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Comparative Viability Analysis of Monolayer Cell Suspension and Multicellular Tumor Spheroids as an In-vitro Tumor Model

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ABSTRACT

Background: Monolayer cell suspension has been used as an in-vitro model for investigating cells characteristics, drug penetration, and tissue research. 3-D multicellular tumor spheroids (3-D MCTS) have attained focus of researchers from the last three decades as a more valuable tool to study tumor biology. The superiority of MCTS has been elucidated here along with the effect of disaggregated spheroids. **Method:** The cellular differentiating fluorophores; calcein-AM and Propidium Iodide (PI) have been exploited for cells viability characteristics, where calcein-AM penetrate viable proliferating cells only, PI penetrate dead cells only, while viable hypoxic cell remains unstained and are resistant to penetration of either fluorophore. Fluorophores uptake in 3, 5, and 7-day old intact spheroids, disaggregated spheroids, and monolayer cell suspension has been investigated. **Results:** The Calcein-AM stained cells and unstained cells fraction in intact, disaggregated, and monolayer cell suspension was statistically non-significant (ANOVA $p = 0.072$ and 0.411 respectively), while the PI-stained cells fraction showed statistical significance (ANOVA, $p = 0.012$). PI-stained cells fraction was greater in intact spheroids followed by disaggregated spheroids with a minimum fraction in monolayer cell suspension. This effect could be due to compact nature, microenvironment, and deficient drainage in spheroids mimicking in-vivo tumor. **Conclusion:** The disaggregated spheroids serve as intermediate between intact spheroids and monolayer cell suspension. The intact spheroids possess superiority over monolayer cell suspension being more compact, and resemblance to in-vivo tumors to elucidate tumor biology.

INTRODUCTION

Monolayer cell suspension or two-dimensional (2-D) cell culture of established cell lines extracted from human tumors have been employed as in-vitro cell culture for investigating cellular biology, tissue research, and drug penetration/screening studies. This in-vitro cell culture possess challenges in term of cell shap, function, cell-to-cell, and cell-to-environment effects in comparison to in-vivo studies [1, 2]. Advancement in invitro cell culture to better mimic in-vivo characteristics was the need of time and has been introduced as three-dimensional (3-D) multicellular tumor spheroids (MCTS). The MCTS are cluster/aggregates of cells possessing defined cell-to-cell and cell-to-environment interactions, production of extracellular matrix (ECM), establishment of gradient in gases/oxygen, growth/signal factors, nutrients, accumulation of catabolites [1-5]. These MCTS demonstrate close similarity in terms of functional, morphological, and physiological characteristics with in-vivo tumors [6, 7]. Furthermore, low concentration of oxygen and glucose in the interior region of MCTS may contribute in establishing of quiescent, hypoxic, anoxic and necrotic cell sub-populations which mimics in-vivo tumors and provide an appropriate tool to investigate tumor biology and drug delivery strategies [8-11].

A critical component of tumor invasion involves enzymatic degradation of the ECM [12]. ECM plays integral role to investigate tumor biology, and its composition apparently varies in intact spheroids and monolayer cell culture. Hence, to establish similarity between *in-vitro* model with *in-vivo* biology, the intact spheroids are of critical importance. Furthermore, intact MCTS possess some distinguished characteristics which

mimic the *in-vivo* tumor, and such features are not available in monolayer cell culture, such as cell-cell contact, cycle times and chronically hypoxic cells population [13]. Intact spheroids are also a useful model to express cellular heterogeneity and microenvironmental similarity with *in-vivo* tumor growth [14].

The MCTS growth, viability, radiation response, and physical characterization has well explored through haemocytometry, confocal microscopy, flow cytometry, and enzymatic assays [15-20]. In this article, the effect of disaggregated spheroids has been explored and a comparative evaluation has been performed among monolayer cell culture, disaggregated spheroids, and intact spheroids to established similarity between the intact spheroids and *in-vivo* tumors.

MATERIALS AND METHODS

Materials

Accessories include T-75cm^2 cell culture flasks, 96 well plates were purchased from Corning (USA), Gilson pipettes of 20µl, 200µl, 500µl and 1000µl capacity (France made), multipipette (Swiss made) (Barloworld Scientific ltd, UK), light microscope (Olympus Optical Co Ltd, Japan). Dulbecco's Modified Eagle's Medium (DMEM), Dulbecco's Modified Eagle's Medium (DMEM) without phenol red, Foetal Bovine Serum (FBS), L-glutamine, trypsin/EDTA were purchased from Invitrogen (UK), penicillin/streptomycin (P/S), phosphate buffer saline (PBS) tablets, dimethylsulfoxide (DMSO), agarose powder were purchased from Sigma (UK).

