Optimization of the formation of embedded multicellular spheroids of MCF-7 cells: How to reliably produce a biomimetic 3D model

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A B S T R A C T
To obtain a multicellular MCF-7 spheroid model to mimic the three-dimensional (3D) of tumors, the microwell liquid overlay (A) and hanging-drop/agar (B) methods were first compared for their technical parameters. Then a method for embedding spheroids within collagen was optimized. For method A, centrifugation assisted cells form irregular aggregates but not spheroids. For method B, an extended sedimentation period of over 24 h for cell suspensions and increased viscosity of the culture medium using methylcellulose were necessary to harvest a dense and regular cell spheroid. When the number was less than 5000 cells/drop, embedded spheroids showed no tight cores and higher viability than the unembedded. However, above 5000 cells/drop, cellular viability of embedded spheroids was not significantly different from unembedded spheroids and cells invading through the collagen were in a sun-burst pattern with tight cores. Propidium Iodide staining indicated that spheroids had necrotic cores. The doxorubicin cytotoxicity demonstrated that spheroids were less susceptible to DOX than their monolayer cells. A reliable and reproducible method for embedding spheroids using the hanging-drop/agarose method within collagen is described herein. The cell culture model can be used to guide experimental manipulation of 3D cell cultures and to evaluate anticancer drug efficacy.

A R T I C L E   I N F O
Article history:
Received 14 June 2016
Received in revised form 13 September 2016
Accepted 3 October 2016
Available online 5 October 2016

Keywords:
MCF-7 cells
Multicellular spheroids
Hanging drop
Liquid overlay
Collagen gels

1. Introduction
Cancer cells in vivo live in a complex microenvironment consisting of basement membrane, extracellular matrix (ECM), vasculature, inflammatory cells and fibroblasts, which have been shown to contribute to cancer development [1,2]. The microvasculature in solid tumors is structurally and functionally abnormal: it is often incompletely formed, arranged in chaotic patterns, and hyper-permeable to the surrounding fluid. Consequently, most solid tumors survive in a microenvironment with oxygen deficiency (hypoxia), elevated interstitial fluid pressure (IFP), nutrient deprivation and low pH. Therefore solid tumors usually exist in a structure with metabolically active cells at the surface and a necrotic core in the center [3].

Traditional two-dimensional (2D) cell culture has been used as an in vitro model to investigate tumor behavior and identify effective antitumor therapies, but it has many limitations such as the lack of cell-cell and cell-ECM interactions and the reduced malignant phenotypes of cancer cells. Moreover, with tumor-extracellular links and elevated IFP, there is a physical barrier to drug diffusion, which is not properly reflected in 2D cell culture [4,5]. Therefore, most of results drawn from experiments done with 2D cell culture were not always in accordance with those from animal studies or clinical trials [6]. Over the past several years, various types of 3D culture systems to generate multicellular tumor spheroid have gained much attention. The 3D spheroid usually has a diameter above 200 μm and consists of peripheral proliferating cells and a central necrotic core [7–9]. In addition, there is
extensive cell-ECM interaction in some 3D spheroid culture models, which is known to be important for signaling between tumor cells [7–9]. As these characteristics are analogous to the natural tumor, the 3D cell culture system more precisely mimics in vivo solid tumors [10], and may bridge the gap between 2D cell culture system and tumors in vivo.

Typically, 3D spheroids are cultured by the liquid overlay method (using plastic culture dishes in the presence of non-adhesive substrates) [6,11] or spinner cell culture method (with specialized equipment) [12]. In these culture systems, cells form spheroids spontaneously or by centrifugation. However, a wide range of sizes and poor reproducibility may result due to the lack of control on initial cell numbers of per spheroid, making subsequent high-throughput drug screening problematic. To address this problem, the hanging-drop/agarose method [13,14] and microwell liquid overlay method [15,16] are often employed to produce spheroids of uniform size. In addition, a user-friendly market available product called AggreWell™ is commonly used, which works similarly to the microwell liquid overlay method in principle. For both methods, cells are grown on non-adherent microwells and then centrifuged to induce cell clustering in the microwells. Although AggreWell™ system is more convenient, its application is usually limited by its high cost and the preformed non-adherent layer in the microwells may not meet different experimental requirements.

