using 3D models to predict in vivo behavior.

Figure 4. Dose-response curves obtained by Photodynamic therapy using Photogem at different concentrations from 6.25 to 50 µg/ml, irradiated at fluency of 18J.cm⁻², for Hep G2 and MDA-MB-231 cells.
5. CONCLUSION

3D cell culturing by magnetic levitation is a valuable tool for characterizing and optimizing the PDT process in vitro, such as evaluating photosensitizer diffusion and predicting the outcome of multisession PDT dosimetry prior to in vivo experimentation. The 3D levitating culture presents important characteristics of real tumor masses and is a bridge between the 2D conventional in vitro models to in vivo model.

In addition, the in vitro models show more advantages than in vivo models using tissues. One of these advantages is the possibility to decrease the variability in the experiments (different sources of the same tissue), reduce the animal experimentation number and in general, way the in vitro methods can be useful controlling all biological interferences.

6. REFERENCES