Methods

Selection of cell line: The cell line HT29 was selected for the cell culture and spheroids generation, because it is widely used for the generation of spheroids (Saleh et al., 2010, Dardousis et al., 2007, Cohen et al., 1999, Baricault et al., 1995). HT29 cell line is of human origin and isolated from primary tumour, colon adrenocarcinoma.

Preparation of media: The Dulbecco's Modified Eagle's Medium (DMEM) was supplemented with 10% Foetal Bovine Serum (FBS) and 1% (v/v) each of penicillin/streptomycin (P/S) and L-glutamine after placing in water-bath at 37°C for half an hour to attain same temperature. This media properly labelled/dated and kept in fridge (2-8°C), which was then ready for further use.

Preparation of monolayer cell suspension: Two flasks of T-75 cm^2 were seeded at the density of 1x10^4 cells/cm^2, placed at 37ºC in incubator with humidified environment and 5% concentration of CO_2. The cell culture media was changed on every alternate day. When the confluence of cell grasped up to 80%, the culture media was pipetted-out and cells rinsed with PBS. Then cells were detached from flak surface using Accutase ® reagent. After detachment, 3ml of media was added to the cell suspension to and pipette the cell suspension several times for segregation. This cell suspension was then centrifuged for 5 minutes at 1000rpm. Decanted the supernatant and re-suspended the pellets in fresh media for onward experimental processing.

Generation of 3-D multicellular tumour spheroids: Liquid overlay technique [21] was adopted for spheroids generation. A cell suspension (200µl) containing 2000 cells/well transferred to each well of 96-well agarose gel coated plate. Cell suspension was composed of cell culture media (DMEM containing 4.5g/l glucose) supplemented with 10% FBS and 1% (v/v) each of P/S and L-glutamine. These 96-well plates were kept in incubator maintained at 37ºC with humidified atmosphere and 5% CO_2 un-interrupted for 3 days. After three days, wells of 96-well plates were observed for the formation and the shape of spheroids. The formed spheroids were exploited for further experimental purposes.

Flow Cytometry: For the discrimination and quantification of distinct cells population in MCTS and monolayer, cell samples (either MCTS or monolayer) were stained with specific marker dyes. Calcein-AM was used to detect the live proliferating cells, Propidium iodide (PI) was used to detect dead cells, while the live hypoxic cells will remain unstained. Stock solutions of each dye (Calcein-AM and PI) were prepared and then working solutions (10µM calcein-AM, while 1µM of PI) were prepared in PBS.

A total of 10 spheroids (intact spheroids) were transferred from 96-well culture plate to an agarose uncoated 96-well plate, washed twice with PBS. A 200 µl of Calcein-AM staining solution was transferred to corresponding set of spheroids. The plate was wrapped in aluminium foil to protect the fluorophore from the effect of light and kept for 1.5 hours at room temperature. The Calcein-AM staining solution was pipetted out and spheroids were washed 3 times with PBS. Then PI staining reagent was added and kept further for 25 minutes at the same conditions. Then PI reagent was pipetted out, spheroids were washed with PBS. These washed spheroids were then transferred to 24-well plate and one ml of Accutase was added to each sample and kept in incubator at 37°C for spheroids disaggregation. After 10 minutes the samples were taken out and pipetted well to facilitate spheroids dispersion into cells suspension. These suspensions were transferred to 1.5ml Eppendorf tubes and centrifuged. The supernatant discarded and the cell pellets were re-suspended in 500 µl of PBS. These samples were then shifted for flow cytometry analysis. Data acquisition of flow cytometry was conducted through flow cytometer. Similar staining procedure was applied for recently disaggregated spheroids as well as for monolayer cell suspension.

To estimate the effect of penetration barrier in spheroids,

three-stage experiments were designed as follow:

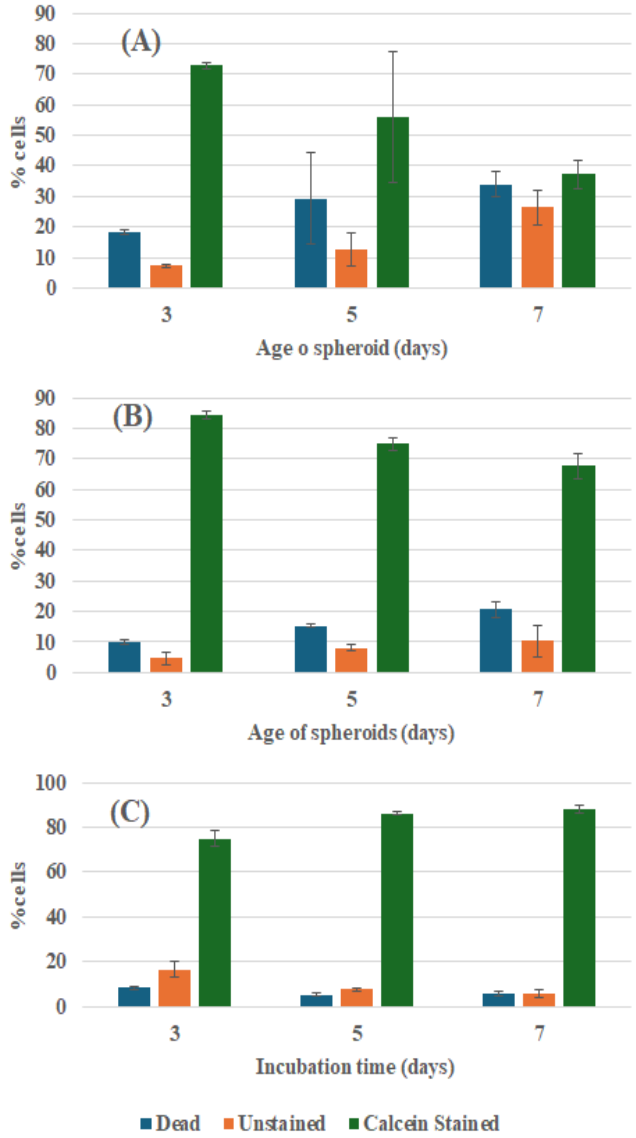
- Viability staining of intact spheroids
- Viability staining of spheroids just after disaggregation
- Viability staining of cells monolayer

RESULTS AND DISCUSSION

Comparison of viability of Intact Spheroids, disaggregated spheroids, and cell monolayer

The distinct cellular distribution in intact spheroids, disaggregated spheroids and in cells monolayer is illustrated in figure 1(A, B, and C) below:

Figure 1



Fraction of distinct cells along the age of intact spheroids (A), disaggregated spheroids (B), and monolayer cell suspension (C). Each value in all three set of experiments is the mean ±SD, n=3.

The live proliferating, live hypoxic and dead cells characteristics of HT29 were studied under various conditions: such as staining of monolayer cell suspension, staining of cells after spheroids disaggregation and staining of intact spheroids. Live proliferating cells stained with calcein-AM, Live hypoxic cells remain unstained, while dead cells were stained with Propidium Iodide (PI).

In figure 1, the post exposure time (days) are taken on x-axis, where 3 indicate 3-day old monolayer sample/spheroids, 5 indicate 5-day old monolayer sample/spheroids, and 7 indicate 7-day old monolayer sample/spheroids. On y-axis, the respective %age of cells has taken, where green colour indicate mean of calcein stained live proliferating cells, red colour indicate mean of calcein unstained live hypoxic cells and blue colour indicate mean of dead cells.

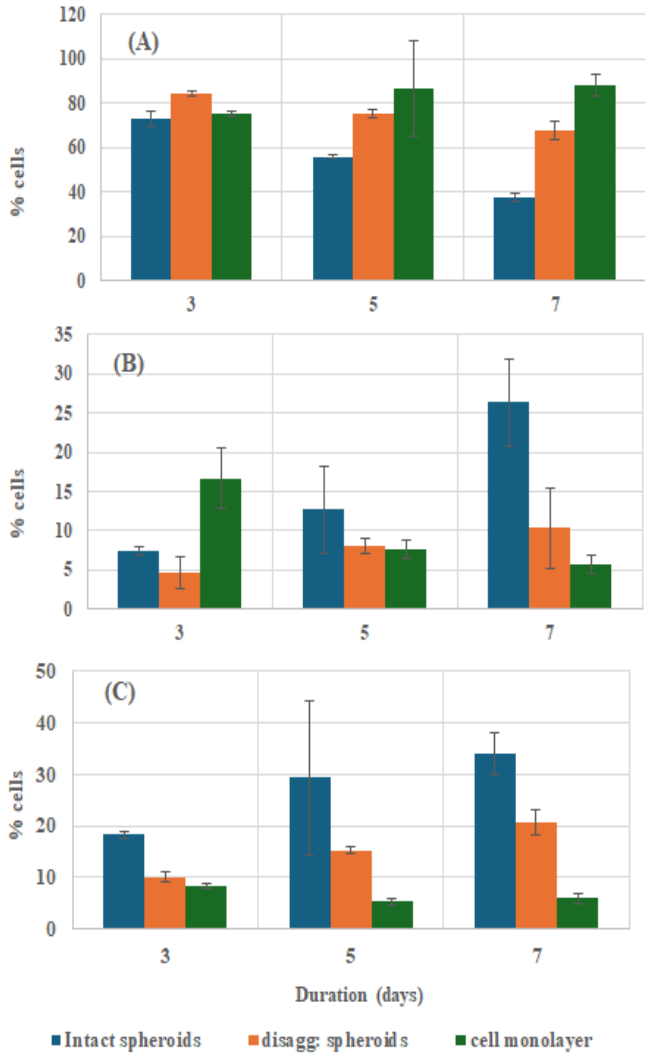
Figure 1 express the calcein-AM stained cells (live proliferating cells) %age in monolayer cells suspension (Figure 1 C) is slightly increasing, while %age of intact and disaggregated spheroids (Figure A, and B respectively) decreases considerably. Similarly, the unstained cell (live hypoxic cells) %age in monolayer cells suspension decreases at a lower rate, while %age in intact and disaggregated spheroids increases at a considerable rate. In case of PI staining, the %age of PI-stained cells (dead cells) along with age of monolayer cells suspension decreases, for which one reason may be subsequent washing steps prior to changing of media. While %age of PI-stained cells in intact and disaggregated spheroids is over all in increasing order.