Although these methods can recreate the steric structure of solid tumors, the cell-ECM interaction cannot be produced in the absence of ECM. Currently, macromolecular polymers including Matrigel™, collagen, and hyaluronic acid were used to mimic ECM [10,17–19]. To construct cell-ECM attachments in spheroids, cell suspensions can be seeded in macromolecular polymers which act as 3D scaffolds. However, there were some limitations of this system. For example, cell spheroids in the 3D matrigel are substantially smaller compared to the in vivo tumor and have heterogeneous size distribution and fail to reproduce the tumor macrostructure which usually consists of a structure with metabolically active cells at the surface and a necrotic core [20,21]. In another method, small cell spheroids can be embedded within the 3D collagen gel and further cultured to generate tight spheroids [22]. This method is more likely to produce large and uniform-sized spheroids with central necrotic cores. The embedded spheroids could be developed from spheroids which are cultured by either microwell liquid overlay or the hanging-drop/agarose method mentioned above.

However, there is little technical information available for methods to culture spheroids. Moreover, protocols to produce reliable, reproducible embedding of spheroids are also not readily available. This study will focus on the techniques to develop 3D cell models that more closely mimic in vivo tumor microenvironment. To generate large, uniform-sized, and single spheroids with a necrotic core using the human breast cancer cell line MCF-7, the microwell liquid overlay and hanging-drop/agarose methods were investigated and some key technical parameters were optimized, such as number of seeded cells, culture medium, centrifugation, and duration of sedimentation of cell suspensions. When adding methylcellulose to culture medium, it could increase cohesive force between the cells to form clusters. The centrifugation could accelerate cell sedimentation and aggregation. An appropriate sedimentation duration period of cell suspensions could harvest mature clusters to further transfer. How these technical parameters effect the formation of spheroids and how to optimize the approaches to reliably produce regular, large (>500 μm), uniform MCF-7 spheroids with extracellular matrix and a necrotic core will be investigated in this paper. The properties of spheroids unembded and embedded in mimic ECM were compared in view of the morphology, the growth characterization, the cell viability. The histology of two types of spheroids was also investigated by Propidium iodide (PI) staining to show central necrosis. The effectiveness of 3D spheroid system was validated by comparing drug resistance in MCF-7 cells spheroid and monolayer cells.

2. Materials and methods

2.1. Materials

All cell culture reagents and dishes were purchased from Wisent Corporation (Nanjing, China) unless specified otherwise. Growth medium was DMEM high glucose medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin solution. Cancer cell line MCF-7 was purchased from ATCC (Manassas, VA). Agarose was purchased from sigma (CAS:9012-36-6). Alamar blue cell viability reagent and Propidium iodide (PI) dye were purchased from HouZai Biotech (Nanjing, China). Methylcellulose was purchased from Colorcon Shanghai Coating Technology Company.

2.2. Two-dimensional cell culture

MCF-7 cells were cultured in a monolayer in DMEM high glucose medium supplemented with 10% fetal calf serum and 1% penicillin-streptomycin solution. Cultures were maintained in a humidified incubator at 37 °C, 5% CO₂. The medium was changed every day. Upon reaching cell confluency, the cells were washed with PBS, trypsinized with 0.25% (w/v) trypsin, and the cell density was regulated for the next spheroid culture [15].

2.3. Parameters affecting spheroid formation

2.3.1. The microwell liquid overlay method

Cell aggregation was induced by growing cell suspensions in agarose-coated 96-well plates. Briefly, 1.5% (w/v) agarose solution underwent high pressure steam sterilization for 25 min and was kept in hot water bath (100 °C) during the well coating process to prevent premature gelation. To prepare the agarose-coated 96-well plates, 50 μl of the hot agarose solution was pipetted into each well and allowed to cool for 30 min at room temperature until solidified. Cell monolayers were detached from their culture flask via a standard trypsinization protocol. Cells were counted and resuspended in medium to the desired density. Next, 150 μl of the cell suspensions were added to each agarose-coated well to allow generation of cellular aggregates.

To obtain a single spheroid of uniform size, cell seeding numbers (3000/5000/8000 cells per well) were compared. To accelerate cell sedimentation and aggregation, the centrifugation step was carried out immediately after cell seeding. Different centrifugation parameters (600 x g, 5 min; 600 x g, 10 min; 1000 x g, 10 min) were compared. Alternatively, different concentrations of MC (0.24% and 0.48% (w/v)) were added into the cell culture medium to increase cell aggregation in the initial 24 h after cell suspensions had been seeded in 96-well plates. Then the medium was replaced every 24 h using standard culture medium (without MC) to further culture. The growth of the spheroids was monitored every day and the optimal parameters were determined based on spheroid morphology.

2.3.2. The hanging-drop/agarose method

The cell suspensions were obtained from cell monolayers with the same procedure described in Section 2.3.1. Then drops of MCF-7 cell suspensions (25 μl) were placed onto the lids of dishes [23], and the lids inverted over dishes containing 10 ml sterile deionized water to prevent the droplets from evaporating. After a sufficient sedimentation duration period, the resultant cellular aggregates on the lids were harvested using a pipette and transferred to agarose-
coated 96-well plates (one droplet in one well) and cultured with 150 μl standard culture medium [24]. Then the medium was replaced every 24 h using standard culture medium to further culture.

The culture medium with different concentrations of MC (0.24% (w/v), 0.48% (w/v)) or without MC, initial hanging drop cell seeding numbers per drop (1000/3000/5000/10,000/15,000 cells), as well as duration period for sedimentation (12 h, 24 h, 36 h) were optimized. An overview of all parameters tested of the above two methods is given in Table 1.

2.4. Spheroids embedding in collagen

2.4.1. Preparation of collagen solution

Frozen rat tails from mature rats (8–9 weeks old) were thawed in 75% (v/v) ethyl alcohol. Then the skin of rat tails was pulled off with the help of clean surgical clamps and scissors. White fibers were separated and soaked with acetic acid solution at pH 3.2. This viscous mixture was centrifuged at 10,000 × g for 10 min. The supernatant was taken out and stored at 4 °C as the collagen solution. The crude collagen solution was salted out to obtain collagen precipitate with 100 g/L sodium chloride solution, then the precipitate was redissolved in 0.1% (v/v) acetic acid solution at pH 3.2 and stored at 4 °C. The collagen solution was neutralized with 0.1 M NaOH before using for embedding cells or cell spheroids [25]. The concentration of collagen was determined as described above. After forming single spheroids, spheroids with cell culture medium were implanted gently in 100 μl collagen solution of each well of a 96-well plate using a pipette.

After collagen solution gelation at 37 °C for 30–60 min, the collagen gels with spheroids embedded in were overlaid with 100 μl of standard culture medium. This culture medium was replaced every 24 h and spheroid invasion was allowed to proceed for 72 h.

2.5. Characterization of spheroid

2.5.1. Spheroid growth assay

In the spheroid formation process, spheroids were imaged using microscopy (Nikon, Tokyo, Japan and photographed on day 1, 2, 3, 5, and 7. After embedding in collagen, spheroids were also imaged at 24, 48, and 72 h. Diameters of embedded spheroids were measured in the resultant images with ImageJ software. The diameters of unembedded spheroids were not measured because of having the fuzzy boundary.

2.5.2. Cell viability of spheroid

MTS (5-(3-carboxymethoxyphenyl)-2-(4(5-dimethylthiazolyl)-3-(4-sulfophenyl)tetrazolium, inner salt) and MTT (3-(4,5-dimethylthiazolyl)-2-yl)-2.5-diphenyltetrazolium bromide) assay are commonly-used methods for assessment of cell viability of 2D cells. When they are used for 3D spheroids, spheroids have to be dissociated to 2D cells due to the poor permeability of MTS or MTT to spheroids [22], which may affect cell viability by using trypsin during cell dissociation. In contrast, there is no need to disaggregate spheroids using alamar blue which has a good permeability in spheroids [14]. Moreover, Yi-Chung Tung had compared cell viability of 3D spheroids based on alamar blue assay and fluorescence microscopy imaging with live/dead stain (Calcein AM and Ethidium homodimer-1). The viability discrepancy obtained from these two methods was less than 10% [14]. Therefore, alamar blue assay was chosen to estimate cell viability for 3D spheroids.

Spheroids proliferation and viability (embedded spheroids and unembedded spheroids) were assessed at day 7 using alamar blue cell viability reagent. Following the manufacturer’s protocol, 2 μl (one-tenth of each culture medium volume) of alamar blue was added to each MCF-7 spheroid sample and incubated for 24 h. After incubation, the medium in each well was transferred to new 96-well plates. Then the plate was read using a Thermo Scientific Microplate Reader (Multiskan MK3) at 570 nm to obtain absorbance intensity readouts which was in direct proportion to cell viability.