The fraction of distinct cells in each set of experiment such as intact spheroids, disaggregated spheroids and cells monolayer suspension shows that only calcein-AM staining reduces due to penetration barrier of intact spheroids. As it has been reported that calcein-AM staining remain restricted to the periphery of spheroids [22], when spheroids are disaggregated, then percentage of calcein-AM staining cells is greater as compared to intact spheroids of same days. While calcein-AM staining of monolayer cell suspension increases along the time (from day-3 to day-7), which may be due to an additional factor of removing the dead cells/fragments from the culture medium at the time of media changing and proceeding to 70% confluence level. This effect may keep the cells microenvironment clean and so enhance healthy growth of cells.

Comparison of calcein-AM stained, unstained and PI-stained cells in Intact Spheroids, disaggregated spheroids, and cell monolayer

To compare each parameter (distinct feature) of all three set of experiments and to better interpret the results, we have taken common parameter (calcein-AM stained cells or unstained cells or PI-stained cells) in a single figure as below:

Figure 2



Fraction of calcein stained live cells along the age of intact, disaggregated spheroids and monolayer cell suspension (A), Fraction of unstained cells along the age of intact, disaggregated spheroids and monolayer cell suspension (B), and Fraction of dead cells along the age of intact, disaggregated spheroids and monolayer cell suspension (C). Each value is the mean ±SD, n=3.

Results show that penetration barrier exist in intact spheroid. The order of calcein-AM staining in monolayer cell suspension > disaggregated spheroids > intact spheroids. On the other hand, the unstained cells and PI-stained cells are at the order of intact spheroids > disaggregated spheroids > monolayer cell suspension.
Multicellular resistance (MCR) is an inherent feature of solid tumors and shows significant barriers to penetration of drugs into tumors[22]. Similarly, intact spheroids as well possess inherent barriers to penetration of molecules. There are established methods to analyze MCTS [23], but to ensure more similarity between *in-vitro* model with *in-vivo* environment is the need and rational of researcher. It has been reported that calcein-AM staining remains restricted to the periphery of spheroids and can't penetrate the deeper layer of spheroids[22], while disaggregated spheroids loose the penetration barriers but exhibit the inherent resistance to penetration of molecules. Therefore calcein-AM staining of disaggregated spheroid is higher than intact spheroids. It has also been reported that difference in response to drug between monolayer culture and spheroids is not only different because of limited drug penetration and existence of hypoxic and proliferating cells region but also there is existence of cell contact effect in spheroids. This cell contact effect result in changes in gene expression, which is lacking in monolayer cell culture, hence response to therapeutic agents varies [24]. Possibly, due to this reason, the monolayer cell culture exhibits more staining of calcein-AM as compared to disaggregated spheroids.
The disaggregated spheroids possess an intermediate level between monolayer cell suspension and intact spheroids. Increase in fraction of calcein-AM stained cells in monolayer cell suspension might be due to removal of dead cells at the time of media changing. Moreover, enhancing the confluence up to 70% might enhance cell-cell interaction, resulting more fraction of proliferating cells and hence more fraction of calcein-AM stained cell. On the other hand, along the time fractions of dead cells (PI stained) and hypoxic cells (unstained) increasing, resulting reducing the fraction of proliferating cells (calcein-AM stained). From these observations we can conclude that 70% confluence of monolayer cell suspension is desirable for experimental purposes, while 7-day old spheroid are desirable for experimental purposes to address the hypoxic cells for penetration studies as well.

CONCLUSION

The difference in distinctive parameters of intact and disaggregated spheroids is minimal, while that of monolayer cell suspension from spheroids is distinctive and remarkable. This difference in monolayer cell suspension and spheroids inheriting from cell-cell contact and microenvironment, where disaggregated spheroids behaved like intermediate between cell monolayer and intact spheroids, and suggesting intact spheroids as a more valuable model for investigating tumor biology.

Declarations

Ethical Approval: This study does not involve human or animals as experimental subject, therefore ethical approval is not applicable.

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