2.5.3. Histological evidence of central necrosis in spheroid by PI staining

Propidium iodide (PI) dye can only diffuse into dead cells, where it combines with DNA to produce red fluorescence. After stained by PI, spheroids exhibiting PI fluorescence in the core indicated that a central necrotic core was formed in the spheroid. For 3D culture systems, 7-day-old spheroids (unembedded and embedded) of different sizes were stained by PI. Specifically, 6 μl PI was added to each spheroid in a 96-well plate and incubated at 4 °C for 90 min to ensure full penetration of PI into the core of the spheroids. After washing with PBS, spheroids were harvested on a pre-cleaned glass microscope slide and visualized under fluorescent microscopy (Nikon, Tokyo, Japan). Fluorescence images were obtained at 488 nm using ImageJ software.

2.5.4. Drug resistance study in spheroids

The effectiveness of 3D spheroid system was validated by

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**Table 1**

The parameters optimized in the microwell liquid overlay (A) and hanging-drop/agarose (B) methods.

<table>
<thead>
<tr>
<th>(A) The microwell liquid overlay method</th>
<th>Centrifugation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell seeding number</td>
<td>Culture media</td>
</tr>
<tr>
<td>3000 cells/well</td>
<td>Standard</td>
</tr>
<tr>
<td>5000 cells/well</td>
<td>Standard</td>
</tr>
<tr>
<td>8000 cells/well</td>
<td>Standard</td>
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<table>
<thead>
<tr>
<th>(B) The hanging-drop/agarose method</th>
<th>Sedimentation duration period</th>
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<tbody>
<tr>
<td>Culture media</td>
<td>Cell seeding number</td>
</tr>
<tr>
<td>Standard</td>
<td>1000 cells/drop</td>
</tr>
<tr>
<td></td>
<td>12 h; 24 h; 36 h</td>
</tr>
<tr>
<td>0.24% (w/v) MC</td>
<td>3000 cells/drop</td>
</tr>
<tr>
<td></td>
<td>12 h; 24 h; 36 h</td>
</tr>
<tr>
<td>0.48% (w/v) MC</td>
<td>5000 cells/drop</td>
</tr>
<tr>
<td></td>
<td>12 h; 24 h; 36 h</td>
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</table>

"Standard" refers to "Standard cell culture medium"; "0.24% (w/v) MC" refers to "cell culture medium containing 0.24% (w/v) MC"; "0.48% (w/v) MC" refers to "cell culture medium containing 0.48% (w/v) MC."
comparing drug resistance in 3D spheroids of MCF-7 cells and their 2D cells. The cytotoxicity study of anticancer drug doxorubicin (DOX) against MCF-7 was performed using alamar blue cell viability assays which could determine viability of cells treated with various concentrations of drug relative to the untreated cells. For 2D culture, MCF-7 cells harvested from growing cells were seeded on a 96-well plate at a density of 6, 000 cells per well and cultured for 24 h. For 3D culture, the embedded and unembedded spheroids were formed by optimized conditions and method as described above and cultured for 7 days. Various concentrations of DOX (1, $10^2$, $10^3$, $10^4$, 5 x $10^4$, 8 x $10^4$, 10 x $10^4$ ng/ml) in DMEM medium of 100 µl were prepared and added to the medium-removed 96-well plate and incubated for 60 h [23,29]. Then alamar blue viability assay was performed following the manufacturer's protocol. Then the plate was read using a Thermo Scientific Microplate Reader (Multiskan MK3) at 570 nm to obtain absorbance intensity readouts. Dose-response curves for 2D and 3D systems were then plotted and the 50% inhibition concentration (IC50) value was calculated through the IBM SPSS 19.0 software.

2.6. Statistical analysis

Data from different experiments were expressed as mean ± standard deviation (SD). Student T-test for data comparison was performed using IBM SPSS 19.0 software. *P* values < 0.05 were considered statistically significant (*) and **p** values < 0.01 were considered extremely significant (****).

3. Results and discussion

3.1. Parameters affecting spheroid formation for the microwell liquid overlay method

3.1.1. The cell seeding number

For the microwell liquid overlay method, cells were prevented from adhering to the plate by agarose and attached to each other to form aggregates. Different cell seeding numbers (3000/5000/8000 cells per well) were tested. However, for all three seeding numbers tested, the aggregates formed were multiple and irregular in shape (Supplemental Fig. 1). To harvest a single spheroid, other improvements, such as adding MC to the culture medium or using a centrifugation step after seeding cell suspensions to 96-well plates, were considered in the following experiments.

3.1.2. Cell culture media

To investigate the effect of MC in cell aggregation, the cell suspensions were adjusted to 5,000 cells per well using different culture media. As shown in Supplemental Fig. 2, adding MC (0.24% or 0.48% (w/v)) to the culture medium did not result in the better cell aggregation compared to the group in standard cell culture medium (without MC). All three conditions tested generated multiple irregular clusters instead of one spheroid when MCF-7 cells were cultivated for 7 days. This could be explained by the following reason. Although MC could increase cohesive force between the cells after forming clusters, it might prevent movement cells towards the center of the well at the beginning due to increased viscosity of the media.

3.1.3. Centrifugation

After adding cell suspensions to the 96-well plates, different centrifugation parameters (600 x g, 5 min; 600 x g, 10 min; 1000 x g, 10 min) were investigated. As shown in Supplemental Fig. 3A, multiple irregular aggregates were generated at day 7 without centrifugation. When the plate was centrifuged for 5 or 10 min at 600 x g, there was no obvious improvement in cell aggregation (Supplemental Fig. 3B and C). A single but irregular aggregate formed at day 7 when centrifuged at 1,000 x g for 10 min after cell seeding (Supplemental Fig. 3D–F). These phenomena showed that cells can be collected in the center of the well by an appropriate centrifugation step. However, the centrifugal force beyond 1,000 x g could damage cells [26], which was proved in our preliminary experiments.

For the microwell liquid overlay method, centrifugation (1,000 x g, 10 min) at the beginning of cell inoculation could facilitate cell aggregate formation. Although the cells formed one aggregate after culture for 7 days, its shape was irregular. This was not the ideal tumor model for high-throughput drug screening due to the poor reproducibility. Therefore, embedding in collagen of cell aggregates formed with this method was not attempted. Thus, a different protocol to reliably produce regular spheroids was explored.

3.2. Parameters affecting spheroid formation for the hanging-drop/agarose method

3.2.1. Cell culture media

In the hanging-drop/agarose method, the cell suspensions were adjusted to the desired cell concentration using different culture media and placed onto the lids of dishes then the lids were inverted. Cell aggregates at the bottom of the drop were observed after cell suspensions were sedimented for 24 h. As shown in Fig. 1A, single cell aggregates in the center of the drop were not observed without MC in the cell culture medium. In contrast, with MC (0.24% or 0.48% (w/v)) in the cell culture medium, cells could form aggregates (Fig. 1B and C). And there was no obvious difference in the shape of aggregates at the two concentrations of MC. The resultant cellular aggregates were harvested from the lids and transferred to agarose-coated 96-well plates and further cultured. After 7 days, a single spherical spheroid was harvested (Fig. 1D). The reliability of this approach could be explained by the following reasons. The gravitational force exerted on the cells in the hanging drop caused cell sedimentation and movement to the center. Moreover, adding MC to the medium could increase the cohesive force between cells to generate a single aggregation during

Fig. 1. The morphology of the MCF-7 cell aggregates or spheroids using different culture media by the hanging-drop/agarose method. 10, 000 cells per drop were plated. (A) Aggregates of MCF-7 cells cultured with standard cell culture medium after 24 h of sedimentation. (B) Spheroid of MCF-7 cells cultured with cell culture medium containing 0.24% (w/v) MC after 24 h of sedimentation. (C) Spheroid of MCF-7 cells cultured with cell culture medium containing 0.48% (w/v) MC after 24 h of sedimentation. (D) A 7-day-old spheroid of MCF-7 cells cultured with cell culture medium containing 0.24% (w/v) MC. Bar equals 200 µm.
sedimentation. In short, the combination of cohesive and gravitational forces induced cell aggregation. To ensure the healthy growing environment for cells, the culture medium with lower concentration of MC was preferred to promote spheroid formation for the subsequent studies.

3.2.2. Sedimentation duration period of cell suspensions

The gravitational force exerted on the cells in the hanging drop caused cell sedimentation, and immature aggregates became visible within the first 12 h. To harvest a dense aggregate, it was necessary to optimize the duration of sedimentation. When the sedimentation duration period was above 24 h, no breakups of the aggregate were observed after transferring them to agarose-coated 96-well plates, implying that robust connections among the cells in the aggregate were generated. However, if the sedimentation duration period was above 36 h, there was not enough nutrient for cell growth in the culture medium. Therefore, a sedimentation duration period of 24 h was adopted subsequently.

3.2.3. The cell seeding number

Seen from Fig. 2A, regular spheroids were all harvested when the number of cells seeded ranged from 1000 to 15,000 cells. So the initial cell seeding number had no effect on the formation of spheroid. As shown in Fig. 2B, the spheroid diameter was linearly dependent on cell seeding number.

For the hanging-drop/agarose method, the key technical parameters include using cell culture medium containing 0.24% (m/v) methylcellulose to adjust the cell suspensions to a initial cell seeding density and a sedimentation duration period of 24 h. The good scalability and reproducibility of spheroid formation by the hanging-drop/agarose method was also demonstrated.

Then the embedded cell spheroids were developed from regular spheroids which were formed by the optimized hanging-drop/agarose method. Then the embedded spheroids or not were compared in view of the morphology, the growth characterization, and the cell viability. To compare drug resistance study of 3D spheroids (embedded and unembedded) of MCF-7 cells and their 2D cells, the doxorubicin cytotoxicity against them was also performed. The spheroids being evaluated in the paper included both the unembedded and the embedded spheroids.

3.3. Spheroid growth assay

As was shown previously, the spheroid formed tended to be irregular in shape by the microwell liquid overlay method, which was not a good model for drug screening (Supplemental Fig. 3). Therefore, only round and regular spheroids cultured by the hanging-drop/agarose method, were embedded in collagen gels for further study. Then the resultant spheroids embedded or not were compared in view of the morphology, the growth characterization, and the cell viability. To monitor the growth characteristics of unembedded spheroids, the morphology and diameter of spheroids were investigated (Fig. 2A and C). Fig. 2A shows representative optical images of spheroids with different cell seeding numbers (1000/3000/5000/10,000 and 15,000 cells). The change of mean spheroid diameter over time in the five groups is shown in Fig. 2C. The spheroid diameter of all groups increased sequentially after an initial slight decrease in the first two days, which could be explained by the following reasons. In the initial stage, cells spontaneously self-assembled to form a cell spheroid with loose intercellular interactions. Then cells began to reorganized themselves by contact and the spheroid became tighter. In addition, the spheroid had a relatively slow growth rate at the beginning, which may have made the spheroid shrink a little in the first two days [4].

As shown in Fig. 3, after spheroids had been embedded in collagen for 72 h, cells at the periphery of the spheroid had invaded into the gel, which indicated that embedded spheroids had invasive activity. Embedded spheroids formed from a cell seeding number of less than 5000 lost their tight cores and a lot of individual cells could be seen invading through the gels after culture for 72 h. Interestingly, when the cell seeding number was bigger (10,000 or 15,000), the invading spheroids exhibited a sun-burst pattern with retention of their tight cores and a few individual cells could be seen peripherally invading through the gels.
3.4. Cell viability of spheroid

The difference of cell viability of the embedded and unembedded spheroids had been investigated. As seen in Fig. 4, when cell seeding number was less than 5,000, 7-day-old embedded spheroids showed significantly higher viability than unembedded spheroids. As indicated in Fig. 3, tight cores of spheroids with cell seeding number less than 5,000 disappeared after culture for 24 h in collagen gels due to the invasion of the MCF-7 cells into the collagen gel. Invasion of individual cells at the periphery of spheroid would lead to a loose connection between cells at the periphery of the spheroid. Therefore, more culture medium was accessible to the peripheral cells, resulting in rapid cell proliferation. However, the cell connection in unembedded spheroids was still very tight. So embedded spheroids showed higher viability than the unembedded. However, cell viability of two kinds of spheroids was not significantly different when cell seeding number was above 5000. This could be explained by the fact that few individual cells at the periphery invaded into the collagen gels (Fig. 3). Therefore, the number and proliferation rate of peripheral cells were similar for unembedded and embedded spheroids. So there was no difference in their cell viability. Although the viability seems to be around the same between the embedded and unembedded spheroids in some situations, the embedded spheroids exist in a cell-ECM attachments microenvironment. The interplay between the ECM and the cells would affect drug response, epigenetic state, and metastasis in cancer cells [27,28].

3.5. Histological evidence of central necrosis in spheroid by PI staining

The tight cores of the embedded spheroids with cell seeding number less than 5000 disappeared after culture for 72 h in collagen gels (Fig. 3). It was inferred that there was no central necrosis in these spheroids. However, as shown under bright field in Fig. 5, embedded spheroids with cell seeding number more than 5000 still contained tight cores and few individual cells were seen invading through collagen after culture for 72 h. However, it is not possible to determine whether the remaining core was viable by

Fig. 3. Images of spheroids embedded within collagen gels at 0 h, 24 h, 72 h. Spheroids with different cell seeding number (1000/3000/5000/10,000 and 15,000 cells) cultured by hanging-drop/agarose method were experimented. Bar equals 200 µm.

Fig. 4. Cellular vitality for 7-day-old MCF-7 spheroids with different cell seeding numbers determined by alamar blue. Abs measurements in the alamar blue assay for different numbers of MCF-7 spheroids had the linearity characterization. n = 6 for each spheroid number group. P values < 0.05 were considered statistically significant (*) and P values < 0.01 were considered extremely significant (**).

Fig. 5. 7-day-old spheroids (unembedded and embedded in collagen gels) were stained with PI (dead cells; red) to demonstrate central necrosis. The left images were under bright field, the right images were under fluorescence at 488 nm. Bars equal 200 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
simple microscopy. Therefore, the dead cells in unembedded and embedded spheroids with cell seeding number of 10,000 and 15,000 were stained by PI to demonstrate the presence of necrotic cores. Seen from Fig. 5, cell death occurred in the center of spheroids and the necrotic cores were similar in size in unembedded and embedded spheroids with the same cell seeding number, which was in accordance with the results obtained from cell viability measurements (Fig. 4). The emergence of necrotic cores may be due to high metabolism combined with nutrient deprivation of cells in the center of spheroid.

3.6. Drug resistance study in spheroids

Alamar blue cell viability assays were performed to determine drug cytotoxicity on monolayer cells and spheroid cells. Spheroids were prepared using the optimized hanging-drop/agarose method with the seeding density of 10, 000 cells per drop.

As shown in Fig. 6, spheroids displayed a higher drug resistance to DOX treatment in comparison to the 2D controls. After 60 h of DOX treatment, the IC50 values obtained from the unembedded and embedded spheroids were 18961.83 ng/ml and 26686.87 ng/ml, respectively, relative to 374.12 ng/ml for the monolayer cells. The IC50 values determined with the unembedded and embedded spheroids were approximately 50.68-fold, 71.33-fold higher than that for monolayer cells. The data demonstrated that spheroids were less susceptible to anticancer drug DOX than their monolayer cells, indicating a high drug resistance. It was also notable that the embedded spheroids exhibited higher drug resistance than the unembedded spheroids. As seen in Fig. 4, cell viability of two kinds of spheroids (the unembedded and embedded) was not significantly different when cell seeding number was 10,000 cells per drop. However, in drug resistance study, the embedded spheroids exhibited a higher IC50 values. This may be because the embedded spheroids exist in a cell-ECM attachments microenvironment which could affect drug response [27,28]. All these results suggested that the embedded spheroid model by the optimized hanging-drop/agarose method has been successfully established, which would be effective for drug screening.

4. Conclusions

In this paper, the technical parameters of microwell liquid overlay and hanging-drop/agarose methods were investigated and subsequently optimized to generate large and uniform-sized MCF-7 spheroids with metabolically active cells at the surface and a necrotic core. The recommended method to prepare 3D spheroids of MCF-7 cells was the optimized hanging-drop/agarose method, in which the key technical parameters include using 0.24% (m/v) methylcellulose in cell culture medium in the initial 24 h after cell suspensions had been seeded in 96-well plates and a sedimentation duration period of 24 h. The spheroids with cell seeding number above 5, 000/drop formed by the hanging-drop/agarose method were recommended embedding in collagen for further culture. The resultant embedded spheroids were proved to be large (>500 μm), single and uniform in size with cell-ECM attachments and central necrosis, which may act as a suitable 3D cell culture model for evaluation of anticancer drug efficacy.

Acknowledgments

This study is financially supported by the National Science Foundation Grant of China (No. 81503005), the Natural Science Foundation of Jiangsu Province (No. BK20140669), and the Priority Academic Program Development of Jiangsu Higher Education Institutions.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ab.2016.10.004.

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